Genetic Diagnostics in Routine Osteological Assessment of Adult Low Bone Mass Disorders

Ralf Oheim[1,$], Elena Tsourdi[2,3], Lothar Seefried[4], Gisela Beller[5], Max Schubach[6], Eik Vettorazzi[7], Julian Stürznickel[1], Tim Rolvien[1,8], Nadja Ehmke[9], Alena Delsmann[1], Franca Genest[4], Ulrike Krüger[10], Tomasz Zemojtel[10], Florian Barvencik[1], Thorsten Schinke[1], Franz Jakob[4], Lorenz C. Hofbauer[2,3], Stefan Mundlos[9,11,12,], Uwe Kornak[9,11,12,13,$]

1) Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

2) Department of Medicine III, Technische Universität Dresden Medical Center, Dresden, Germany

3) Center for Healthy Aging, Technische Universität Dresden Medical Center, Dresden, Germany

4) Orthopedic Center for Musculoskeletal Research, Orthopedic Department, University of Würzburg, Germany

5) Centre of Muscle and Bone Research, Charité-Universitätsmedizin Berlin, Berlin, Germany

6) Berlin Institute of Health at Charité – Universitätsmedizin Berlin, Berlin, Germany

7) Department of Medical Biometry and Epidemiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

8) Department of Orthopaedics and Trauma Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

9) Institute of Medical Genetics and Human Genetics, Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

10) Core Facility Genomics, Berlin Institute of Health (BIH), Berlin, Germany

11) BIH Center for Regenerative Therapies, Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

12) Max Planck Institute for Molecular Genetics, Berlin, Germany

13) Institute of Human Genetics, Universitätsmedizin Göttingen, Göttingen, Germany

$ Corresponding Authors

Uwe Kornak, PhD., Institute of Human Genetics, Universitätsmedizin Göttingen, Heinrich-Düker-Weg 12, 37073 Göttingen, Germany, Phone: +49-551-39-69011, Email: uwe.kornak@med.uni-goettingen.de, ORCID: 0000-0002-4582-9838

Ralf Oheim, MD, Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, Lottestraße 59, 22529 Hamburg, Germany, Phone: +49-40-7410-56373, Email: r.oheim@uke.de, ORCID: 0000-0001-9449-5013

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Abstract

Context: Many different inherited and acquired conditions can result in premature bone fragility / low bone mass disorders (LBMD). **Objective:** We aimed at elucidating the impact of genetic testing on differential diagnosis of adult LBMD and at defining clinical criteria for predicting monogenic forms. **Methods:** Four clinical centers broadly recruited a cohort of 394 unrelated adult women before menopause and men younger than 55 years with a bone mineral density (BMD) Z-score $\leq$-2.0, and/or pathological fractures. After exclusion of secondary causes or unequivocal clinical/biochemical hallmarks of monogenic LBMD all participants were genotyped by targeted next-generation sequencing. **Results:** In total 20.8% of the participants carried rare disease-causing variants (DCV) in genes known to cause osteogenesis imperfecta (*COL1A1, COL1A2*), hypophosphatasia (*ALPL*), and early-onset osteoporosis (*LRP5, PLS3,* and *WNT1*). In addition, we identified rare DCV in *ENPP1, LMNA, NOTCH2*, and *ZNF469*. Three individuals had autosomal recessive, 75 autosomal dominant, and four X-linked disorders. 9.7% of the participants harbored variants of unknown significance. A regression analysis revealed that the likelihood of detecting a DCV correlated with a positive family history of osteoporosis, peripheral fractures (>2), and a high normal BMI. In contrast, mutation frequencies did not correlate with age, prevalent vertebral fractures, BMD, or biochemical parameters. In individuals without monogenic disease-causing rare variants, common variants predisposing for low BMD, e.g. in *LRP5*, were overrepresented. **Conclusion:** The overlapping spectra of monogenic adult LBMD can be easily disentangled by genetic testing and the proposed clinical criteria can help to maximize the diagnostic yield.

Mini Abstract

We evaluated the impact of genetic testing in 394 participants with adult low bone mass disorders. Disease-causing variants (DCV) were found in 20.8% and variants of unknown significance in 9.7% of the participants. DCV detection correlated with positive family history, peripheral fractures (>2), and high normal BMI.

Keywords: osteoporosis, low bone mass disorder, monogenic disorder, genetic risk score, rare genetic variant, genotype-phenotype correlation

Abbreviations: BMD, bone mineral density; BMI, body mass index; CADD, Combined Annotation Dependent Depletion; DCV, disease-causing variant; LBMD, low bone mass disorder; gnomAD, The Genome Aggregation Database; GRS, genetic risk score; VUS, variant of unknown significance.

Introduction

Genetic testing is increasingly integrated into the diagnostic workup in all medical disciplines due to the availability of efficient genetic screening methods based on next-generation sequencing. Such analyses allow for further disease classification, the identification of rare monogenic diseases and thereby individualized treatment. However, discipline-specific criteria for the identification of individuals with rare monogenic disorders are needed to maximize the diagnostic yield of genetic testing. Especially challenging is the recognition of mildly affected individuals with a disease onset in adulthood lacking the classical clinical signs of the corresponding early-onset rare disorder (1).

In clinical routine rare low bone mass disorders (LBMD) in adults are primarily diagnosed by DXA and often subsumed under the umbrella term osteoporosis, although this term in principle denotes low bone mass and impaired microarchitecture. But indeed a plethora of different pathologies, also including mineralization disorders, can cause metrological 'low bone mass'. In addition, after secondary forms of LBMD have been ruled out, it can be difficult to distinguish acquired and inherited forms.

Severe rare monogenic LBMD with congenital or infantile onset and common age-related (postmenopausal or senile) osteoporosis with multifactorial pathogenesis can be regarded as a continuum of clinical phenotypes with many genetic and pathogenetic overlaps (2,3). This principle is illustrated by the different forms of bone fragility caused by mutations in *WNT1* or *LRP5*. Biallelic recessive mutations in *WNT1* and *LRP5* cause severe congenital bone fragility that presents as osteogenesis imperfecta with variable extra-skeletal manifestations (4-7). However, monoallelic dominant mutations in *WNT1* and *LRP5* cause a nonsyndromic LBMD with often adult onset, which lies in between congenital monogenic LBMD and age-related osteoporosis (8-12). Such late-onset monogenic LBMD are difficult to diagnose clinically within this heterogeneous patient group and it is currently unclear when and which type of genetic testing should be applied (13).

Genome-wide association studies (GWAS) have identified more than 500 genetic loci harboring common genetic variants influencing bone mineral density (BMD), a major risk factor for age-related osteoporosis, and lifetime fracture risk (14). BMD shows a heritability of 50-85% and common genetic variants explain about 25% of the BMD variance (14,15). Also, common polymorphisms in the *LRP5*, *LRP6* and *WNT1* gene loci predispose to low BMD (16). Genetic risk scores (GRS, also called polygenic risk scores) based on such predisposing polymorphisms have been developed for many common conditions and are ready to be integrated into clinical routine (17). In the osteoporosis field it was shown that a comprehensive GRS outperformed

common clinical and radiological scoring methods in the prediction of osteoporotic fracture risk (15). In addition, testing for monogenic LBMD was suggested in cases with severe osteoporosis with early onset in spite of a disparately low GRS (18). Adult LBMD affect men and women alike and its management is a challenge due to the lack of guidelines and approved drugs. Early diagnosis and possibilities for treatment stratification are therefore needed.

In this study we wanted to evaluate to which extent premature low bone mass and fragility, after exclusion of secondary forms, have an underlying monogenic cause. Second, we aimed at identifying clinical criteria predicting monogenic forms of adult LBMD. Third, we wanted to investigate the contribution of common genetic variants to the pathogenesis of premature low bone mass in participants without monogenic cause. To this end, we performed genetic analysis of a cohort of 394 affected individuals.

