

# Bone Mineral Density in Prader Willi Syndrome: A Search for Genetic Markers

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## Abstract

**Introduction:** Prader-Willi syndrome (PWS) (is caused by lack of paternally expressed imprinted genes at chromosome 15q11.2-q13. Diminished (BMD) and osteoporosis are common in PWS. The purpose of this study was to determine whether polymorphisms in genes previously shown to correlate with bone mineral density (BMD), might explain the variable expression of abnormal BMD in PWS.

**Material and methods:** Blood samples were collected from 96 PWS individuals aged 3.5-47.9 (median 14.4) years. DNA samples were tested for 12 polymorphisms in 8 candidate genes: interleukin-1 (IL1-alfa, IL1-beta and IL1RN), CYP1A1, Low Density Lipoprotein Receptor-Related Protein 5 (LRP5), vitamin D receptor (VDR), RANK and RANKL. All patients underwent BMD measurements at the femoral neck and lumbar spine using a hologic dual energy x-ray absorptiometry (DXA) machine.

**Results:** Abnormal BMD was defined as Z-score <-1.5. Severe reduced BMD as Z-score <-2.5. 67 subjects (70%) had abnormal BMD (youngest 3.7 years old), 25 (26%) had severely reduced BMD (youngest 6.8 years old). BMD correlated negatively with age ( $p < 0.001$ ) and BMI ( $p = 0.006$ ). BMD showed significant correlations with genotypes IL1 alpha C889T ( $p = 0.031$ ), Cyp1A1 C4887A ( $p = 0.04$ ) and VDR FOK I (ff /Ff/FF) ( $p = 0.002$ ); FF genotype has a protective effect.

**Conclusion:** Individuals with PWS have low BMD/osteoporosis at a markedly younger age than the general population. The significant correlation between VDR genotypes and BMD is not specific for PWS. Recommendations including vitamin D, calcium, exercise and specific drugs which slow bone loss or build new bone and hormone replacement should be considered in PWS individuals, particularly patients with the high-risk genetic polymorphisms.

**Keywords:** Prader-Willi syndrome; Osteoporosis; Osteopenia; Genetic markers of osteoporosis; Bone mineral density; DXA

## Introduction

Prader-Willi syndrome (PWS) is a complex genetic disorder caused by lack of expression of genes on the paternally inherited chromosome 15q11.2-q13 region. The estimated prevalence is 1/10,000-1/30,000 [1]. Infants with PWS suffer from severe hypotonia, feeding difficulties, and failure to thrive (FTT) followed in later infancy or early childhood by excessive appetite with gradual development of obesity, short stature, hypogonadism, intellectual disabilities and behavioral problems [2].

There are three main genetic subtypes in PWS: paternal 15q11-q13 deletion in 65-75%, maternal uniparental disomy (UPD) in 20-30% and imprinting center defect in 1-3% of all cases [3,4]. PWS is the most common syndromic cause of life-threatening obesity and the first recognized disorder related to genomic imprinting. Muscle mass is decreased by 25-37%, which might partly explain the hypotonia [5]. Diabetes and scoliosis are common features of PWS [6]. Hypothalamic dysfunction has been implicated in many manifestations of this syndrome including hyperphagia, high pain threshold, sleep-disordered breathing, and multiple endocrine abnormalities including hypogonadism [7,8]. The etiology of hypogonadism in PWS (primary gonadal or hypothalamic) is heterogeneous [5].

Increased fat mass and decreased lean body mass are characteristics of PWS. Several studies have demonstrated that individuals with PWS have lower bone mineral density (BMD) compared to normal controls [9]. Hypotonia, hypogonadism, growth hormone deficiency, and limited mineral and vitamin intake due to diet restrictions may contribute to the

decreased BMD and bone mineral content (BMC) in PWS. Recently it has been shown that PW Snord 116 knockout mice show reduced bone formation indicating a possible link between the PW critical region and osteoporosis [10]. Since bone density and mineral content are highly variable among PWS individuals, other factors may contribute to the development of osteopenia and/or osteoporosis. Identifying risk factors for developing osteopenia or osteoporosis may allow early intervention prior to severe bone loss. The purpose of our study was to investigate polymorphisms of candidate genes previously shown to correlate with osteoporosis and/or bone disease and determine if similar associations are present in a PWS population.

