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Novel *PLS3* variants in X-linked osteoporosis: exploring bone material properties

Running Title: Osteoporosis and *PLS3*

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ABSTRACT

Background: Idiopathic Juvenile Osteoporosis (IJO) is a condition that refers to significantly lower than expected bone mass manifesting in childhood, for which there is no identifiable aetiology. IJO classically presents in early pubertal period with multiple fractures including metaphyseal and vertebral crush fractures, and low bone-mass. However, with advances in genetic screening, several causes of IJO have been recently reported in literature.

Methods: Here we describe two patients and provide information on their clinical phenotype, genotype and bone material analysis in one of the patients.

Results: Patient 1: 40-year old adult male diagnosed with IJO in childhood who re-presented with a hip fracture as an adult. Genetic analysis identified a pathogenic *PLS3* hemizygous variant, c.1765del in exon 16. Patient 2: 15-year old boy with multiple vertebral fractures and bone biopsy findings suggestive of IJO who also has a diagnosis of autism spectrum disorder. Genetic analysis identified a maternally inherited *PLS3* pathogenic c.1295T>A variant in exon 12. Analyses of the transiliac bone sample revealed severe reduction of trabecular volume and bone turnover indices and elevated bone matrix mineralisation.

Discussion: We propose that genetic testing for *PLS3* should be undertaken in patients presenting with a current or previous history of IJO as this has implications for genetic counselling and cascade screening of wider family members. The extensive evaluation of the transiliac biopsy sample of Patient 2 revealed a novel bone phenotype.

Conclusion: This report includes a review of IJO and genetic causes of osteoporosis, and suggests that existing cases of IJO should be screened for *PLS3* in addition to other candidate genes known to cause childhood-onset primary osteoporosis. Through analysis of bone material properties in Patient 2, we can conclude that *PLS3* does have a role in bone mineralisation.

INTRODUCTION

Idiopathic juvenile osteoporosis (IJO) is the descriptive term used to define primary osteoporosis of unknown aetiology presenting in childhood. It is usually associated with vertebral and metaphyseal fractures in the years leading to puberty. It has been previously reported to not be associated with heritable genetic variants and usually diagnosed by a process of elimination through exclusion of other causes of decreased bone density [Rauch et al., 2000]. The classic pathophysiology of IJO involves a dysfunction in cancellous bone formation in two ways: fewer remodelling cycles and reduction in quantity of bone formed in each of these cycles. This leads to thin trabeculae by the time of maturity [Rauch et al., 2000].

Hartikka et al., 2005 reported heterozygous variants in *LRP5* in a small number of children with IJO more than a decade ago [Hartikka et al., 2005]. Genetic causes of osteoporosis have now been increasingly identified due to advances in genetic screening, with variants in genes with dominant, recessive and X-linked recessive inheritance patterns [Biha et al., 2016; Fahiminiya et al., 2014; Kampe et al., 2015; Roch-Braz et al., 2016].

PLS3 (OMIM 300131) has 16 exons and is located on Xq23, it encodes a highly conserved plastin 3 that binds actin and shown to have an effect on bone mineral density. Plastin 3 is ubiquitously expressed in solid tissues and involved in the binding and bundling of actin filaments in the cytoskeleton. Variants in *PLS3* lead to significantly reduced bone mineral density [Laarschot et al., 2016; Laine et al., 2015; McGovern et al., 2015]. Although, the exact function of *PLS3* in bone is still unknown, suggested roles of Plastin 3 includes the processes of mechanosensing, converting applied mechanical loading forces into molecular signals that are interpreted by the cells. More recently, it has been suggested to have a role in the mineralisation process. Being located on the X chromosome, PLS3-induced osteoporosis

has a more severe effect on males than females, although heterozygous carrier females are also known have early-onset osteoporosis.

Here, we report two patients initially diagnosed clinically as IJO in whom *PLS3* pathogenic variants were identified. Although, there have been several large pedigrees reported with plastin-induced osteoporosis in the literature, bone material properties have not been extensively studied and results have been varied. In the current report, we demonstrate this variability further and attempt genotype-phenotype correlation in order to untangle the results on detailed bone material analyses.

MATERIALS AND METHODS

Clinical Report

Patient 1: The first patient is a 40-year old Caucasian male who initially presented to a Paediatric Metabolic Bone service outside of our centre at the age of 12 years, with a history of musculoskeletal aches and pains and morning stiffness.

On examination at that time, he was noted to be short in the trunk, and showed difficulty moving from a position of forward flexion to an upright position. X-rays were reported as showing generalised osteopenia with compression of almost all lower thoracic and lumbar vertebrae, consistent with a clinical diagnosis of 'Osteoporosis' or 'Osteogenesis Imperfecta'. He had an elevated alkaline phosphatase but no other abnormality was noted on investigation. He was given a diagnosis of IJO at age 13. He was instructed to wear a supportive back brace but received no specific therapy. It is of note that his mother, then in her 40s, had already been diagnosed with osteoporosis (Figure 1A). He was subsequently lost to follow-up.

Aged 38, this patient was referred to the Adult Metabolic Bone service, following a fracture of his hip, which occurred during an accident at work. Additionally, he gave a history of a traumatic foot fracture. On examination, he had white sclerae, was not facially dysmorphic with no other features suggestive of OI or other metabolic bone disease. During diagnostic work-up, he was noted to have multiple vertebral fractures and recalled the history of IJO. Investigations did not identify any other contributory causes. His bone density was below the expected range for age; lumbar spine T-score was -4.8 (although the presence of vertebral fractures affects the reliability of the result), Z-score was -4.8, total hip T score was -3.5 with a Z-score of -3.6. Spinal imaging demonstrated fractures of all lumbar vertebrae and most of the thoracic vertebrae (with the exception of T3) (Figure 1B and C). He was prescribed Alendronate, supplementary calcium and vitamin D.

This patient was referred to Genetics, aged 39. A detailed family pedigree was taken which confirmed that his mother, now in her 70's, had been diagnosed with osteoporosis at an early age. More recently, after long term bisphosphonate treatment, she had sustained bilateral atypical femoral fractures (two years apart). He has two teenage sons who have had no concerns regarding their bone health. He had targeted gene testing for *LRP5* and *PLS3*.

His family history was then expanded from his mother's osteoporosis to include the osteoporosis of his maternal uncle. His sister had sustained no fractures. Although, family studies have been requested, no samples have been forthcoming.

Patient 2: The second patient is a 15-year old boy referred to the Metabolic Bone service with a history of recurrent fractures. He is the first child of healthy, non-consanguineous Caucasian parents with no significant family history apart from osteoporosis in maternal aunt and maternal grandmother. He has a younger sibling who is fit and well (Figure 2A).

His initial fracture aged 2 was of his left forearm following minor trauma, then aged 6 of his right forearm and wrist. Aged 12 years, when he was first seen in the Metabolic Bone clinic, his back was noted to be straight but his spine imaging showed multiple vertebral crush fractures both in the lumbar and thoracic spine affecting 7 vertebrae altogether (Figure 2B). Both his lumbar spine and total body less head BMD (Bone mineral density) were below the normal range for age with aBMD (arealBMD) Z-scores of -2.7 and -2.6 respectively. Bone biopsy performed prior to starting treatment with bisphosphonates showed features detailed below including reduced osteoblast and osteoclast surfaces, trabecular loss and reduced connectivity, and cortical thinning. He was also noted to have elevated bone specific alkaline phosphatase for age, which subsequently normalised, possibly reflecting recent fractures. He was commenced on treatment with three-monthly Pamidronate infusions at 1mg/kg on three successive days with good initial response.