Materials and Methods

Study population

All participants recruited within this prospective observational study were adult outpatients seen in the four participating clinical centers in Germany (Berlin, Dresden, Hamburg, Würzburg) between 2016 and 2018. Inclusion criteria were: DXA Z-score $\leq$-2.0 and/or clinical evidence for bone fragility such as history of low-energy/osteoporotic fractures assessed by patient history and available medical records. Family history of osteoporosis (i.e. known osteoporosis, hip fractures or other major low energy fractures in first degree relatives) was recorded as part of the comprehensive medical history. The onset of the phenotype had to be premenopausal in women and before the age of 55 years in men. Participants suffering from conditions known to cause secondary osteoporosis such as multiple myeloma, systemic mastocytosis, systemic steroid therapy (>7.5 mg per day for > 3 months), malabsorption and malassimilation, eating disorders, alcoholism, endocrine diseases such as thyrotoxicosis or primary hyperparathyroidism, chronic inflammatory bowel diseases, as well as chronic liver and kidney diseases were excluded. The diagnostic workup was performed according to current guidelines and included the assessment of BMD by dual-energy X-ray absorptiometry (DXA scan) at the lumbar spine and hip. Blood samples were taken for biochemical analyses of serum concentrations of calcium, phosphate, creatinine, 25-hydroxycholecalciferol (25-OH-D_3), parathyroid hormone (PTH), alkaline phosphatase (AP), bone-specific alkaline phosphatase (BAP), and osteocalcin. Urine samples were taken for analyses of

deoxypyridinoline/creatinine (DPD). In addition, grip strength was performed as part of a neuromuscular assessment. All recruited participants gave written informed consent and the study was approved by the ethics committee (EA04/060/16) of Charité-Universitätsmedizin Berlin.

Genotyping

Genomic DNA was isolated from EDTA blood samples, fragmented by an ultrasound device (Covaris, Woburn, MA, USA) and used for library preparation. For enrichment of target sequences different versions of a custom design Sure Select XT (Agilent, Santa Clara, CA, USA) gene panel (skeletal disease associated genome, sDAG) containing selected noncoding regions containing SNPs (in total 20 SNPs) associated with BMD and the core genes for relevant disorders with low and high BMD were used as described before (8). Additionally, in ten individuals all coding exons were enriched (Human all exon V6, Agilent, Santa Clara, CA, USA). Sequencing was performed on MiSeq, NextSeq, and HiSeq machines (Illumina, San Diego, CA, USA) with a coverage of >100X leading to >20X coverage of >98% of the target region.

Bioinformatics and evaluation of gene panel data

After mapping (BWA-MEM) and variant calling (GATK) data was analyzed by the software MutationDistiller in a phenotype-driven manner (19). The software Sequence Pilot (JSI medical systems, Ettenheim, Germany) was used for unbiased variant prioritization. Global copy number analysis was performed using the software clearCNV (manuscript under review). The pathogenicity of the prioritized variants was judged using MutationTaster version 2, and the freely accessible version of Varsome (20,21). Variants were compared to databases (ExAC, GnomAD and HGMD professional, Quiagen, Hilden, Germany). Variant classification followed the American College of Medical Genetics (ACMG) recommendations (22).

Further statistical analysis of sequence variants

Samples with less than 85% of 20x coverage over the panel target region or with many off target reads were removed. Because multiple panel enrichments were used, the intersection of all target enrichments (extended -±20 bp) was used when data were jointly analyzed. Two control cohorts were selected for comparison: variants from 503 individuals of European origin from the 1000 Genomes Project (cohort 1KG EUR; Controls) and 59

individuals from an in-house high BMD cohort, sequenced with the same gene panels (cohort high BMD) (23). To exclude outliers, principle component analysis (PCA) of all three cohorts together was performed on variants located in the final target region using the python package 'allele'. LD pruning was performed with a window size of 200 bp and 20 variants to advance to the next window. Finally, outliers were identified from the first four components and were removed in downstream analysis.

Rank sum test of rare variants according to CADD scores

Enrichments of potential deleterious variants in genes known to be associated with low BMD (Supplement Table S1)(24) were computed using the raw Combined Annotation Dependent Depletion (CADD) score (25). Here, the enrichment of CADD scores of LBMD individuals compared to other cohorts was tested using the rank sum test. Variants were annotated with Jannovar v0.34 using the RefSeq database, as well as CADD v1.4 scores and gnomAD exome v2.0.1 allele frequencies. Variants were classified into different groups defined by Jannovar's variant effect classes and allele frequency of gnomAD nonFinish Europeans (NFE). The following groups were used: all variants, rare variants (NFE<0.01), MODERATE (missense, inframe indel) and HIGH (stop gain/lost, in-frame indel, splice donor/acceptor) effect variants, rare MODERATE and HIGH effect variants (NFE<0.01), and ultrarare MODERATE and HIGH effect variants (not in gnomAD). Genes involved only in high BMD were used as a negative control set. For multiple test correction, the Benjamini-Hochberg (FDR) method was used.

Individual common variant frequency difference and genetic risk score calculation

Genetic risk scores (GRS) per sample were calculated using SNPs associated with BMD in Europeans and located in the high coverage target regions from the GWAS catalog (download date 11/20/2019; see Supplement Table S2)(24,26). For each sample, the beta values (negative beta values have BMD-lowering and positive beta values BMD-increasing effects) of the hits were summarized for each effect allele present in a sample. Homozygous variants were counted twice. Negative beta values account for low BMD and positive beta values account for high BMD. For comparison between LBMD with and without DCV and the two control cohorts, mean SNP effects on BMD were computed and normalized to the 1KG EUR controls. Receiver operating characteristic (ROC) plots were generated to analyze the separation between cohorts. GRS of the different

cohorts were compared using one-way ANOVA. For evaluation of frequencies of individual SNPs in the different cohorts we used the Fischer exact test

Statistical analysis of phenotype-genotype correlation

Baseline characteristics are summarized as the number of participants (%) for categorical variables and as the mean (SD) and/or median (range) for continuous variables. Groups were compared using chi-squared tests and Student's t tests, respectively. Univariate associations between continuous variables and the presence of a class 4 or 5 variant were investigated using logistic regression models. A classification and regression tree analysis was performed to detect cut-offs of predictive variables. All analyses were performed using R 3.6.1 (R Core Team, Vienna, Austria).

Results

Rare disease-causing variants are a frequent finding in adult LBMD

A total of 394 unrelated adult index participants that fulfilled the inclusion criteria (DXA Z-score $\leq$-2.0 and/or clinical evidence for bone fragility, disease onset premenopausal in women and <55 years in men, no underlying condition known to cause secondary osteoporosis) were recruited in four clinical centers (Table 1). Their average age at analysis was 48.1$\pm$11.8 years; 45.4% were women and 54.6% were men. Three hundred fifteen (80%) of these had experienced one or multiple fractures at the time of diagnosis. A positive family history of osteoporosis was evident in 124 (31.5%) participants. We performed targeted sequencing using a custom gene panel and additional exome sequencing in ten individuals with negative gene panel results and a clinical phenotype and/or family history strongly suggesting a monogenic disease. Genotyping revealed 85 disease-causing variants (DCV), (ACMG variant class IV or V), in 82 (20.8%) individuals (Table 2, Supplement Table S3)(24). Variants of unknown significance (VUS) (ACMG class III) were present in 28 (7%) participants (Table2, Supplement Table S4)(24). Thus, we identified bona fide DCV in about 1/5 of all participants with broadly defined premature low bone mass and bone fragility.

Clinical hallmarks of individuals with monogenic LBMD

Due to our broad recruitment criteria our cohort comprised a large spectrum of adult LBMD. This permitted a statistical search for criteria for late-onset monogenic LBMD that are of use in a clinical real-life setting. A comparison of individuals with and without DCV revealed significantly more peripheral fractures (5.7±10.8 vs. 1.8±3; p<0.001), a higher frequency of a family history for osteoporosis (47.9% vs. 27.2%; p<0.001), and a higher body mass index (BMI, 24.8±4.9 vs. 23.6±4.2 kg/m^2; p=0.033) in presence of a DCV (Table 1). However, no significant differences regarding height and weight were detected between groups (171.8±10.7 cm vs. 169.6±12.0 cm, p=0.111; 70.01±15.35 kg vs. 71.49±15.88 kg, p=0.461, respectively). Statistical analyses of biochemical parameters revealed slightly increased serum calcium levels in mutation carriers (2.33±0.17 vs. 2.28+0.15 mM; p=0.025)(Supplement Table S5)(24) and significantly reduced maximal grip strength in 82 tested individuals (30.2±10.2 N vs. 36.2±12.5 N; p=0.024)(Supplement Figure S1)(24). No significant differences were apparent for age, sex, vertebral fractures or DXA Z-scores at the lumbar spine and hip (Table 1 and Supplement Figure S1)(24).