## Materials and Methods

DNA samples were collected from 96 of 103 individuals above the age of 3 years with PWS followed at the Israeli national multidisciplinary PWS clinic at Shaare Zedek Medical Center, Jerusalem, Israel. There were

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46 males and 50 females (ages 3.5-47.9 years). Four families refused to participate in this study and three families failed to return for follow-up visits. The demographic parameters, age, weight, height and BMI, were retrieved from patients' charts. The following parameters were obtained for each patient: fat mass, lean and bone mass composition, levels of vitamin D, PTH, alkaline phosphatase and calcium, testosterone (for males) and estradiol (for females). Clinical data regarding demographic parameters was retrieved from patients' charts.

This study was approved by the institutional review board of the Shaare Zedek Medical Center, Jerusalem, Israel. Written informed consent was obtained from participants and/or legal guardians. All patients underwent BMD measurements using a Hologic Dual Energy x-ray absorptiometry (DXA) machine (Discovery 4500 with DICOM), as part of their routine follow-up. BMD was recorded as T-scores (compared to healthy 21 years old) and z-scores (compared to age-matched healthy controls) at the femoral neck (FN) and lumbar spine (LS) [11]. For qualitative analysis, the cut-off point for DXA values was -2.5 for both T- and Z-scores. Reduced BMD (osteopenia) was defined as Z score less than -1.5 for two out of three tested regions (lumbar spine, right hip and left hip). Severely reduced BMD was defined as Z score less than -2.5 for two out of three tested regions.

A systematic search of the literature for gene polymorphisms revealed 11 polymorphisms in 7 different candidate genes that have been shown to be significantly correlated with osteoporosis and/or bone disease: interleukin 1 (IL1 $\alpha$ , IL1 $\beta$  and IL1RN) is involved in both osteoclast and osteoblast differentiation, with the following 4 polymorphisms associated with osteoporosis: C889T in the promoter of IL1 $\alpha$  gene; C511T in the promoter of IL1 $\beta$  gene; C3954T in exon 5 of IL1 $\beta$ ; and VNTR (Variable Number of Tandem Repeats) in IL1RN gene [12]. Cytochrome P450 *CYP1A1* C4887A polymorphism was shown to be related to estrogen metabolism and bone density [13]. LRP5 (Low Density Lipoprotein Receptor-Related Protein 5) is implicated in osteoblast differentiation during growth [14] with 2 polymorphisms, C135242T and G138351A, associated with osteoporosis. Expression and nuclear activation of the vitamin D receptor (VDR) are necessary

for the effects of vitamin D with the C $\text{FokIT}$  polymorphism significantly associated with bone disease [15].

RANK polymorphisms rs1805034, rs35211496 and RANKL rs2277438 were found to be significantly associated with bone density [16,17]. All of these polymorphisms were analyzed in our study. PCR reactions were performed using standard protocols (Table 1). Polymorphisms were detected by restriction enzyme assays, sequencing or capillary electrophoresis (Table 2).

All restriction enzymes were obtained from New England Biolabs Israel (<http://www.ornat.co.il/>). For the IL1RN VNTR polymorphism, the forward primer in the PCR reaction was fluorescently labeled (Applied Biosystems) and the reaction products were diluted and run on an ABI Prism 3100 Avant automated sequencer and analyzed using Genotyper (software ABI). All restriction enzymes were obtained from New England Biolabs Israel (<http://www.ornat.co.il/>). For the IL1RN VNTR polymorphism, the forward primer in the PCR reaction was fluorescently labeled (Applied Biosystems) and the reaction products were diluted and run on an ABI Prism 3100 Avant automated sequencer and analyzed using Genotyper (software ABI).

### Statistical Analysis

The polymorphisms were compared using ANOVA (both parametric and non-parametric) and for the genotypes and multi-variance logistic regression. A p-value of 0.05 was considered statistically significant.