Eight months after starting treatment, his lateral vertebral analysis showed 6 vertebrae with reduced height; his age-matched lumbar spine aBMD Z-score had risen from -2.7 prior to treatment to -1.4 and his total body less head aBMD Z-score rose from -2.6 to -1.7. Further treatment for 2.5 years with Pamidronate followed, with a switch to Zoledronic acid thereafter. His lumbar spine aBMD continued to increase despite reducing the dose of treatment to 60% of the regular dose. His aBMD rose to +1.3 and his current DXA image shows significant vertebral endplate sclerosis with some improvement in the overall shape and height of the previously crush-fractured vertebrae (Figure 2C).

He was referred to the Genetics clinic in view of his bone fragility and a diagnosis of autism spectrum disorder (ASD). A detailed family pedigree was taken which confirmed that his mother had sustained a fracture of her forearm but there was a more significant fracture history in a maternal aunt who had been diagnosed with osteoporosis in her 30's. He was

enrolled to a research study looking at the association of bone fragility and autism with appropriate consent and trio whole exome sequencing was undertaken.

Bone histomorphometry and quantitative Backscattered Electron Imaging (qBEI):

A transiliac bone biopsy was obtained from Patient 2 before the initiation of bisphosphonate (BP) treatment, following double labeling with tetracycline to allow for dynamic measurement of bone formation. Sample preparation and histomorphometric analyses were performed using standard procedures[10]. Results were compared to reference data of healthy age-matched controls and children with OI type I [Glorieux et al., 2000; Rauch et al., 2000]. Subsequently, the residual block was prepared to assess bone mineralization density distribution (BMDD), reflecting the calcium content of cortical and trabecular bone matrix, by qBEI as described elsewhere [Roschger et al., 2008]. The BMDD parameters values were compared to healthy controls and children with OI type I [Fratzl-Zelman et al., 2009; Fratzl-Zelman et al., 2016; Roschger et al., 2008].

RESULTS

Patient 1: Targeted genomic sequencing of exons 2-16 of *PLS3* detected a hemizygous deletion, c.1765del in exon 16 of *PLS3*. This likely pathogenic variant was predicted to result in a p.Ala589fs change. The predicted result for this variant was a frameshift, leading to the creation of a premature termination codon 21 amino acids downstream. This particular variant had not been reported on the LOVD database entry on *PLS3* but other such frameshift variants in *PLS3* have been reported in association with osteoporosis.

Patient 2: Targeted sequencing for genes associated with OI including autosomal dominant panel (*COL1A1/A2* & *IFITM5*) and *LRP5* testing were negative. Previous genetic testing also included normal 60Kb arrayCGH in view of his diagnosis of ASD.

This patient was then enrolled to a research study studying the association of autism and OI funded by Newlife Foundation. This study had been approved by the South Yorkshire Research Ethics Committee and appropriate institutional boards and the research has been performed in accordance with the 1964 Helsinki Declaration. Trio whole exome sequencing showed he was hemizygous for the c.1295T>A pathogenic variant in exon 12 of *PLS3*. This variant was predicted to result in a p.Leu432* change and although, not previously reported in literature, similar variants in *PLS3* have been reported in association with osteoporosis. Further testing showed that his mother carried the same *PLS3* variant.

The qualitative assessment of the bone biopsy sample obtained from the patient prior initiation of bisphosphonate therapy revealed very small and isolated trabecular features; one very trabecularised cortex and one well delineated cortical plate (see Figure 3A). Under polarized light, cortical and trabecular bone showed an ordered lamellar pattern. In particular, the parallel lamellar organisation of the cortical plates suggested that they were formed by primary bone apposition through processes of bone modeling and modeling drift (Figure 3B). The bone histomorphometric analyses confirmed that the amount of trabecular (bone volume per tissue volume) was severely reduced compared to aged-matched controls and rather in the range of OI patients (See Table 1). However, in contrast to OI bone, all parameters of bone formation and bone resorption were also found to be very low. Compared to healthy control values, osteoid thickness, mineral apposition rate and the mineralising surface were decreased. The percentage of trabecular surfaces covered either by osteoblasts or osteoclasts was reduced by about 80 and 90 percent, respectively (Figure 3C). It should be noted that more osteoclasts and osteoblasts were viewed in the cortical areas (not shown) mirroring an active trabecularisation process.

The qBEI analyses revealed a hypermineralisation of the bone matrix similar as in OI bone. In both, the trabecular and cortical compartment, the BMDD curve was shifted towards

higher mineral content compared to healthy controls (Figure 3D, E). Consistently, the average calcium content of the matrix (CaMean), the most frequently occurring calcium concentration (CaPeak) and the portion of highly mineralized bone (CaHigh) were elevated in both bone compartments, while the percentage of lowly mineralised matrix (CaLow) was markedly reduced (See Table 2).

DISCUSSION

Osteoporosis is a complicated diagnosis to make, due to the range of factors that can contribute to its pathogenesis. It is characterised by reduced bone mass and an increased predisposition for bone fractures. Current definition of childhood-onset primary osteoporosis requires a clinically significant fracture history and BMD Z-score at or below -2.0 [Kämpe et al., 2015]. A clinically significant fracture history can be defined as two or more long bone fractures aged 10 years or below and three or more long bone fractures aged 19 years or below. However, vertebral compression fractures often are alone enough to make this diagnosis even with a normal BMD. Diagnostic workup should include exclusion of other co-morbidities such as celiac disease, inflammatory bowel disease, eating disorders, Vitamin D and calcium deficiency.

Current strategies for genetic testing for childhood-onset osteoporosis include targeted gene panels including genes that cause OI, *LRP5, WNT1* and *PLS3*. However, with costs of genetic testing coming down and more genes being discovered, whole exome/ genome sequencing is likely to replace targeted gene panels as first-line testing in the near future.

PLS3 (OMIM number: 300131) is located on chromosome Xq23. It contains 16 exons and spans 90kb. It encodes plastin 3 protein, which is an actin binding protein found in intestinal microvilli, hair cell stereocilia and fibroblast filopodia. Additionally, it seems to

have a role in development of bone, an influence on growth of axons, and expressed in circulating cells in cases of colorectal cancer [Laarschot et al., 2016; Laine et al., 2015; McGovern et al., 2015].

The bone regulatory actions of *PLS3* are not fully known. It has been demonstrated that overexpression protects against spinal muscular atrophy. It has also been observed in zebrafish that knockout pls3 fish develop craniofacial bone structure malformations, which was reversible using human PLS3 mRNA [McGovern et al., 2015]. Dijk et al., 2013 observed that the chicken homologue for PLS3 (fimbrin) is highly expressed in osteocyte dendrites responsible for mechanosensation [Dijk et al., 2013]. The authors propose loss of mechanosensation as a possible mechanism for the osteoporosis effects.

Whilst the particular variants exhibited by both these patients in this study have not been published elsewhere, other frameshift variants in this gene have been found to cause X-linked osteoporosis. Maternal heterozygotes have been shown to be either unaffected or show early osteoporosis symptoms, as was the case with these patients [Dagleish et al., 2017].

Since its first description of PLS3-osteoporosis in five families with apparent X-linked osteoporosis, there have been other case reports adding to the literature and expanding the phenotype of this condition [Dijk et al., 2013; van de Laarschot et al., 2016; Kannu et al., 2017; Lv et al., 2017; Kämpe et al., 2017]. From the literature so far and corroborated by our patients, the phenotype consists of vertebral compression fractures, peripheral including long bone fractures and low BMD but without features such as blue sclerae, short stature, joint hyperlaxity or facial features typical of 'Classical OI' which is helpful in terms of genotype-phenotype correlation. Therefore, in patients with predominant history of vertebral fractures and/or low BMD without features of OI and family history suggestive of X-linked osteoporosis, PLS3-osteoporosis remains the top differential diagnosis. It is also important to

make this diagnosis as carrier females albeit more mildly affected than males must be screened and kept under appropriate follow-up.