We next performed a regression tree analysis to determine the clinical criteria to predict monogenic forms of LBMD. A solid threshold was seen for the first group with more than ten peripheral fractures (p<0.001). 76.9% (10/13) of these participants carried a mutation (Fig. 1A). The majority of these severely affected individuals harbored loss-of-function mutations in *COL1A1*, typical for osteogenesis imperfecta (OI) type 1, but did not show other characteristic signs of this disease such as short stature, dentinogenesis imperfecta and/or blue sclerae. The second group were individuals with ten or less peripheral fractures with a positive family history for LBMD of which 29% (34/117) showed a DCV (p<0.01) while in a third group with a negative family history for LBMD and three to ten fractures DCVs had a frequency of 23.7% (18/76, p=0.006) (Fig. 1A). Interestingly, additional subgroups were defined by the BMI (Fig. 1B). In those with a positive family history mutation frequency was significantly higher when BMI was greater 23.5 kg/m^2 (p=0.025), while the threshold was 26.9 kg/m^2 in those with negative family history (p=0.02). Only 10.6% (20/188) of individuals of the fourth group with two or less peripheral fractures carried a mutation. Thus, the number of peripheral fractures, a positive family history for LBMD, and a higher BMI are highly predictive for monogenic forms of LBMD. Nevertheless, except for a high preponderance of type 1 collagen-associated bone diseases among participants with >10 fractures, it was not possible to clinically distinguish the different genetically defined subforms.

A broad spectrum of genes is involved in monogenic LBMD

Of the 85 DCV identified in 82 participants, 53 (62%) had been described before, and 32 were novel (Supplement Table S3)(24). Seventy-five (88%) were heterozygous, six (7.1%) were compound heterozygous, and another four (4.7%) were hemizygous. Fifty-one (60%) DCV were missense variants or in-frame deletions, eight (9.4%) primarily affected splicing, 25 (29%) were either nonsense or frameshift variants and one variant was a larger deletion. In 23 cases, segregation analysis was performed, showing a *de novo* status in two (8.6%), while 21 (91.4%) were inherited from an affected parent.

Among the thirteen most severely affected participants with more than ten peripheral fractures DCV in the genes encoding type I collagen (*COL1A1* and *COL1A2*) predominated (Table 3, Supplement Table S3)(24). Besides monoallelic DCV in *LRP5* and *WNT1*, the only individual with biallelic variants in *WNT1* was found in this group. In the second group with ten or less peripheral fractures and positive family history (n= 117) DCV in *COL1A1* or *COL1A2* were again most frequent, followed by *LRP5*, *WNT1*, *ALPL*, and three hemi- and heterozygous *PLS3* variants. In addition, DCV in *CASR, ENPP1, EXT2, SCL34A3, and ZNF469* were detected. Also, in the third group with negative family history and three to ten peripheral fractures (n= 76) DCV in *COL1A1* or *COL1A2* were most frequent while *LRP5* mutations were the second most common cause. An *ALPL*-associated case was compound heterozygous. In the fourth group with two or less peripheral fractures and negative family history (n= 188) *LRP5* variants were most frequent, followed by monoallelic *ALPL* variants. The participants with Hajdu-Cheney syndrome caused by a *NOTCH2* variant and with a *LMNA*-associated skeletal and cardiac disorder also belonged to this group. The genes *CASR*, *EXT2*, *LMNA*, and *SLC34A3* are currently not known as LBMD disease genes but are involved in pathways broadly influencing skeletal development and homeostasis.

In addition to typical LBMD genes (*ALPL*, *COL1A1*, *COL1A2*, *LRP5*), VUS were found in several genes associated with connective tissue disorders (*FBN1*, *FBN2*, *TGFBR1*) and Wnt pathway-associated genes (*DVL1*, *LRP6*) (Supplement Table S4)(24). Similar to *LRP5*, *LRP6* has been implicated in BMD regulation (27). The only monogenic LBMD solved by additional exome sequencing was a brittle cornea syndrome caused by compound heterozygous DCV in *ZNF469* (28). This gene was not contained in the first gene panel design. The only larger deletion detected in the cohort was (c.1309+198_1548del) in the gene *ALPL* encoding alkaline phosphatase.

To achieve a complementary and unbiased prioritization, variants in low BMD disease genes were automatically classified using the Combined Annotation-Dependent Depletion (CADD) score. CADD is a single score for the prediction of variant pathogenicity integrating conservation and a broad array of functional scores (25). However, in contrast to ACMG criteria for classifying rare variants, it does neither consider phenotypic aspects nor familiar inheritance (22). An enrichment of pathogenic variants with high CADD score in our premature LBMD cohort was computed in comparison to 503 European control datasets derived from the 1000 Genomes Project (1KG EUR; Controls). The enrichment increased with decreasing variant frequency, which is in line with the general notion that variant frequency is inversely correlated to variant impact (Supplement Table S6)(24). After removing the 82 individuals with monogenic LBMD from the analysis only few ultrarare variants with high CADD score remained, which correspond to the 28 VUS, of which 13 are not listed in the gnomAD database. The specificity of the analysis was confirmed by observing no enrichments for variants in genes associated with high BMD disorders (Supplement Table S7)(24). We therefore conclude that our mutation identification was comprehensive.

The role of common variants in the pathogenesis of adult LBMD in individuals without monogenic causes

We wanted to further investigate the genetic contribution to the LBMD pathogenesis in the 312 participants without a detected DCV. We speculated that common variants predisposing for low BMD and/or fracture rate might be enriched in this cohort. For proof of concept, twenty single nucleotide polymorphisms (SNPs) were used to calculate a basic genetic risk score (GRS) and predicted its effect on BMD relative to healthy controls (Supplement Table S2)(24,29,30). While LBMD individuals without a DCV had a significantly lower predicted BMD (0.90±0.46; vs. 1KG EUR p=0.014; vs. high BMD p=0.001), LBMD individuals with a DCV (1.00±0.49) and the control cohort (mean 1.00±0.45) had identical values. The predicted BMD for the high BMD cohort was highest (1.15±0.40) (Fig. 2A). Accordingly, the ROC curves showed a weak distinction from random for the comparison of the GRS of LBMD individuals without DCV vs. the 1KG EUR control cohort and the high BMD cohort, respectively (AUROC; LBMD no DCV vs. 1KG EUR: 0.55, LBMD no DCV vs. high BMD: 0.64) (Fig. 2B). In addition, the *LRP5* coding SNP rs4988321 (c.1999G>A) with a proven BMD-lowering effect was significantly overrepresented in the LBMD no DCV cohort (7.3% vs. 4.1%; p=0.007) (Fig. 2C)(16). These results demonstrate that common variants associated with low BMD contributed to the pathogenesis in LBMD individuals without a DCV, underlining the importance of the genetic background and proving that premature LBMD are a mixture of monogenic and complex diseases.

Discussion

Our prospective multicenter study aimed at establishing the frequency of monogenic low bone mass disorders (LBMD) among participants with a premature low bone mass and bone fragility. In addition, we investigated the cohort for specific clinical criteria that might predict the presence of a monogenic LBMD. Overall, we detected variants causing monogenic bone disease in more than 20% of individuals. In the specific group of participants with 0-10 peripheral fractures and a positive family history disease causing variants were even present in 29%. So far, the genetics of unspecific LBMD and/or bone fragility have been mainly investigated in pediatric cohorts (31,32). In one of the few studies explicitly focusing on a smaller cohort of adult primary osteoporosis, a DCV frequency of 21% was described, which is similar to our cohort (11). However, whereas in our cohort DCV in the genes *COL1A1* and *COL1A2* were more frequent (8.6%) than in *LRP5* and *WNT1* (6.6%), these authors detected most variants in *LRP5* and *WNT1* (16%) and far less in *COL1A2* (3.3%). This possibly mirrors differences in recruitment as well as differences in clinical routine with respect to genetic testing. Our cohort was recruited within a real-life setting by physicians that were no rare disease specialists or human geneticists. The recruitment strategy was explicitly broad, covering the whole spectrum of mild and severe cases not showing the classical syndromic presentation of monogenic LBMD, after exclusion of secondary forms of osteoporosis. We are thus confident that our conclusions are of general relevance for many clinical disciplines confronted with these individuals. Interestingly, also for other musculoskeletal phenotypes screening of heterogeneous cohorts resulted in mutation detection rates around 20% (33,34).