### Results

Characteristics of the PWS patients are presented in Table 3. The mean values for DXA T-scores and Z-scores are presented in Table 4. The patients were divided into two age groups (<18 years and >18 years old). The mean age of the 58 patients younger than 18 was 9.9+3.9 years (3.5-18 years). The mean age of the 38 patients older than 18 was 26.9+6.2 (18.1-49.7 years). Sixty-seven patients had abnormal BMD (70%). For the group of patients <18 years old, 24 (41%) had normal bone mineral density, 28 (49%) had mild-moderate reduced BMD and

Gene	Polymorphism	Primer	Primer sequence 5'...3'	Annealing temperature
Interleukin 1 $\alpha$	C889T	F	TTACATATGAGCCTTCCATG	57°C
		R	AAGCTTGTTCTACCACCTGAACATAGGC	
	C511T	F	GTTTAGGAATCTTCCACTT	51°C
		R	TGGCATTGATCTGGTTCATC	
Interleukin 1 $\beta$	C3954T	F	GCTTTTTTGCTGTGAGTCCCG	57°C
		R	CTCAGGTGTCTCGAAGAAATCAAA	
Interleukin 1RN	VNTR	F	CTCAGCAACTCCTAT	51°C
		R	TCCTGGTCTGCAGGTAA	
<i>CYP1A1</i>	C4887A	F	CTGTCTCCCTCTGGTTACAGGAAGC	61°C
		R	TTCCACCCGTTGCAGCAGGATAGCC	
Lipoprotein receptor- related protein 5	C135242T	F	GGAGGACAAGCTCCCGCACATTCT	61°C
		R	CATGCAAGTCTGCATGGCTGATCC	
	G138351A	F	TTGTGTGGCTTGCCCGCACCC	63°C
		R	CCTCCTTGACGCCCGTGAGC	
Vitamin D receptor	C $\text{fokIT}$	F	AGTGCCCTGGCACTGACTCTGCTCT	61°C
		R	ATGGAAACACCTTGCTTCTTCCCTC	
RANK	rs1805034 C>T	F	CCAAAGCACTGAACCACCTT	61°C
		R	CTGGGGCACATCTATCAACC	
	rs35211496 C>T	F	GTACCACTGGAGCCAGGACT	61°C
		R	GGCTGTATCTCGTGGAAAA	
RANKL	rs9533156 C>T	F	TCCTGACTGTTGGGTGAGC	61°C
		R	TCCCAAATCCCTATTTCTGC	

**Table 1:** Primers used for PCR for each polymorphism (Note: F: forward; R: reverse).

Gene	Polymorphisms	Restriction Enzyme
Interleukin 1 $\alpha$	C-889T	NcoI
Interleukin 1 $\beta$	C-511T	AvaI
	C3954T	TaqI
CYP1A1	C4887A	Eco31I
Lipoprotein receptor	C135242T	Ava1
-related protein 5	G138351A	Stu1
Vitamin D receptor	CfokI	FokI
RANK rs1805034	C>T	SsiI (Acil)
RANK rs35211496	C>T	BseYI
RANKL	C>T	TspR1

**Table 2:** Restriction enzyme used for each of the analyzed polymorphisms.

No. Females/No. Males	50/46
Age (years) (mean $\pm$ SD)	16.6 $\pm$ 9.7
No. patients with UPD/deletion	37/57
BMI (kg/m <sup>2</sup> ) (mean $\pm$ SD)	25 $\pm$ 13.4
BMI SD (mean $\pm$ SD)	1.5 $\pm$ 1.0
Vitamin D (ng/ml) 3.0 year < (mean $\pm$ SD)	N=82 25.91 $\pm$ 11.55 RANGE: 5-81.6
Calcium (mg/dL) 3.0 year < (mean $\pm$ SD)	N=87 9.528 $\pm$ 0.415 RANGE: 7.9-10.4
Testosterone (ng/ml) (mean $\pm$ SD)*	
3.0-12.0 year	N=17 0.12 $\pm$ 0.05 RANGE: <0.1-0.31
12.1-18.0 year	N=8 0.42 $\pm$ 0.43 RANGE: <0.1-1.39
>18.1 year	N=21 1.84 $\pm$ 1.66 RANGE: <0.1-7.5
Estradiol (pg/ml)** >15.0 year (mean $\pm$ SD)	N=19 50.80 $\pm$ 27.95 RANGE: <37-107.63

**Note:** \*For statistical purposes, values below assay sensitivity (0.1 ng/ml) were assigned 0.1 ng/ml. \*\*For statistical purposes, values below assay sensitivity (37 pg/ml) were assigned 37 pg/ml.