Patient 2 had features of autism spectrum disorder in addition to bone fragility and was recruited to a research study to explore this association further. Exome data has not identified any other variants of interest apart from the *PLS3* variant which is X-linked. So far, *PLS3* patients have been reported to have normal intellectual development. Interestingly, reports of PLS3-osteoporosis patients include those paediatric patients labelled as having 'spastic cerebral palsy', 'waddling gait' and there has been a suggestion that *PLS3* is linked to spinal muscular atrophy (SMA) [Hosseinbarkooie at al., 2017]. It is therefore, important to assess neuromuscular function and CNS assessment in further detail in these patients. Plastin 3 is said to be a protective modifier in SMA and reported to have a role in neuromuscular synapse maintenance which adds further weight to the suggestion that in addition to the role in bone mineralisation, this plastin may also have a role in intellectual development. It remains to be seen whether he has an alternate diagnosis to explain the ASD or the *PLS3* variant is somehow contributing to his diagnosis of ASD. Further trio genome sequencing studies are ongoing to search for an alternate aetiology.

The extensive evaluation of the transiliac biopsy sample of Patient 2 revealed a novel bone phenotype associated with *PLS3* variant. Fahiminiya et al., 2014 first reported pediatric patients with normal bone formation and normal BMDD [Fahiminiya et al., 2014]. In a more recent study from Kämpe et al., 2017, data from a *PLS3* variant carrier was presented that showed increased osteoid formation and concomitant hypomineralization of the bone matrix [Kämpe et al., 2017]. In sharp contrast, Patient 2 of the present study had low bone turnover, low osteoid formation compared to healthy controls and very low values compared to OI as shown on Table 1. Indeed, children with OI tend to have elevated indices of bone formation and resorption that was definitively not observed in the present case [Rauch et al., 2000].

However, this *PLS3* patient shares with OI patients, the characteristic decreased trabecular bone volume and the abnormally high mineral content of the bone matrix as revealed by qBEI (Table 2) [Rauch et al., 2000]. This suggests that the increased matrix mineralisation in our *PLS3* patient (Patient 2) results primarily from a long history of very low bone turnover.

Indeed, the mineralisation of bone tissue increases with time and it takes years for a newly formed bone packet to become fully mineralized. Thus, in a situation where little or no bone is remodeled, one would expect a rising of the mineral content of the bone matrix and high bone matrix mineralisation making the bone tissue stiffer, harder and more brittle [Roschger et al., 2008; Bishop et al., 2016]. It has to be underlined that during skeletal growth, bone turnover is generally increased and the bone matrix tends to be rather lower mineralised than hypermineralised [Fratzl-Zelman et al., 2009]. Interestingly, the highly trabecularised cortical envelopes observed in our patient suggest that bone modeling remains active in the *PLS3* patient. An ongoing cortical bone formation represents possibly a compensatory mechanism to counteract the lack of an adequate amount of trabecular bone. It seems very likely that bone fragility in Patient 2 results from a decrease in bone mass that can only be inadequately compensated by increased primary bone formation and from alteration of bone material properties similar to that seen in children with OI.

Genotype-phenotype correlation in terms of bone material properties is difficult as there is limitation in obtaining a transiliac bone biopsy from all patients due to its invasive nature. Phenotypic variability even with the same family is a well-observed genetics phenomenon and often complex to explain. PLS3-osteoporosis reports so far include large pedigrees with a combination of whole gene deletions, intragenic deletions (likely resulting in haploinsufficiency in females or complete absence of plastin 3 in males), frameshift, splice site and missense variants (likely resulting in truncated or abnormal protein) which makes direct correlation of bone material properties difficult. From the *PLS3* patients in who bone

material analyses has been undertaken so far, we could cautiously conclude that *PLS3* does have a role in bone mineralisation, although the effect of it may be dependent on age and timing of bone biopsy. However, further reports of this nature are required to reproduce the effect of *PLS3* on bone material and elucidate the exact function of *PLS3* in bone.

Lindert et al., 2016 identified variants in *MBTPS2* in two families with several affected males identifying another X-linked gene resulting in heritable bone fragility [Lindert et al., 2016]. The phenotype of these patients was not in keeping with the diagnosis of IJO and was suggestive of a severe OI phenotype. In addition to the implicit benefit of screening for *PLS3*, the literature suggests that it would also be beneficial to screen *LRP5/6* and *WNT1* in patients with a presumed diagnosis of IJO. The proteins encoded by these genes form part of a major bone anabolic pathway; variants in genes for other factors interacting with the pathway including *WNT3a*, *DKK1* and *LRP4* have all also been associated with low bone mass and fracture. Variations in *LRP5* can cause opposing effects on the bone density of the patient; a loss-of-function variant causes osteoporosis-pseudoglioma (OPPG) [Hartikka et al., 2005; Biha et al., 2016; Palsgaard et al., 2016], whereas a gain-of-function variant can cause dramatic increases in bone density [Niziolek et al., 2015]. Confirming the genetic aetiology in IJO/ childhood-onset primary osteoporosis is important due to implications for patients' diagnosis and treatment; for the wider family in terms of cascade screening especially in a X-linked condition, providing accurate information on recurrence risk (as in Patient 1 where there is no risk for his unaffected sons which is reassuring for the family).

In conclusion, IJO/ childhood-onset primary osteoporosis can be a complicated diagnosis, resulting from early onset bone fragility. We recommend that all young male patients with this prospective diagnosis undergo diagnostic analysis of *PLS3*, either as part of targeted gene panel testing for bone fragility or a single gene testing where there is reduced access to genomic sequencing technologies. Given the emerging phenotype of PLS3-

osteoporosis, further case reports of this nature are important to expand on the spectrum of clinical presentation and add to the *PLS3* mutation database.

REFERENCES

Biha N, Ghaber SM, Hacen MM, Collet C. Osteoporosis-Pseudoglioma in a Mauritanian Child due to a Novel Mutation in LRP5. 2016. Case Rep Genet. 2016:9814928.

Bishop N. Bone Material Properties in Osteogenesis Imperfecta. J Bone Miner Res. 2016 Apr;31(4):699-708.

Dalgleish R. 2017. Osteogenesis Imperfecta Variant Database: PLS3. Available from: https://oi.gene.le.ac.uk/home.php?select_db=PLS3 [Accessed 15th December 2017]

Dijk, F.S. van, Zillikens, M.C., Micha, D., Riessland, M., Marcelis, C.L., de Die-Smulders, C.E., Milbradt, J., Franken, A.A., Harsevoort, A.J., Lichtenbelt, K.D., Pruijs, H.E., Rubio-Gozalbo, M.E., Zwertbroek, R., Moutaouakil, Y., Egthuijsen, J., Hammerschmidt, M., Bijman, R., Semeins, C.M., Bakker, A.D., Everts, V., Klein-Nulend, J., Campos-Obando, N., Hofman, A., te Meerman, G.J., Verkerk, A.J., Uitterlinden, A.G., Maugeri, A., Sistermans, E.A., Waisfisz, Q., Meijers-Heijboer, H., Wirth, B., Simon, M.E., and Pals, G., 2013. PLS3 mutations in X-linked osteoporosis with fractures. *N Engl J Med.* Oct 17;369(16):1529-36.

Fahiminiya S, Majewski J, Al-Jallad H, Moffatt P, Mort J, Glorieux FH, Roschger P, Klaushofer K, Rauch F. Osteoporosis caused by mutations in PLS3: clinical and bone tissue characteristics. 2014. J Bone Miner Res. 29(8):1805-14.

Fratzl-Zelman N, Roschger P, Misof BM, Pfeffer S, Glorieux FH, Klaushofer K, Rauch F. 2009. Normative data on mineralization density distribution in iliac bone biopsies of children, adolescents and young adults. Bone. 44(6):1043-8.