Previous results obtained in smaller cohorts demonstrated the difficulties of predicting monogenic LBMD on clinical grounds (11,13). However, our larger cohort described here allows to determine clinical criteria due to higher statistical power. The evaluation of the clinical data showed that the likelihood of detecting a mutation was interestingly neither influenced by BMD assessed by DXA nor by vertebral fracture frequency. In contrast, our results underline the importance of the clinical feature ‘peripheral fracture’ from a diagnostic point of view as the strongest correlations with mutation frequency were found for this basic parameter and not for radiological or biochemical parameters. The second most important predictive parameter was family history for a LBMD/osteoporosis. This underlines the value of investing a few minutes into a family anamnesis and not only into costly radiological and laboratory investigations. The positive correlation of mutation frequency and BMI is in line with the known correlation of BMI and BMD (35). Hence, low BMD and/or fractures in spite of high normal BMI, which physiologically should entail high normal BMD, is an indicator of rare variants lowering BMD in the absence of secondary causes. The fact that the likelihood of detecting a mutation did not

increase with lower age of the individuals is congruent with other studies describing that most identified genetic factors influencing BMD do not have an age-dependent effect (36). Genes associated with low BMD/osteoporosis obviously blunt the peak bone mass already at younger ages (37). In addition, grip force was found to be significantly reduced in the group of individuals with monogenic LBMD. Although assessed only in a sub-cohort, this finding underlines the close interplay of bone and muscle and the relevance of a neuromuscular assessment in the diagnostic workup of osteoporosis patients (38).

The four main groups defined by regression analysis comprise a wide disease spectrum. Group 1 (>10 peripheral fractures) mostly contains individuals with oligosymptomatic OI type 1 due to type 1 collagen mutations. However, two individuals with heterozygous *LRP5* and *WNT1* mutations are also in this category, underlining the difficulty to distinguish between dominant early-onset (primary) osteoporosis and OI. Likewise, in the less severe group 2 (<10 peripheral fractures and positive family history) more cases were explained by mutations in *LRP5*, *PLS3*, and *WNT1* than in *COL1A1* and *COL1A2*. *ALPL* variants were found in groups 2 to 4, which corroborates the described heterogeneous impact, especially of heterozygous mutations (39). Our comprehensive genotyping approach also revealed mutations in genes beyond the OI/early-onset osteoporosis spectrum, e.g. *SLC34A3* and *ENPP1*. While most of the genes harboring heterozygous mutations are known for dominant disorders, *ENPP1* and *SLC34A3* are associated with recessive disorders (40). However, osteopenia, elevated FGF23 as well as low serum phosphate were found in individuals carrying heterozygous variants in both genes (41). Although we cannot exclude the potential presence of an undetected second variant beyond the coding region of these genes, the positive segregation analysis and the phenotypic differences to the recessive forms of the disorders suggest a mild dominant effect of these mutations. Mutations in *CASR* influence BMD via the dysregulation of serum calcium levels (42), whereas mutations in *EXT2* and *LMNA* are better known for causing exostoses and myopathies, respectively, but do also cause low BMD which might contribute to phenotype of the affected individuals here (43,44). The exostoses were few and not regarded relevant by the participant.

From the ten cases without causative variants in the gene panel analysis that were subsequently analyzed by exome sequencing only one could be solved by detection of biallelic variants in *ZNF469* (28). Closer clinical investigations of this participant revealed indeed a moderate decrease in corneal thickness in addition to the documented blue sclerae and generalized joint hypermobility, thus demonstrating the value of identifying the exact genetic cause. This low rate of additional mutation detection is in line with a previous study in which exome sequencing in osteogenesis imperfecta cases not solved by gene panel sequencing failed to identify

causative variants (45). Overall, our results show that premature LBMD can be considered a genetic disease in a substantial number of participants for which testing of a broad spectrum of genes is warranted.

We next calculated a genetic risk score (GRS) based on 20 SNPs associated with BMD. Whereas individuals with a DCV were not different from the control cohort, those without a DCV showed a higher number of common variants predisposing for low BMD. An AUC of 0.55 in the ROC analysis of our GRS further underlined an involvement of common variants in LBMD and is similar to other GRS with low numbers of SNPs (46). Recent analyses show an AUC of 0.72 if more than 22,000 common SNPs are included (47). The clinical use for such an osteoporosis GRS is the preselection of individuals at high risk for appropriate osteoporosis check-ups and prevention of unnecessary examinations in individuals at low risk (15). However, currently the most comprehensive SNP-based GRS only explains about 25% of the BMD variability. It is likely that its predictive value can be improved by including rare variants; however, their rarity impedes a determination of pathogenic effect sizes by genetic association studies. Therefore, alternative evaluation strategies are warranted. The ACMG criteria for evaluation of rare variants are very useful for monogenic disorders, but the result is a classification, not a score (22). In contrast, the CADD score predicts deleteriousness for all coding and noncoding variants based on evolutionary conservation as well as multiple annotations (25). As a proof of concept, we found in our LBMD cohort an enrichment of variants with high CADD scores in low BMD disease genes but not in high BMD disease genes. While the universal properties of the CADD score are ideal for bridging the worlds of rare and common variants, additional adjustments are required before the CADD score can be integrated into a general GRS for low BMD.

There are several limitations to our study. The cohort consists of individuals from one country with mainly Western European population background, which might not be representative for other populations. Genetic testing was restricted to disease genes with known role in bone and skeletal biology and the genetic risk score comprised only a limited number of SNPs. Moreover, the availability of genetic testing in the health care systems of other countries varies considerably. In addition, segregation analysis was only possible in 23 individuals with DCV and genotype-phenotype analyses were limited by a relatively small sample size. Also participants with vitamin D deficiency were not excluded *per se* as vitamin D deficiency is common in Germany, vitamin D levels were equally distributed between groups and no significant impact on fracture incidence or BMD was seen. Finally, not for all reported fractures radiographs were available and family history of osteoporosis was only assessed by comprehensive anamnesis in most cases.

In summary, a genetic cause for LBMD should be considered after the exclusion of causes for secondary osteoporosis if the onset is premenopausal or below 55 years. Frequent (>2) peripheral fractures, a positive family history for fractures/osteoporosis, and a high normal BMI are further indicators of a genetic type of osteoporosis. Genetic testing should become part of the routine diagnostic workup in this patient cohort. A genetically proven diagnosis can be crucial since i) it ends the resource-consuming search for other causes of the disease, ii) it allows for a more precise prognosis, iii) it facilitates early identification of family members at risk, and iv) it may be the basis for a specific precision therapy in the near future. Clearly, such a tailored therapy depends on the availability of drugs specifically influencing the pathway affected by the rare variants.

(4401 words)

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Author Information FJ, LH, MA, SM, Uko designed the study. ET, FB, FG, GB, JS, LS, RO, TM, TR recruited participants. EV, JS, AD, RO, MS, TS, Uko analyzed data. Ukr, TZ organized labwork. LH, MS, NE, RO, SM, TS, Uko interpreted the data. MS, RO, SM, Uko wrote the manuscript. ET, FB, FG, FJ, JS, LH, LS, RO, SM, TS, TZ, Uko, Ukr critically reviewed the manuscript.

Ethics Declaration The study was approved by the ethics committee (EA04/060/16) of Charité-Universitätsmedizin Berlin.