Fratzl-Zelman N, Barnes AM, Weis M, Carter E, Hefferan TE, Perino G, Chang W, Smith PA, Roschger P, Klaushofer K, Glorieux FH, Eyre DR, Raggio C, Rauch F, Marini JC. 2016. Non-Lethal Type VIII Osteogenesis Imperfecta Has Elevated Bone Matrix Mineralization. J Clin Endocrinol Metab. 101(9):3516-25.

Glorieux FH, Travers R, Taylor A, Bowen JR, Rauch F, Norman M, Parfitt AM. Normative data for iliac bone histomorphometry in growing children. 2000.Bone.26(2):103-9.

Hartikka H, Mäkitie O, Männikkö M, Doria AS, Daneman A, Cole WG, Ala-Kokko L, Sochett EB. Heterozygous mutations in the LDL receptor-related protein 5 (LRP5) gene are associated with primary osteoporosis in children. 2005. J Bone Miner Res. 20(5):783-9.

Hosseinibarkooie S, Schneider S, Wirth B. Advances in understanding the role of disease-associated proteins in spinal muscular atrophy. Expert Rev Proteomics. 2017. 14(7):581-592

Kämpe AJ, Mäkitie RE, Mäkitie O. New Genetic Forms of Childhood-Onset Primary Osteoporosis. 2015. Horm Res Paediatr. 84(6):361-9.

Kämpe AJ, Costantini A, Mäkitie RE, Jäntti N, Valta H, Mäyränpää M, Kröger, H, Pekkinen M, Taylan F, Jiao H, Mäkitie O. PLS3 sequencing in childhood-onset primary osteoporosis identifies two novel disease-causing variants. Osteoporos Int. 2017. 28(10):3023-3032.

Kämpe AJ, Costantini A, Levy-Shraga Y, Zeitlin L, Roschger P, Taylan F, Lindstrand A, Paschalis EP, Gamsjaeger S, Raas-Rothschild A, Hövel M, Jiao H, Klaushofer K, Grasemann C, Mäkitie O. PLS3 Deletions Lead to Severe Spinal Osteoporosis and Disturbed Bone Matrix Mineralization. J Bone Miner Res. 2017 Aug 4. doi: 10.1002/jbmr.3233. [Epub ahead of print]

Kannu P, Mahjoub A, Babul-Hirji R, Carter MT, Harrington J. PLS3 Mutations in X-Linked Osteoporosis: Clinical and Bone Characteristics of Two Novel Mutations. 2017. Horm Res Paediatr. Jun 12. doi: 10.1159/000477242. [Epub ahead of print]

Laarschot DM, van de, and Zillikens, M.C. Atypical femur fracture in an adolescent boy treated with bisphosphonates for X-linked osteoporosis based on PLS3 mutation. 2016. Bone. 91:148-51.

Laine CM, Wessman M, Toiviainen-Salo S, Kaunisto MA, Mäyränpää MK, Laine T, Pekkinen M, Kröger H, Välimäki VV, Välimäki MJ, Lehesjoki AE, Mäkitie O. A novel splice mutation in PLS3 causes X-linked early onset low-turnover osteoporosis. 2015. J Bone Miner Res. 30(3):510-8.

Lindert U, Cabral WA, Ausavarat S, Tongkobpetch S, Ludin K, Barnes AM, Yeetong P, Weis M, Krabichler B, Srichomthong C, Makareeva EN, Janecke AR, Leikin S, Röthlisberger B, Rohrbach M, Kennerknecht I, Eyre DR, Suphapeetiporn K, Giunta C, Marini JC, Shotelersuk V. MBTPS2 mutations cause defective regulated intramembrane proteolysis in X-linked osteogenesis imperfecta. 2016. Nat Commun. 7:11920.

Lv F, Ma M, Liu W, Xu X, Song Y, Li L, Jiang Y, Wang O, Xia W, Xing X, Qiu Z, Li M. A novel large fragment deletion in PLS3 causes rare X-linked early-onset osteoporosis and response to zoledronic acid. 2017. Osteoporos Int. Jun 16. doi: 10.1007/s00198-017-4094-0. [Epub ahead of print]

McGovern VL, Massoni-Laporte A, Wang X, Le TT, Le HT, Beattie CE, Rich MM, Burghes AH. Plastin 3 Expression Does Not Modify Spinal Muscular Atrophy Severity in the Δ7 SMA Mouse. 2015. PLoS One. 10(7):e0132364.

Niziolek PJ, Bullock W, Warman ML, Robling AG. Missense Mutations in LRP5 Associated with High Bone Mass Protect the Mouse Skeleton from Disuse- and Ovariectomy-Induced Osteopenia. 2015. PLoS One. 10(11):e0140775.

Palsgaard J, Emanuelli B, Winnay JN, Sumara G, Karsenty G, Kahn CR. Cross-talk between insulin and Wnt signaling in preadipocytes. Role of Wnt Co-receptor LDL Receptor-Related Protein-5 (LRP5). J Biol Chem. 2016. 291(32):16878.

Rauch F, Travers R, Norman ME, Taylor A, Parfitt AM, Glorieux FH. Deficient bone formation in idiopathic juvenile osteoporosis: a histomorphometric study of cancellous iliac bone. 2000. J Bone Miner Res. 15(5):957-63.

Rauch F, Travers R, Parfitt AM, Glorieux FH. Static and dynamic bone histomorphometry in children with osteogenesis imperfecta. 2000. Bone. 26(6):581-9.

Roch-Braz MGM and Ferraz-de-Souza B. Genetics of osteoporosis: searching for candidate genes for bone fragility. 2016. Arch. Endocrinol. Metab. 60(4):391-401.

Roschger P, Paschalis EP, Fratzl P, Klaushofer K. Bone mineralization density distribution in health and disease. 2008. Bone. 42(3):456-66.

Roschger P, Fratzl-Zelman N, Misof BM, Glorieux FH, Klaushofer K, Rauch F. Evidence that abnormal high bone mineralization in growing children with osteogenesis imperfecta is not associated with specific collagen mutations. Calcif Tissue Int. 2008 Apr;82(4):263-70

van de Laarschot, DM, Zillikens MC. Atypical femur fracture in an adolescent boy treated with bisphosphonates for X-linked osteoporosis based on PLS3 mutation. 2016. Bone. 91:148-51.

FIGURE LEGENDS

Figure 1A, B and C: Family pedigree of Patient 1; Spinal imaging in Patient 1 at 38-years of age demonstrating vertebral fractures of all lumbar vertebrae and most of the thoracic vertebrae (with the exception of T3) and residual deformities; low BMD on DXA imaging.

Figure 2A, B and C: Family pedigree of Patient 2; Pretreatment lateral DXA image of the spine at 12-years of age showing multiple vertebrae with reduced height and altered shape; Lateral DXA image of the spine at 15-years of age showing endplate sclerosis and minor improvement in the overall size and shape of vertebrae following 3.5 years of bisphosphonate treatment.

Figure 3A-E: Bone histology and BMDD in Patient 2

A: Backscattered electron image from the entire transiliac bone biopsy sample. Note the one highly trabecularised abnormal cortical plate, (Trabecularised Ct) in contrast to the well delineated normal cortex (Ct) on the other side and the isolated and small trabecular features in the cancellous bone compartment.

B: Cortical plate, histological section, Goldner´s stained viewed under polarised light. Note the parallel lamellar arrangement of the collagen fibrils.

C: Trabecular bone, histological section, Goldner´s stained (mineralised bone is green, non-mineralised is osteoid red). Trabeculae appear very thin, isolated and without an osteoid seam.

D and E: BMDD curves obtained from cortical bone (D) and cancellous bone (E): both curves are shifted towards higher values of the mineral content in comparison to the reference cohort of healthy children.

ACKNOLWEDGEMENTS: We would like to thank the families for consenting to publication of their details; Newlife Foundation for Disabled Children (for Patient 2) Grant Reference: SG/14-15/05 for their generous funding to this study.

AUTHOR ROLES: All authors contributed to preparation and critical review of manuscript; MB: Study design; writing up manuscript, recruitment of patients; phenotyping; NFZ, PR, KK: bone biopsy analyses; ROS, MB, NP, NJB, RJ, EM: clinical data; RP, KS: genotyping.

Table 1:

Histomorphometric results in the Patient with *PLS3* variant compared to age-matched control values

Histomorphometric variables	References Healthy children*	P2	Difference vs. healthy controls (%)	References OI type I**
Age (years)	11-13.9	12		7.6 ± 3.8
Structural parameters:				
Bone volume per Tissue volume (%)	24.4 ± 4.3	7.4	-69.7	11.0 ± 5.2
Trabecular thickness (µm)	148 ± 23	75.7	-48.9	105.0 ± 25
Trabecular number (1/mm)	1.66 ± 0.22	0.9	-45.8	1.3 ± 0.39
Cortical Width (mm)	0.90 ± 0.33	0.8#	-11.2	0.52 ± 0.2
Static parameters of bone formation				
Osteoid thickness (µm)	6.7 ± 1.7	2.7	-59.7	5.5 ± 1.7
Osteoid surface per bone surface (%)	22.1 ± 7.8	19.2	-13.1	48 ± 14
Osteoid volume per bone volume (%)	2.12 ± 1.0	1.4	-34.0	5.2 ± 2.6
Osteoblast surface per bone surface (%)	6.7 ± 4.5	1.1	-83.6	19.4 ± 9.5
Dynamic parameters of bone formation				
Mineralizing surface per bone surface (%)	11.07 ± 5.0	6.8	-38.6	48 ± 16
Mineral apposition rate (µm/d)	0.87 ±0.09	0.72	-17.2	0.73 ± 0.18
Adjusted apposition rate (µm/d)	0.46 ± 0.10	0.25	-45.7	0.35 ± 0.14
Bone formation rate per bone surface (µm/y)	37.3 ± 16.7	17.7	-52.6	77 ± 34
Bone formation rate per bone volume (%/y)	49.9 ± 21.4	50.4	+1.0%	116 ± 62
Mineralization lag time (d)	14.5 ± 3.00	10.8	-25.52	16.5 (12.5 -19.8)
Static parameters of bone resorption				
Eroded surface per bone surface (%)	14.9 ± 5.6	0.9	- 94.0	15.6 (13.7 - 21.8)
Osteoclast surface per bone surface (%)	1.14 ± 0.74	0.1	- 91.2	1.37 (1.05 - 1.70)
Number of osteoclasts per bone surface (1/mm)	0.29 ± 0.14	0.05	- 82.8	0.047 ± 0.29

Reference data are given as mean ±SD or median with interquartile range (25%; 75%); * Published values from Glorieux et al., 2000[10] ; ** Published values from Rauch et al., 2000[11] ; # Thickness was only evaluated in the one well-delineated cortical plate

Table 2:

BMDD in the patient with *PLS3* variant (Patient 2) compared to control values

BMDD variables	References values from healthy children and adolescents *	Patient 2	Difference vs. healthy controls (%)	References OI type I **, ***
Cancellous bone				
CaMean (weight % Ca)	20.95 (0.57)	22.91	+ 9.4	22.43 (0.63)
CaPeak (weight % Ca)	21.66 (0.52)	23.92	+10.4	23.39 (0.57)
CaWidth (Δ weight % Ca)	3.47 (3.12; 3.64)	3.81	+9.8	3.08 (0.28)
CaLow (%)	6.14 (4.90; 7.99)	5.72	-6.8	5.94 (2.05)
CaHigh (%)	0.89 (0.43; 1.47)	19.00	21-fold increased	7.54 (5.00; 11.82)
Cortical bone				
CaMean (weight % Ca)	20.45 (19.69; 21.04)	22.52	+10.1	22.51 (0.46)
CaPeak (weight % Ca)	21.14 (20.62; 21.75)	23.31	+10.3	23.29 (0.48)
CaWidth (Δ weight % Ca)	3.81 (3.38; 4.38)	3.73	-2.1	3.28 (0.25)
CaLow (%)	9.06 (6.22; 15.00)	4.33	-52.2	4.40 (0.80)
CaHigh (%)	0.46 (0.28; 1.22)	8.49	18-fold increased	8.60 (4.00)

Reference data are given as mean ±SD or median with interquartile range (25%; 75%);

Definition of BMDD variables from Roschger et al., 2008[12] CaMean: the mean calcium concentration (weighted mean); CaPeak: the most frequently occurring calcium concentration (the peak position of the BMDD) in the sample; CaWidth: the width of the BMDD distribution (full width at half maximum) reflecting the heterogeneity in matrix mineralization; CaLow: the percentage of low mineralized bone area, which is mineralized below 17.68 weight% calcium, normally reflecting bone areas undergoing primary mineralization; CaHigh: the percentage of highly mineralized bone matrix, having the calcium content above 25.30 weight% calcium.

* Published values from Fratzl-Zelman et al., 2009[13]; ** Published values from Roschger et al., 2008[14] (for cancellous OI bone); Fratzl-Zelman et al., 2016[15] (for cortical OI bone).

Subject: **AJMG: MS 18-0025** Revision Required

Dear Dr Bhoj (Elizabeth),

Thank you for considering our manuscript "Novel PLS3 variants causing a phenotype of childhood-onset osteoporosis: exploring bone material properties in this X-linked form of osteoporosis" which has been reviewed by the American Journal of Medical Genetics.

We have addressed the comments raised by the reviewers and hope the revised manuscript meets your approval. We hope you are able to consider our work favourably.

Sincerely,

Meena Balasubramanian

Response to Editor's Comments:

Response to Associate Editor

Thank you very much for submitting your manuscript "Novel PLS3 variants causing a phenotype of childhood-onset osteoporosis: exploring bone material properties in this X-linked form of osteoporosis", we are pleased to report that the reviewers found the information presented to be compelling and of high interest to the readership of AJMG. They have provided some minor suggestions for revision, which we would ask you to carefully consider. We look forward to your resubmission of this very interesting article.

Response: Thank you, I hope the modifications meet your approval.

Response to Reviewer's Comments:

Response to Reviewer: 1

Comments to the Author

This paper is very well presented and provides further evidence that PLS3 accounts for some cases of X-linked osteoporosis. The authors build a compelling argument for seeking a molecular genetic aetiology for Idiopathic Juvenile Osteoporosis (IJO), not only to determine the pathology/treatment for individual patients, but also to advise their families on who else is at risk. The imaging clearly demonstrates the radiological findings in this condition. The histological studies are helpful to describe the impact of PLS3 variants on bone structure. The authors acknowledge that the cellular mechanisms of this are not fully understood at present.

Response: Thank you for the kind feedback.

Suggestions for the manuscript:

Title: This could be made simpler, for example, Novel PLS3 variants in X-linked osteoporosis: exploring bone material properties.

P4L46: Sentence does not flow, suggest minor change to-the suggested role of Plastin 3 includes the process of…

P5L46: Include level of alkaline phosphatase if known.

P6L38-42: The sentence about the results could be deleted as they are included in the next section.

P7L19: Define aBMD.

P8L10-11: Delete results as above.

P8L47: Use term pathogenic variant rather than mutation, to be consistent with title and other sections of the manuscript.

P8L49: Check notation of variant, if frameshift is downstream.

P12L56: In patients with pathogenic PLS3 variants.

P13L16: Shares.

P14L36: X-linked gene rather than pattern of inheritance.

Figure 1C: Data only just visible, but legibility depends on the size of the image in the final version.