Data Availability Statement Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

References

1. Sawyer SL, Hartley T, Dyment DA, Beaulieu CL, Schwartzentruber J, Smith A, Bedford HM, Bernard G, Bernier FP, Brais B, Bulman DE, Warman Chardon J, Chitayat D, Deladoëy J, Fernandez BA, Frosk P, Geraghty MT, Gerull B, Gibson W, Gow RM, Graham GE, Green JS, Heon E, Horvath G, Innes AM, Jabado N, Kim RH, Koenekoop RK, Khan A, Lehmann OJ, Mendoza‐Londono R, Michaud JL, Nikkel SM, Penney LS, Polychronakos C, Richer J, Rouleau GA, Samuels ME, Siu VM, Suchowersky O, Tarnopolsky MA, Yoon G, Zahir FR, Majewski J, Boycott KM. Utility of whole‐exome sequencing for those near the end of the diagnostic odyssey: time to address gaps in care. *Clinical Genetics*. 2016;89(3):275-284.
2. Hendrickx G, Boudin E, Van Hul W. A look behind the scenes: the risk and pathogenesis of primary osteoporosis. *Nat Rev Rheumatol*. 2015;11(8):462-474.
3. Costantini A, Makitie O. Value of rare low bone mass diseases for osteoporosis genetics. *Bonekey Rep*. 2016;5:773.
4. Keupp K, Beleggia F, Kayserili H, Barnes AM, Steiner M, Semler O, Fischer B, Yigit G, Janda CY, Becker J, Breer S, Altunoglu U, Grunhagen J, Krawitz P, Hecht J, Schinke T, Makareeva E, Lausch E, Cankaya T, Caparros-Martin JA, Lapunzina P, Temtamy S, Aglan M, Zabel B, Eysel P, Koerber F, Leikin S, Garcia KC, Netzer C, Schonau E, Ruiz-Perez VL, Mundlos S, Amling M, Kornak U, Marini J, Wollnik B. Mutations in WNT1 cause different forms of bone fragility. *Am J Hum Genet*. 2013;92(4):565-574.
5. Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, Lu JT, Pekkinen M, Wessman M, Heino TJ, Nieminen-Pihala V, Aronen M, Laine T, Kroger H, Cole WG, Lehesjoki AE, Nevarez L, Krakow D, Curry CJ, Cohn DH, Gibbs RA, Lee BH, Makitie O. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. *N Engl J Med*. 2013;368(19):1809-1816.
6. Pyott SM, Tran TT, Leistritz DF, Pepin MG, Mendelsohn NJ, Temme RT, Fernandez BA, Elsayed SM, Elsobky E, Verma I, Nair S, Turner EH, Smith JD, Jarvik GP, Byers PH. WNT1 mutations in families affected by moderately severe and progressive recessive osteogenesis imperfecta. *Am J Hum Genet*. 2013;92(4):590-597.
7. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell*. 2001;107(4):513-523.
8. Butscheidt S, Delsmann A, Rolvien T, Barvencik F, Al-Bughaili M, Mundlos S, Schinke T, Amling M, Kornak U, Oheim R. Mutational analysis uncovers monogenic bone disorders in women with pregnancy-associated osteoporosis: three novel mutations in LRP5, COL1A1, and COL1A2. *Osteoporos Int*. 2018;29(7):1643-1651.
9. Luther J, Yorgan TA, Rolvien T, Ulsamer L, Koehne T, Liao N, Keller D, Vollersen N, Teufel S, Neven M, Peters S, Schweizer M, Trumpp A, Rosigkeit S, Bockamp E, Mundlos S, Kornak U, Oheim R, Amling M, Schinke T, David JP. Wnt1 is an Lrp5-independent bone-anabolic Wnt ligand. *Sci Transl Med*. 2018;10(466):eaau7137.
10. Sturznickel J, Rolvien T, Delsmann A, Butscheidt S, Barvencik F, Mundlos S, Schinke T, Kornak U, Amling M, Oheim R. Clinical Phenotype and Relevance of LRP5 and LRP6 Variants in Patients With Early-Onset Osteoporosis (EOOP). *J Bone Miner Res*. 2021;36(2):271-282.
11. Collet C, Ostertag A, Ricquebourg M, Delecourt M, Tueur G, Isidor B, Guillot P, Schaefer E, Javier RM, Funck-Brentano T, Orcel P, Laplanche JL, Cohen-Solal M. Primary Osteoporosis in Young Adults: Genetic Basis and Identification of Novel Variants in Causal Genes. *JBMR Plus*. 2018;2(1):12-21.
12. Makitie RE, Haanpaa M, Valta H, Pekkinen M, Laine CM, Lehesjoki AE, Schalin-Jantti C, Makitie O. Skeletal Characteristics of WNT1 Osteoporosis in Children and Young Adults. *J Bone Miner Res*. 2016;31(9):1734-1742.
13. Rolvien T, Sturznickel J, Schmidt FN, Butscheidt S, Schmidt T, Busse B, Mundlos S, Schinke T, Kornak U, Amling M, Oheim R. Comparison of Bone Microarchitecture Between Adult Osteogenesis Imperfecta and Early-Onset Osteoporosis. *Calcif Tissue Int*. 2018;103(5):512-521.
14. Morris JA, Kemp JP, Youlten SE, Laurent L, Logan JG, Chai RC, Vulpescu NA, Forgetta V, Kleinman A, Mohanty ST, Sergio CM, Quinn J, Nguyen-Yamamoto L, Luco AL, Vijay J, Simon MM, Pramatarova A, Medina-Gomez C, Trajanoska K, Ghirardello EJ, Butterfield NC, Curry KF, Leitch VD, Sparkes PC, Adoum AT, Mannan NS, Komla-Ebri DSK, Pollard AS, Dewhurst HF, Hassall TAD, Beltejar MG, andMe Research T, Adams DJ, Vaillancourt SM, Kaptoge S, Baldock P, Cooper C, Reeve J, Ntzani EE, Evangelou E, Ohlsson C, Karasik D, Rivadeneira F, Kiel DP, Tobias JH, Gregson CL, Harvey NC, Grundberg E, Goltzman D, Adams DJ,

Lelliott CJ, Hinds DA, Ackert-Bicknell CL, Hsu YH, Maurano MT, Croucher PI, Williams GR, Bassett JHD, Evans DM, Richards JB. An atlas of genetic influences on osteoporosis in humans and mice. *Nat Genet*. 2018;51(2):258-266.
15. Forgetta V, Keller-Baruch J, Forest M, Durand A, Bhatnagar S, Kemp JP, Nethander M, Evans D, Morris JA, Kiel DP, Rivadeneira F, Johansson H, Harvey NC, Mellstrom D, Karlsson M, Cooper C, Evans DM, Clarke R, Kanis JA, Orwoll E, McCloskey EV, Ohlsson C, Pineau J, Leslie WD, Greenwood CMT, Richards JB. Development of a polygenic risk score to improve screening for fracture risk: A genetic risk prediction study. *PLoS Med*. 2020;17(7):e1003152.
16. van Meurs JB, Trikalinos TA, Ralston SH, Balcells S, Brandi ML, Brixen K, Kiel DP, Langdahl BL, Lips P, Ljunggren O, Lorenc R, Obermayer-Pietsch B, Ohlsson C, Pettersson U, Reid DM, Rousseau F, Scollen S, Van Hul W, Agueda L, Akesson K, Benevolenskaya LI, Ferrari SL, Hallmans G, Hofman A, Husted LB, Kruk M, Kaptoge S, Karasik D, Karlsson MK, Lorentzon M, Masi L, McGuigan FE, Mellstrom D, Mosekilde L, Nogues X, Pols HA, Reeve J, Renner W, Rivadeneira F, van Schoor NM, Weber K, Ioannidis JP, Uitterlinden AG, Study G. Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. *JAMA*. 2008;299(11):1277-1290.
17. Torkamani A, Wineinger NE, Topol EJ. The personal and clinical utility of polygenic risk scores. *Nat Rev Genet*. 2018;19(9):581-590.
18. Lu T, Zhou S, Wu H, Forgetta V, Greenwood CMT, Richards JB. Individuals with common diseases but with a low polygenic risk score could be prioritized for rare variant screening. *Genetics in medicine : official journal of the American College of Medical Genetics*. 2021;23(3):508-515.
19. Hombach D, Schuelke M, Knierim E, Ehmke N, Schwarz JM, Fischer-Zirnsak B, Seelow D. MutationDistiller: user-driven identification of pathogenic DNA variants. *Nucleic acids research*. 2019;47(W1):W114-W120.
20. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nature methods*. 2014;11(4):361-362.
21. Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Albarca Aguilera M, Meyer R, Massouras A. VarSome: the human genomic variant search engine. *Bioinformatics*. 2019;35(11):1978-1980.
22. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, Committee ALQA. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in medicine : official journal of the American College of Medical Genetics*. 2015;17(5):405-424.
23. Genomes Project C, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR. A global reference for human genetic variation. *Nature*. 2015;526(7571):68-74.
24. Oheim R, Tsourdi E, Seefried L, Beller G, Schubach M, Vettorazzi E, Stürznickel J, Rolvien T, Ehmke N, Delsmann A, Genest F, Krüger U, Zemojtel T, Barvencik F, Schinke T, Jakob F, Hofbauer LC, Mundlos S, Kornak U. Supplement From: Genetic Diagnostics in Routine Osteological Assessment of Adult Low Bone Mass Disorders. *Figshare*. Posted February 05, 2022. doi:10.6084/m6089.figshare.19127825.
25. Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic acids research*. 2019;47(D1):D886-D894.
26. Buniello A, MacArthur JAL, Cerezo M, Harris LW, Hayhurst J, Malangone C, McMahon A, Morales J, Mountjoy E, Sollis E, Suveges D, Vrousgou O, Whetzel PL, Amode R, Guillen JA, Riat HS, Trevanion SJ, Hall P, Junkins H, Flicek P, Burdett T, Hindorff LA, Cunningham F, Parkinson H. The NHGRI-EBI GWAS Catalog of published genome-wide association studies, targeted arrays and summary statistics 2019. *Nucleic acids research*. 2019;47(D1):D1005-D1012.
27. Mani A, Radhakrishnan J, Wang H, Mani A, Mani MA, Nelson-Williams C, Carew KS, Mane S, Najmabadi H, Wu D, Lifton RP. LRP6 mutation in a family with early coronary disease and metabolic risk factors. *Science*. 2007;315(5816):1278-1282.
28. Rolvien T, Kornak U, Linke SJ, Amling M, Oheim R. Whole-Exome Sequencing Identifies Novel Compound Heterozygous ZNF469 Mutations in Two Siblings with Mild Brittle Cornea Syndrome. *Calcif Tissue Int*. 2020;107(3):294-299.
29. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, Oei L, Albagha OM, Amin N, Kemp JP, Koller DL, Li G, Liu CT, Minster RL, Moayyeri A, Vandenput L, Willner D, Xiao SM, Yerges-Armstrong LM, Zheng HF, Alonso N, Eriksson J, Kammerer CM, Kaptoge SK, Leo PJ, Thorleifsson G, Wilson SG, Wilson JF, Aalto V, Alen M, Aragaki AK, Aspelund T, Center JR, Dailiana Z, Duggan DJ, Garcia M, Garcia-Giralt N, Giroux S, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Khusainova R, Kim GS, Kooperberg C, Koromila T, Kruk M, Laaksonen M, Lacroix AZ, Lee SH, Leung PC, Lewis JR, Masi L, Mencej-Bedrac S, Nguyen TV, Nogues X, Patel MS, Prezelj J, Rose LM, Scollen S, Siggeirsdottir K, Smith AV, Svensson O, Trompet S, Trummer O, van Schoor NM, Woo J, Zhu K, Balcells S, Brandi ML, Buckley