Figure 3A: Clarify which are normal and abnormal findings in the legend.

Response: Thank you for these suggestions which we have fully incorporated; apologies, we are unable to obtain the precise ALP values for Patient 1 as this is going back several years now and at a different healthcare provider. We will also submit high resolution images for publication as unable to upload as JPEG.

Response to Reviewer: 2

Comments to the Author

Balasubramanian and co-authors report on two patients with novel PLS3 variants associated with juvenile osteoporosis and describe bone biopsy findings in one of the patients. The manuscript adds to previously reported PLS3 mutations and gives new insights to the underlying bone pathology. The manuscript is very well written but there are some possibilities to further improve the presentation:

Response: Thank you for the kind feedback.

Abstract: Conclusions and the title do not match very well, the biopsy findings are not mentioned in the Abstract Discussion and Conclusions at all.

The Discussion is very long and should be significantly shortened. I would suggest to leave out the paragraphs discussing genotype-phenotype correlations on bone tissue level (page 14, 1st paragraph) and the following paragraph on MBTPS2 and on other genetic forms (until page 15 line 5) which seem unnecessary here.

Response: Thank you for these suggestions which we have fully incorporated; we have also shortened the discussion section but kept the salient points.

I hope you are able to consider our work favourably.

Yours sincerely,

Meena Balasubramanian

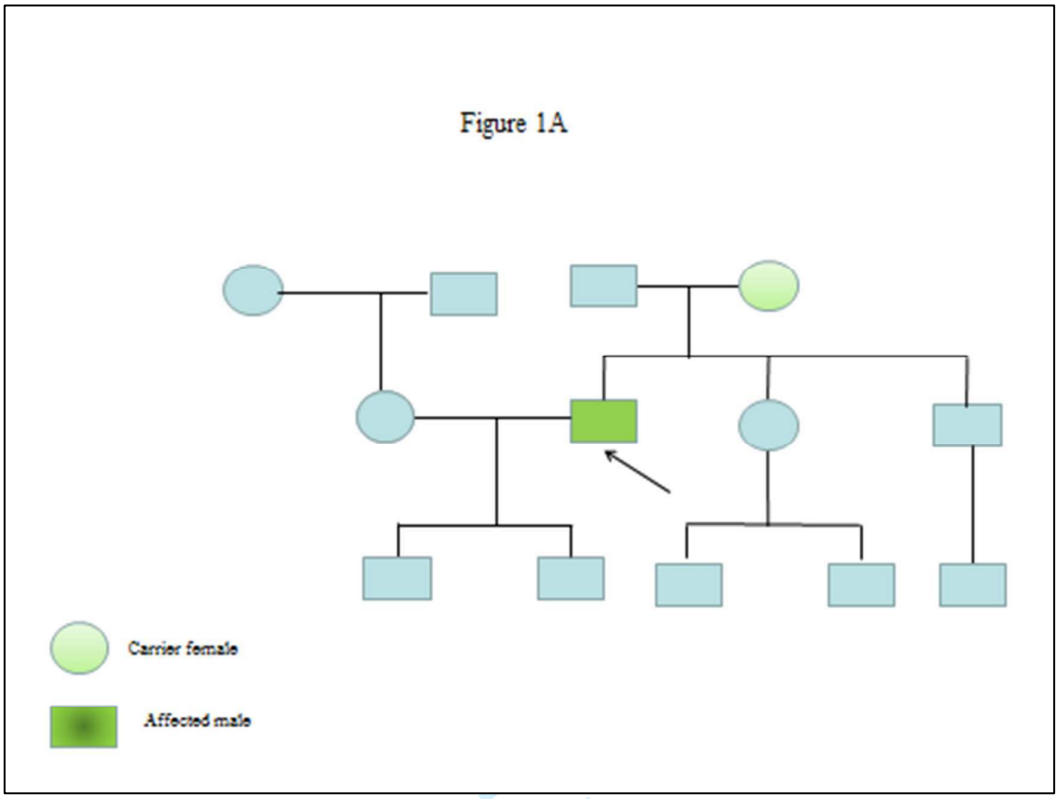


Figure 1B:

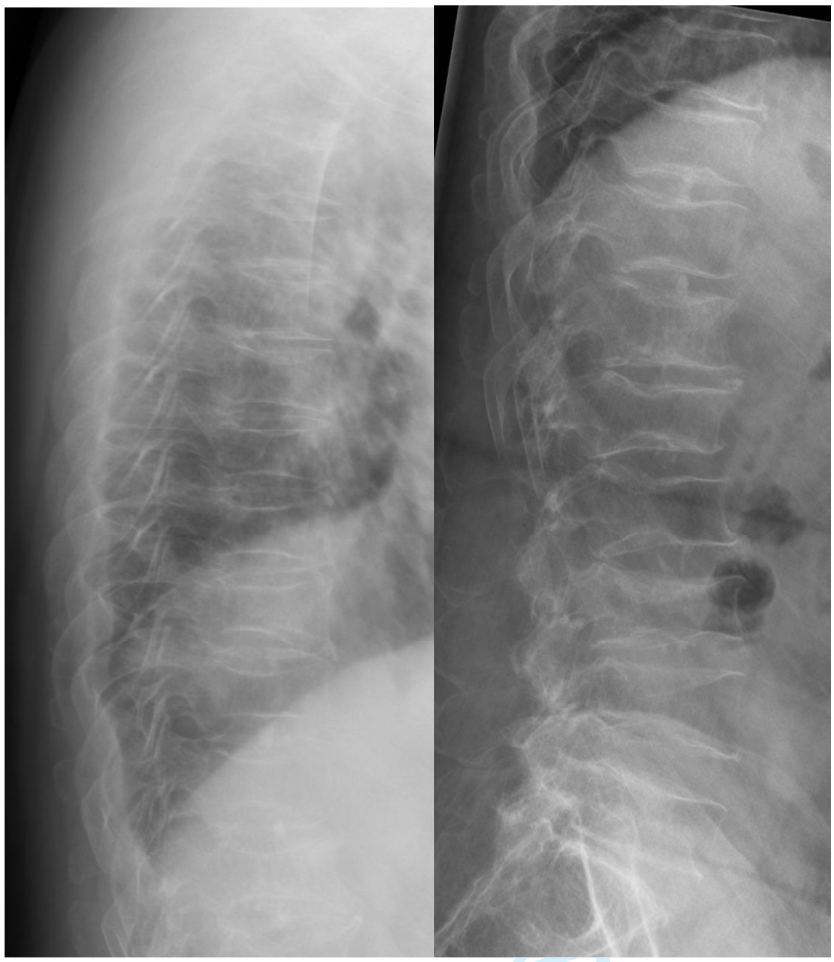
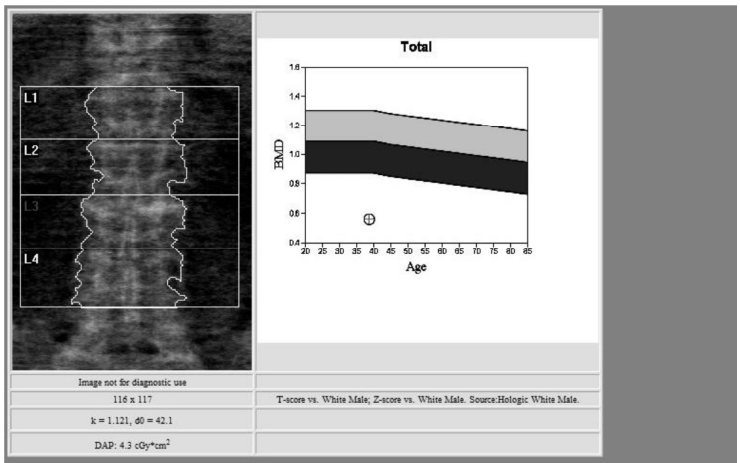