BM, Cheng S, Christiansen C, Cooper C, Dedoussis G, Ford I, Frost M, Goltzman D, Gonzalez-Macias J, Kahonen M, Karlsson M, Khusnutdinova E, Koh JM, Kollia P, Langdahl BL, Leslie WD, Lips P, Ljunggren O, Lorenc RS, Marc J, Mellstrom D, Obermayer-Pietsch B, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Ridker PM, Rousseau F, Slagboom PE, Tang NL, Urreizti R, Van Hul W, Viikari J, Zarrabeitia MT, Aulchenko YS, Castano-Betancourt M, Grundberg E, Herrera L, Ingvarsson T, Johannsdottir H, Kwan T, Li R, Luben R, Medina-Gomez C, Palsson ST, Reppe S, Rotter JI, Sigurdsson G, van Meurs JB, Verlaan D, Williams FM, Wood AR, Zhou Y, Gautvik KM, Pastinen T, Raychaudhuri S, Cauley JA, Chasman DI, Clark GR, Cummings SR, Danoy P, Dennison EM, Eastell R, Eisman JA, Gudnason V, Hofman A, Jackson RD, Jones G, Jukema JW, Khaw KT, Lehtimaki T, Liu Y, Lorentzon M, McCloskey E, Mitchell BD, Nandakumar K, Nicholson GC, Oostra BA, Peacock M, Pols HA, Prince RL, Raitakari O, Reid IR, Robbins J, Sambrook PN, Sham PC, Shuldiner AR, Tylavsky FA, van Duijn CM, Wareham NJ, Cupples LA, Econs MJ, Evans DM, Harris TB, Kung AW, Psaty BM, Reeve J, Spector TD, Streeten EA, Zillikens MC, Thorsteinsdottir U, Ohlsson C, Karasik D, Richards JB, Brown MA, Stefansson K, Uitterlinden AG, Ralston SH, Ioannidis JP, Kiel DP, Rivadeneira F. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet*. 2012;44(5):491-501.

30. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, Andrew T, Falchi M, Gwilliam R, Ahmadi KR, Valdes AM, Arp P, Whittaker P, Verlaan DJ, Jhamai M, Kumanduri V, Moorhouse M, van Meurs JB, Hofman A, Pols HAP, Hart D, Zhai G, Kato BS, Mullin BH, Zhang F, Deloukas P, Uitterlinden AG, Spector TD. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. *The Lancet*. 2008;371(9623):1505-1512.

31. Laine CM, Koltin D, Susic M, Varley TL, Daneman A, Moineddin R, Cole WG, Makitie O, Sochett E. Primary osteoporosis without features of OI in children and adolescents: clinical and genetic characteristics. *Am J Med Genet A*. 2012;158A(6):1252-1261.

32. Bardai G, Ward LM, Trejo P, Moffatt P, Glorieux FH, Rauch F. Molecular diagnosis in children with fractures but no extraskeletal signs of osteogenesis imperfecta. *Osteoporos Int*. 2017;28(7):2095-2101.

33. Sentchordi-Montane L, Benito-Sanz S, Aza-Carmona M, Diaz-Gonzalez F, Modamio-Hoybjor S, de la Torre C, Nevado J, Ruiz-Ocana P, Bezanilla-Lopez C, Prieto P, Bahillo-Curieses P, Carcavilla A, Mulero-Collantes I, Barreda-Bonis AC, Cruz-Rojo J, Ramirez-Fernandez J, Bermudez de la Vega JA, Travessa AM, Gonzalez de Buitrago Amigo J, Del Pozo A, Vallespin E, Solis M, Goetz C, Campos-Barros A, Santos-Simarro F, Gonzalez-Casado I, Ros-Perez P, Parron-Pajares M, Heath KE. High prevalence of variants in skeletal dysplasia associated genes in individuals with short stature and minor skeletal anomalies. *Eur J Endocrinol*. 2021.

34. Renner S, Schuler H, Alawi M, Kolbe V, Rybczynski M, Woitschach R, Sheikhzadeh S, Stark VC, Olfe J, Roser E, Seggewies FS, Mahlmann A, Hempel M, Hartmann MJ, Hillebrand M, Wieczorek D, Volk AE, Kloth K, Koch-Hogrebe M, Abou Jamra R, Mitter D, Altmuller J, Wey-Fabrizius A, Petersen C, Rau I, Borck G, Kubisch C, Mir TS, von Kodolitsch Y, Kutsche K, Rosenberger G. Next-generation sequencing of 32 genes associated with hereditary aortopathies and related disorders of connective tissue in a cohort of 199 patients. *Genetics in medicine : official journal of the American College of Medical Genetics*. 2019;21(8):1832-1841.

35. Ravn P, Cizza G, Bjarnason NH, Thompson D, Daley M, Wasnich RD, McClung M, Hosking D, Yates AJ, Christiansen C. Low body mass index is an important risk factor for low bone mass and increased bone loss in early postmenopausal women. Early Postmenopausal Intervention Cohort (EPIC) study group. *J Bone Miner Res*. 1999;14(9):1622-1627.

36. Medina-Gomez C, Kemp JP, Trajanoska K, Luan J, Chesi A, Ahluwalia TS, Mook-Kanamori DO, Ham A, Hartwig FP, Evans DS, Joro R, Nedeljkovic I, Zheng HF, Zhu K, Atalay M, Liu CT, Nethander M, Broer L, Porleifsson G, Mullin BH, Handelman SK, Nalls MA, Jessen LE, Heppe DHM, Richards JB, Wang C, Chawes B, Schraut KE, Amin N, Wareham N, Karasik D, Van der Velde N, Ikram MA, Zemel BS, Zhou Y, Carlsson CJ, Liu Y, McGuigan FE, Boer CG, Bonnelykke K, Ralston SH, Robbins JA, Walsh JP, Zillikens MC, Langenberg C, Li-Gao R, Williams FMK, Harris TB, Akesson K, Jackson RD, Sigurdsson G, den Heijer M, van der Eerden BCJ, van de Peppel J, Spector TD, Pennell C, Horta BL, Felix JF, Zhao JH, Wilson SG, de Mutsert R, Bisgaard H, Styrkarsdottir U, Jaddoe VW, Orwoll E, Lakka TA, Scott R, Grant SFA, Lorentzon M, van Duijn CM, Wilson JF, Stefansson K, Psaty BM, Kiel DP, Ohlsson C, Ntzani E, van Wijnen AJ, Forgetta V, Ghanbari M, Logan JG, Williams GR, Bassett JHD, Croucher PI, Evangelou E, Uitterlinden AG, Ackert-Bicknell CL, Tobias JH, Evans DM, Rivadeneira F. Life-Course Genome-wide Association Study Meta-analysis of Total Body BMD and Assessment of Age-Specific Effects. *Am J Hum Genet*. 2018;102(1):88-102.

37. Stagi S, Cavalli L, Seminara S, de Martino M, Brandi ML. The ever-expanding conundrum of primary osteoporosis: aetiopathogenesis, diagnosis, and treatment. *Ital J Pediatr*. 2014;40:55.

38. Rupp T, Butscheidt S, Jahn K, Simon MJ, Mussawy H, Oheim R, Barvencik F, Amling M, Rolvien T. Low physical performance determined by chair rising test muscle mechanography is associated with prevalent fragility fractures. *Arch Osteoporos*. 2018;13(1):71.

39. Alonso N, Larraz-Prieto B, Berg K, Lambert Z, Redmond P, Harris SE, Deary IJ, Pugh C, Prendergast J, Ralston SH. Loss-of-Function Mutations in the ALPL Gene Presenting with Adult Onset Osteoporosis and Low Serum Concentrations of Total Alkaline Phosphatase. *J Bone Miner Res*. 2020;35(4):657-661.
40. Lorenz-Depiereux B, Benet-Pages A, Eckstein G, Tenenbaum-Rakover Y, Wagenstaller J, Tiosano D, Gershoni-Baruch R, Albers N, Lichtner P, Schnabel D, Hochberg Z, Strom TM. Hereditary hypophosphatemic rickets with hypercalciuria is caused by mutations in the sodium-phosphate cotransporter gene SLC34A3. *Am J Hum Genet*. 2006;78(2):193-201.
41. Oheim R, Zimmerman K, Maulding ND, Sturznickel J, von Kroge S, Kavanagh D, Stabach PR, Kornak U, Tommasini SM, Horowitz MC, Amling M, Thompson D, Schinke T, Busse B, Carpenter TO, Braddock DT. Human Heterozygous ENPP1 Deficiency Is Associated With Early Onset Osteoporosis, a Phenotype Recapitulated in a Mouse Model of Enpp1 Deficiency. *J Bone Miner Res*. 2020;35(3):528-539.
42. Mouly C, Vargas-Poussou R, Lienhardt A, Silve C, Hureaux M, Magdelaine C, Buffet A, Grunenwald S, Kuhn JM, Brue T, Reznik Y, Tabarin A, Martin-Coignard D, Haymann JP, Tack I, Bennet A, Caron P, Linglart A, Vezzosi D, Reference Centre for Rare Diseases of Calcium PM. Clinical characteristics of familial hypocalciuric hypercalcaemia type 1: A multicentre study of 77 adult patients. *Clin Endocrinol (Oxf)*. 2020;93(3):248-260.
43. Rankin J, Ellard S. The laminopathies: a clinical review. *Clin Genet*. 2006;70(4):261-274.
44. Matsumoto K, Ogawa H, Nozawa S, Akiyama H. An analysis of osteoporosis in patients with hereditary multiple exostoses. *Osteoporos Int*. 2020.
45. Mrosk J, Bhavani GS, Shah H, Hecht J, Kruger U, Shukla A, Kornak U, Girisha KM. Diagnostic strategies and genotype-phenotype correlation in a large Indian cohort of osteogenesis imperfecta. *Bone*. 2018;110:368-377.
46. Eriksson J, Evans DS, Nielson CM, Shen J, Srikanth P, Hochberg M, McWeeney S, Cawthon PM, Wilmot B, Zmuda J, Tranah G, Mirel DB, Challa S, Mooney M, Crenshaw A, Karlsson M, Mellstrom D, Vandenput L, Orwoll E, Ohlsson C. Limited clinical utility of a genetic risk score for the prediction of fracture risk in elderly subjects. *J Bone Miner Res*. 2015;30(1):184-194.
47. Kim SK. Identification of 613 new loci associated with heel bone mineral density and a polygenic risk score for bone mineral density, osteoporosis and fracture. *PLoS One*. 2018;13(7):e0200785.

Figure Legends

Figure 1. Statistical analysis of clinical thresholds predicting monogenic forms of adult low bone mass disorders (LBMD). Regression tree analysis of the performed classification showing clinical thresholds determining disease-causing variant (DCV) frequency, starting from the entire cohort of n=394. Clinical criteria and p-values are shown in circles, boxes show percentages and total numbers of LBMD individuals with a DCV falling under the respective criteria. Thresholds are peripheral fractures >10, family history for fractures/osteoporosis, peripheral fractures >2. Further subdivision followed according to BMI.

Figure 2. Role of common BMD-associated and rare deleterious variants in LBMD individuals without monogenic disease-causing variant. (A) Comparison of BMD predicted by a genetic risk score (GRS) relative to healthy controls between LBMD without DCV (LBMD noDCV; 312), the LBMD with DCV (LBMD DCV; n=82), the 1KG EUR control cohort (1KG EUR; n=503), and individuals with high BMD (high BMD; n=56). (B) Receiver operating characteristic (ROC) curves of genetic risk scores (GRS) of common variants associated with BMD. The GRS of individuals with LBMD without DCV (LBMD noDCV) was compared to 1KG EUR (red) and high BMD (blue). AUC= area under the curve. An AUC of 0.5 (dashed line) shows a random partitioning. Full partitioning using GRS would be achieved at an AUC of 1.0. (C) The frequency of one of the strongest BMD-influencing common variants, SNP rs4988321, in *LRP5* is significantly increased in LBMD noDCV compared to controls (*p<0.05, **p<0.01).

Table 1: Baseline characteristics of the low bone mass disorder (LBMD) study population subdivided according to disease causing variant (DCV) status.

	No DCV (n=312)	DCV (n=82)	Total (n=394)	p value
Age* [years]				0.739
mean (SD)	48.2 (11.4)	47.7 (13.2)	48.1 (11.8)	
Sex				0.574
male	168 (53.8%)	47 (57.3%)	215 (54.6%)	
female	144 (46.2%)	35 (42.7%)	179 (45.4%)	
Family history				**<0.001**
Positive	85 (27.2%)	39 (47.6%)	124 (31.5%)	
Vertebral fractures [n]				0.870
mean (SD)	1.3 (2.4)	1.3 (2.6)	1.3 (2.5)	
Peripheral fractures [n]				**<0.001**
mean (SD)	1.8 (3.0)	5.7 (10.8)	2.6 (5.8)	
BMI [kg/m^2]				**0.033**
n	306	79	385	
mean (SD)	23.6 (4.2)	24.8 (4.9)	23.9 (4.3)	
Weight (kg (SD))	70.01 (15.35)	71.49 (15.88)	70.34 (15.46)	0.461
Height (m (SD))	171.8 (10.7)	169.6 (12.0)	171.3 (11.0)	0.111

Z-score (Hip)			0.369
n	304	78	382
mean (SD)	-2.3 (1.0)	-2.4 (1.1)	-2.3 (1.0)
Z-score group (Hip)			0.460
(< -2.5)	128 (42.1)	34 (43.6%)	162 (42.4%)
(-2.5 – -1.0)	152 (50.0%)	41 (52.6%)	193 (50.5%)
(> -1)	24 (7.9%)	3 (3.8%)	27 (7.1%)
Z-score (Spine)			0.842
n	301	81	382
mean (SD)	-2.9 (1.1)	-2.9 (1.4)	-2.9 (1.1)
Z-score group (Spine)			0.418
(< -2.5)	206 (68.4%)	59 (72.8%)	265 (69.4%)
(-2.5 – -1.0)	79 (26.2%)	16 (19.8%)	95 (24.9%)
(> -1)	16 (5.3%)	6 (7.4%)	22 (5.8%)

DCV - disease causing variant (ACMG class IV and V), n - number, SD - standard deviation, BMI - body mass index, *= age at analysis

Table 2: Frequencies and classification of rare variants and affected genes identified in low bone mass disorder (LBMD) study population.