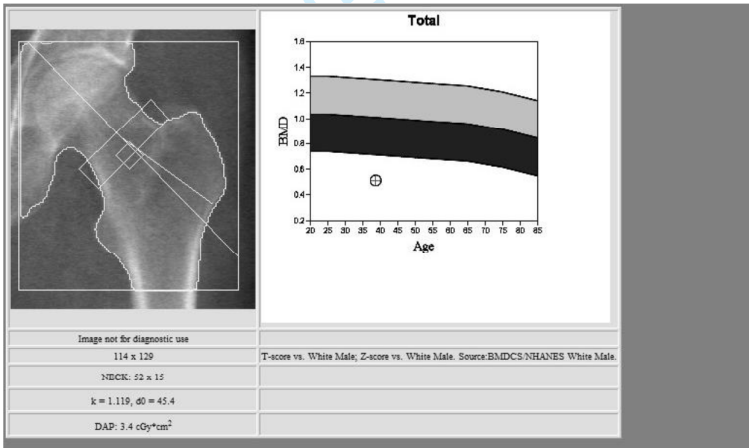
Figure 1C:



Results Summary:

Region	Area[cm²]	BMC[(g)]	BMD[g/cm²]	T-score	PR (Peak Reference)	Z-score	AM (Age Matched)
L1	13.21	7.30	0.552	-4.7	51	-4.7	51
L2	13.67	7.47	0.547	-5.0	50	-5.0	50
L4	17.39	10.03	0.577	-4.7	53	-4.7	53
Total	**44.28**	**24.81**	**0.560**	**-4.8**	**52**	**-4.8**	**52**

Total BMD CV 1.0%, ACF = 1.037, BCF = 1.014, TH = 9.245



Results Summary:

Region	Area[cm²]	BMC[(g)]	BMD[g/cm²]	T-score	PR (Peak Reference)	Z-score	AM (Age Matched)
Neck	6.28	2.70	0.430	-3.7	46	-3.2	49
Troch	14.05	4.26	0.303	-3.8	39	-3.5	40
Inter	28.60	18.06	0.631	-3.1	53	-3.0	53
Total	**48.94**	**25.02**	**0.511**	**-3.5**	**49**	**-3.3**	**51**
Ward's	1.15	0.20	0.173	-4.3	22	-3.6	25

Total BMD CV 1.0%, ACF = 1.037, BCF = 1.014, TH = 6.592

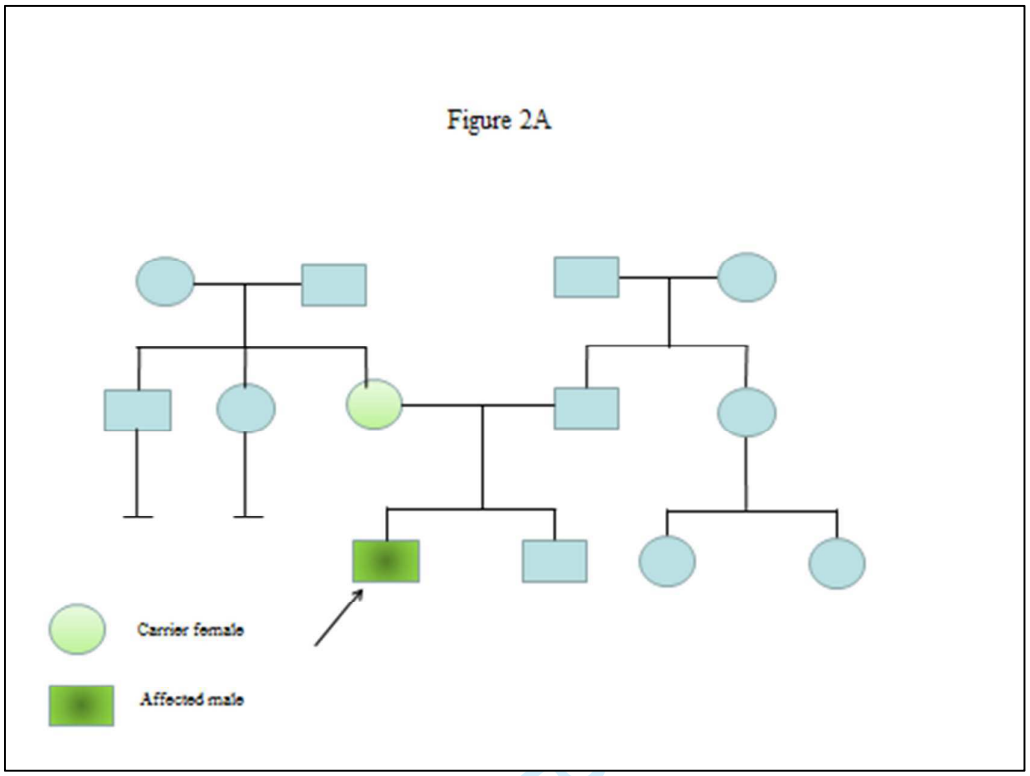


Figure 2B:

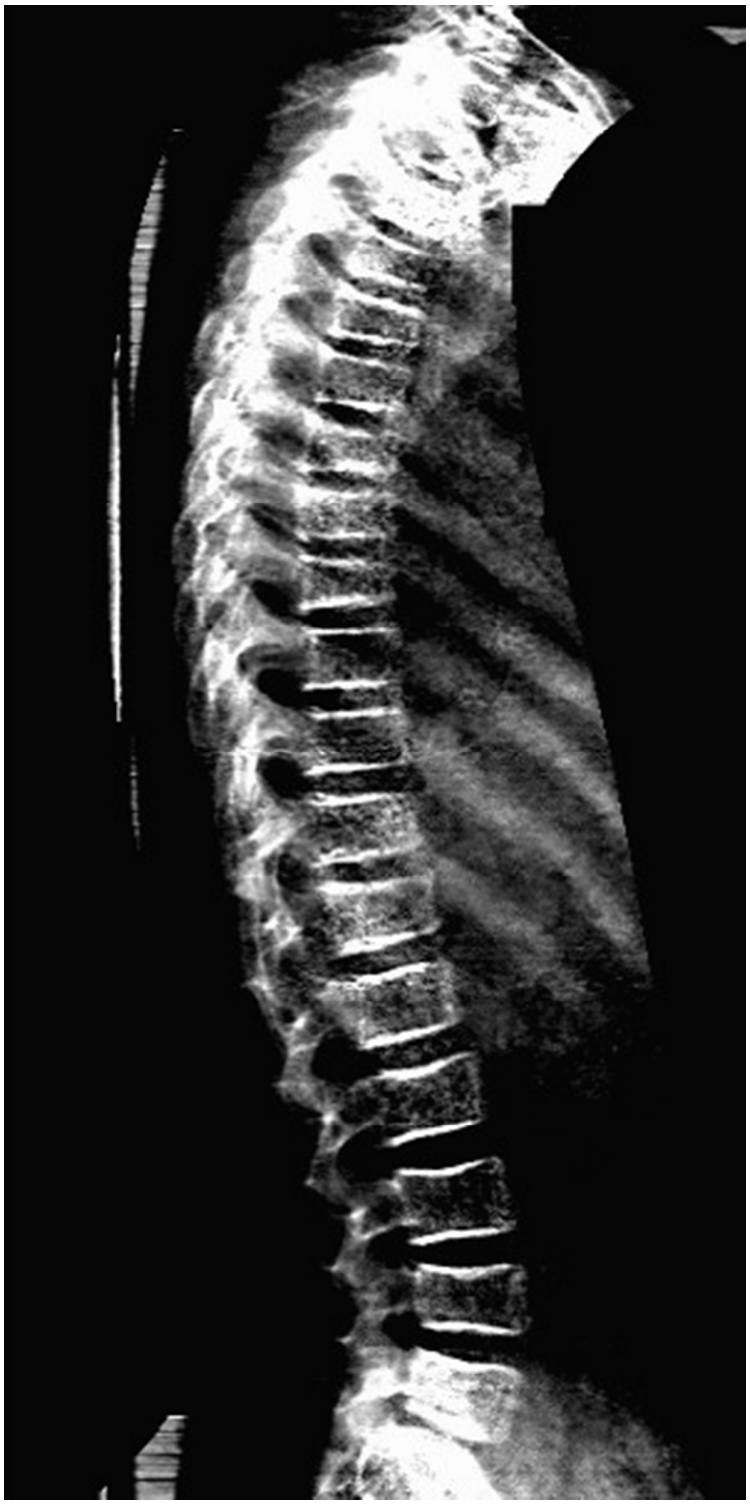


Figure 2C:

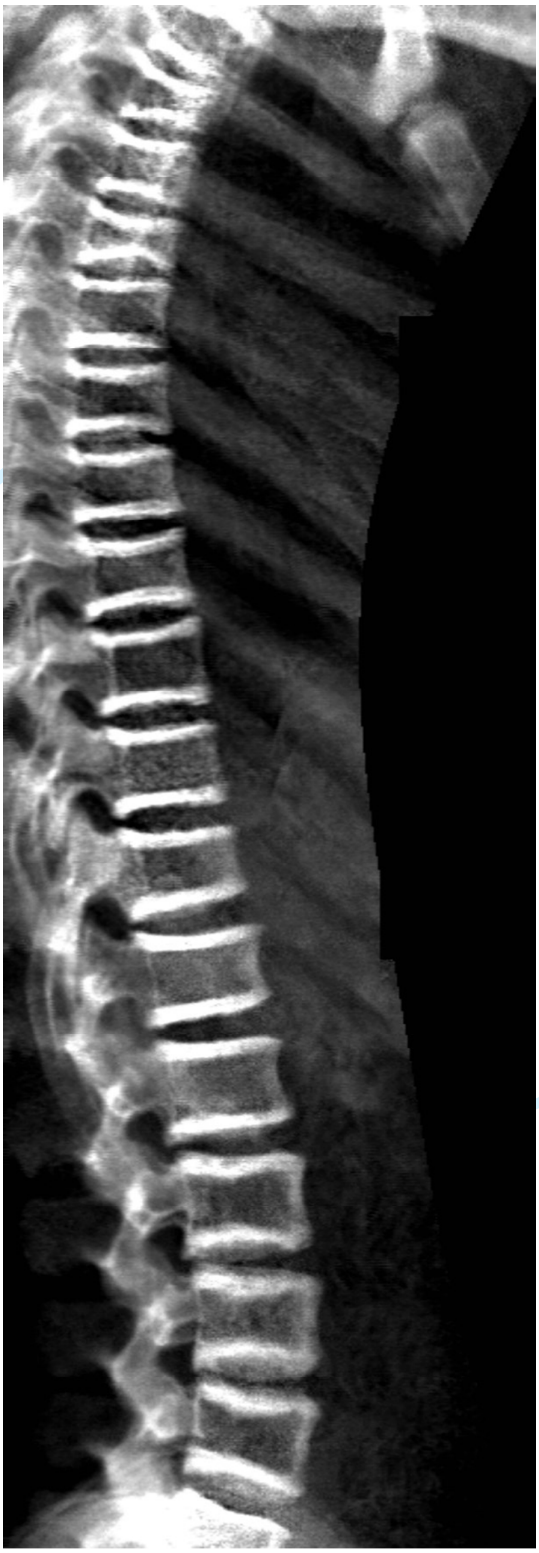
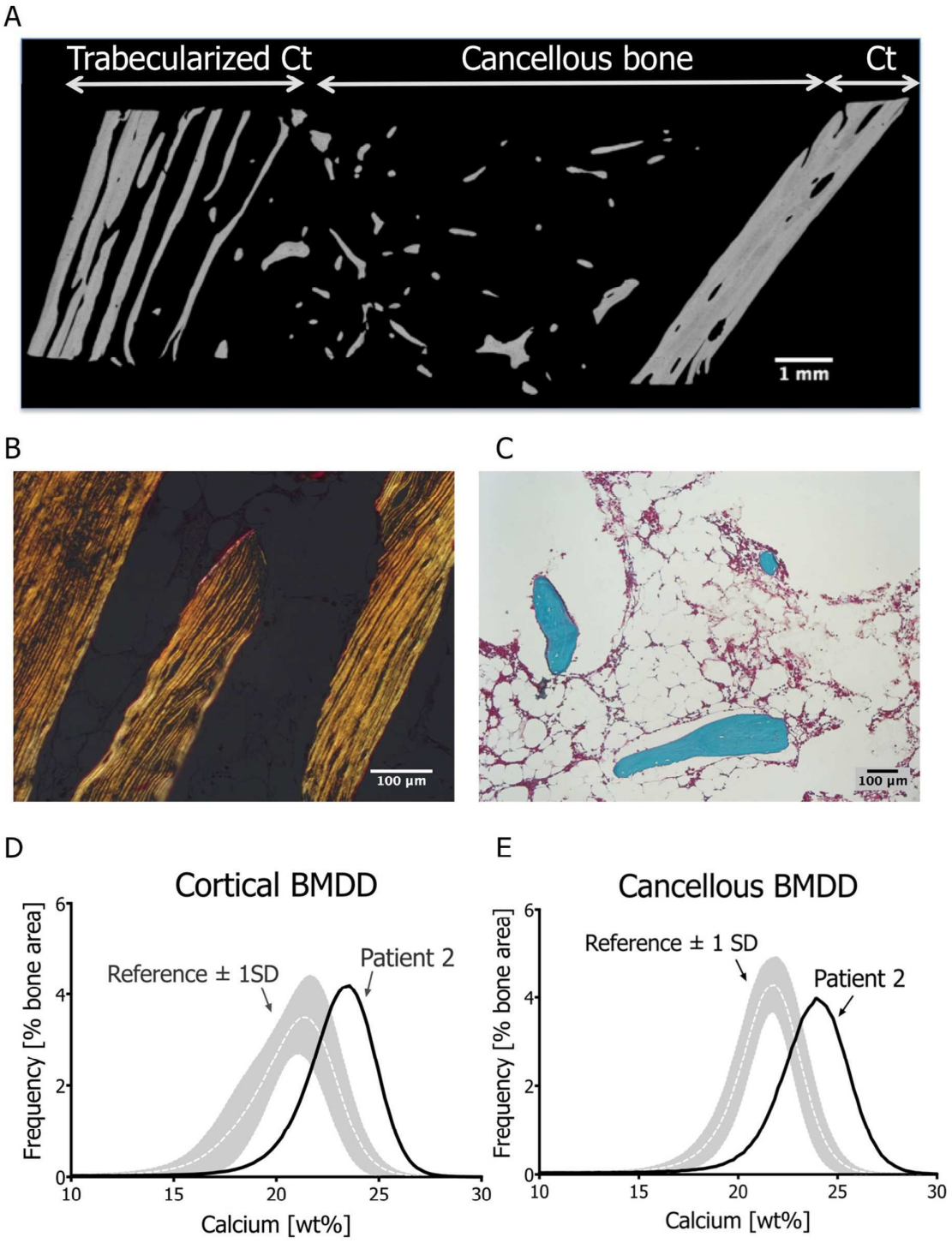


Figure 3


A
Trabecularized Ct
Cancellous bone
Ct
1 mm
B
100 µm
C
100 µm
D
Cortical BMDD
Reference ± 1SD
Patient 2
Frequency [% bone area]
Calcium [wt%]
E
Cancellous BMDD
Reference ± 1 SD
Patient 2
Frequency [% bone area]
Calcium [wt%]