Pathway	Gene name	LBMD (n=394)	
		DCV	VUS
ECM	*BMP1*		1
	COL1A1	22	4
	COL1A2	12	4
	EXT2[#]	1	
	PLOD1		1
mineralization	*ALPL*	8	
	ENPP1	2	2
	CASR[#]	1	1
	SLC34A1		2
	SLC34A3[#]	3	
Wnt	*DVL1*[#]		1
	LRP5	17	1
	LRP6		3
	WNT1	9	
TGF-β	*FBN1*		3
	FBN2		3
	TGFBR2		1
other	*LMNA*[#]	1	1
	NOTCH2	1	
	PLS3	4	
	ZNF469	1	

total		**82** **20.8%**	**28** **7.0%**

ECM – extracellular matrix, LBMD - low bone mass disorder, DCV - disease causing variant (ACMG class IV and V), n – number, Wnt – genes involved in Wnt signaling, TGF-β - genes involved in TGF-β signaling. # - no currently known LBMD gene, but involvement in skeletal development / homeostasis.

Table 3: Frequencies of disease-causing variants in the clinically defined subgroups.

Gene name	Group 1 >10 PF	Group 2 0-10 PF FH+	Group 3 3-10 PF FH-	Group 4 0-2 PF FH-
ALPL		2.6%	1.3%	2.1%
CASR		0.9%		
COL1A1	38.5%	6.0%	11.8%	1.1%
COL1A2	15.4%	4.3%	3.9%	1.1%
ENPP1		0.9%		0.5%
EXT2		0.9%		
LMNA				0.5%
LRP5	7.7%	6.0%	3.9%	3.2%
NOTCH2				0.5%
PLS3		2.6%	1.3%	
SLC34A3		0.9%		0.5%
WNT1	15.4%	3.4%	1.3%	1.1%
ZNF469		0.9%		
total	76.9%	29.1%	23.7%	10.6%

PF = peripheral fractures, FH = family history, + = positive, - = negative

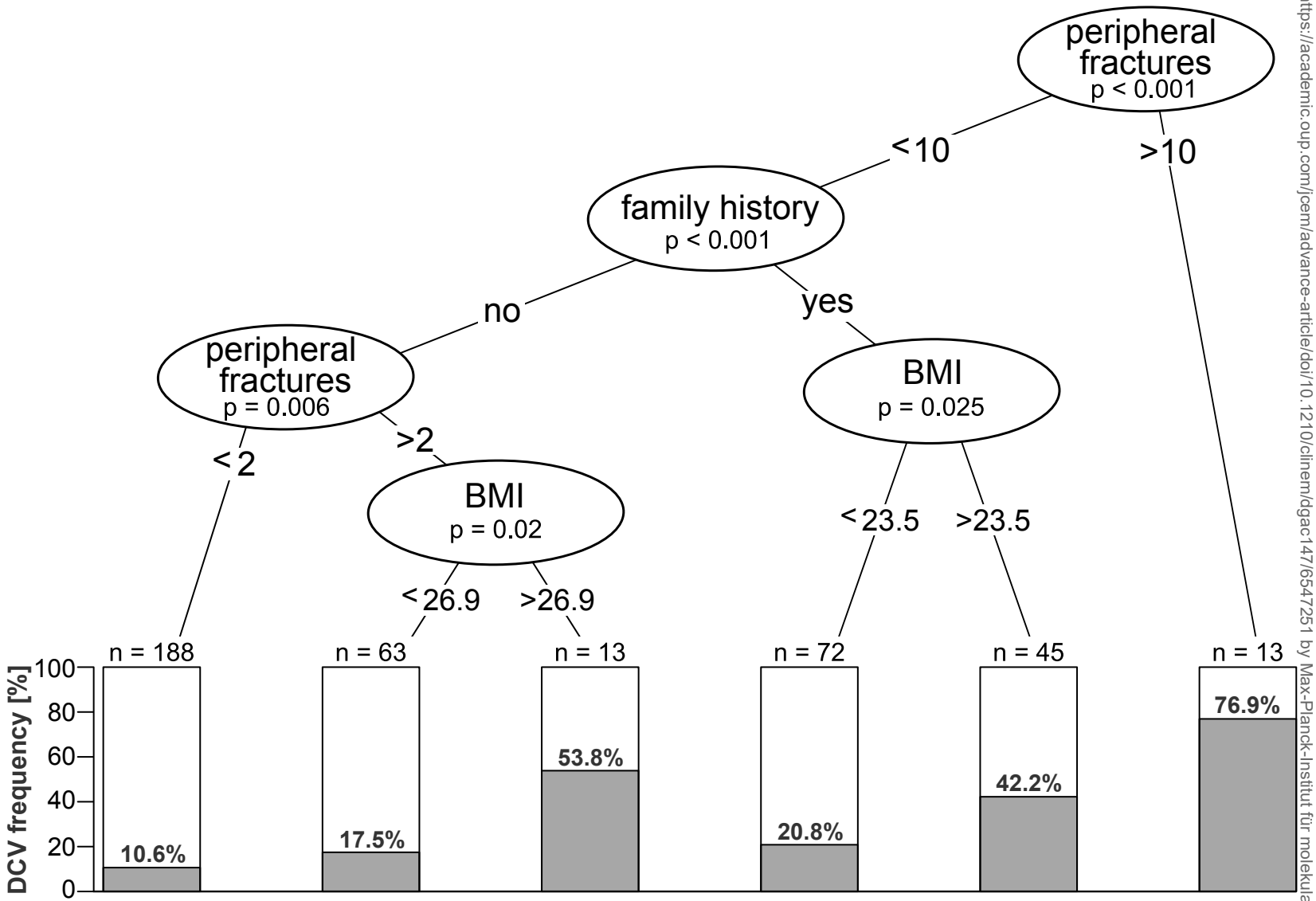

Figure 1

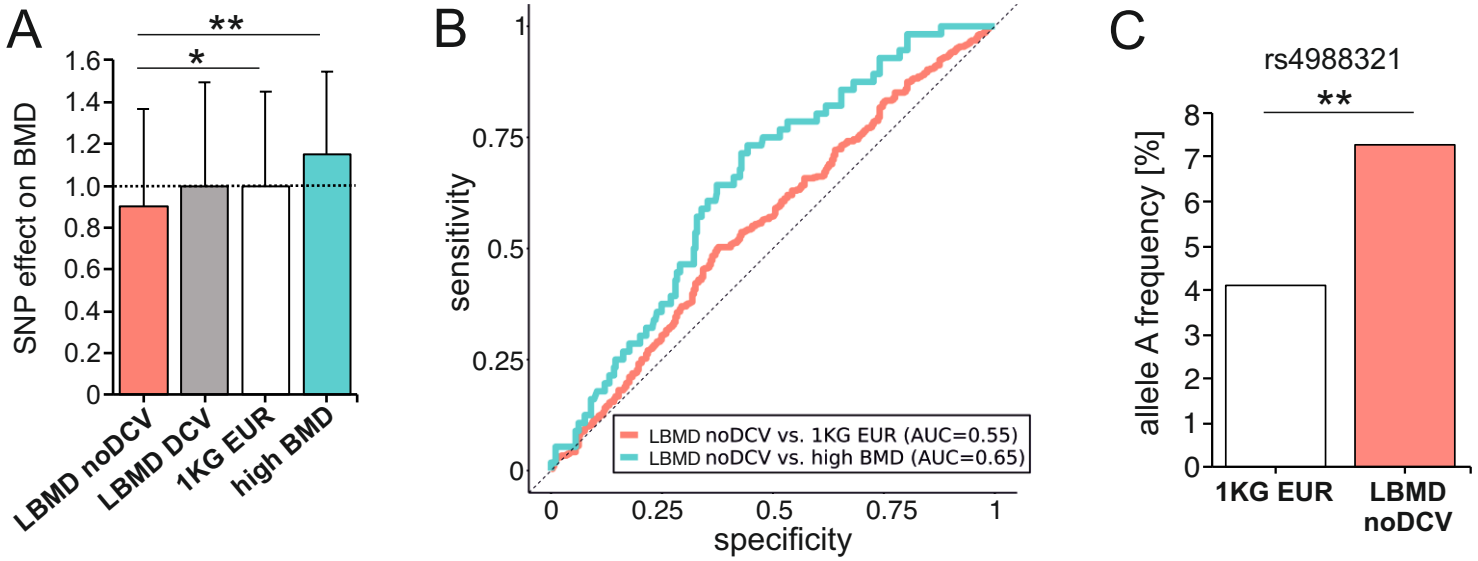

Figure 2