**Table 3:** Patient characteristics.

DXA measurements	Mean $\pm$ SD
Right Hip T score	-2.1 $\pm$ 1.1
Right Hip Z score	-2.0 $\pm$ 1.3
Right Hip BMD gr/cm <sup>2</sup>	0.7 $\pm$ 0.1
Left Hip T score	-1.6 $\pm$ 1.1
Left Hip Z score	1.2 $\pm$ 1.3
Left Hip BMD gr/cm <sup>2</sup>	0.7 $\pm$ 0.2
Lumbar spine T score	-1.9 $\pm$ 1.1
Lumbar spine Z score	-0.8 $\pm$ 0.5
Lumbar spine BMD gr/cm <sup>2</sup>	-0.8 $\pm$ 0.6

**Table 4:** Mean DXA measurements.

6 had severely reduced BMD (10%). For the group of patients >18 years old, only 4 (10%) had normal BMD, 15 (39%) severely reduced BMD and 19 (51%) had mild-moderate reduced BMD.

A significant negative correlation was found between BMD and age ( $p < 0.001$ ) and BMI ( $p = 0.006$ ), but no correlation was found between sex and BMD. No correlation was found between the levels of serum estradiol (in females) or testosterone (in males) and BMD. The youngest patient with severely reduced BMD was 6.8 years old and with moderate reduced BMD 3.7 years old.

We found significant correlations between vitamin D receptor genes and the presence of reduced BMD in our PWS cohort. The distribution of the Vitamin D receptor Fok I genotypes for PWS patients and controls were: 39 (40.6%) patients were homozygous FF and 28% of controls, 35 (36.5%) patients were heterozygous Ff compared to 58.5% in controls and 22 (22.9%) patients were homozygous for the minor allele ff (13.8% in controls) [17] (Table 5). Vitamin D receptor genotypes (FF/Ff/ff)

were found to be significantly correlated to reduced BMD ( $p = 0.002$ ) (Table 6a). The minor allele f (Ff and ff) was found to be a risk factor for osteopenia/osteoporosis in this cohort (Table 6b). These results remain significant with age correction. Statistically significant correlations were also found between the genotype of the IL1 alpha C889T and the T score left hip ( $p = 0.031$ ) and between CYP1A1 C4887A and T score left hip ( $p = 0.04$ ). No significant correlations with BMD were found for the other genotypes.

## Discussion

The majority of PWS individuals in our study had abnormal BMD, even in children younger than age 10 years. We found that genetic factors, not located on chromosome 15, contribute to the early decrease of bone density in PWS.

Osteoporosis is a recognized clinical feature of PWS [18]. In our study, 70% had reduced BMD, 26% had severely reduced BMD ( $Z$ -score  $< 2.5$ ). While osteopenia/osteoporosis is rare in normal children, we observed moderate reduced BMD in a 3-7/12 year's old child and severely reduced BMD in a 6-8/12 years old child. Previous studies also reported that among patients with PWS, 61.5% had low BMD [19]. Hoybe et al. [20] found that the majority of PWS adults were classified as having osteopenia or osteoporosis. BMD was low in their population compared with age- and sex-matched controls.

GH treatment has been shown to significantly improve BMD [21]. Sex hormone replacement unexpectedly appears to have no effect on bone mineral density in one study [20], however, Longi et al [22] showed that bone strength was lower in adult PWS patients who were treated with sex steroids but did not receive GH replacement [22].

The expression of abnormal BMD in PWS is variable and there are no known factors indicating which individuals are at increased risk for early development of osteoporosis or osteopenia. Therefore we studied polymorphisms known to be associated with abnormal BMD in other populations [19] in order to determine if similar correlations occurred in a PWS cohort. We found that out of the 11 polymorphisms in our study, vitamin D receptor FOK polymorphism correlated with BMD in PWS patients.

Genotypes	PWS	Controls*
Vit D receptor FokI		
FF	39 (40.6%)	28%
Ff	35 (36.5%)	58.8%
ff	22 (22.9%)	13.4%

**Note:** (\*15)

**Table 5:** Distribution of VDR genotypes in patients and controls.

Genotypes	Osteoporosis/osteopenia	
	Yes (No pts/%)	No (No pts/%)
Vit D receptor FokI		
FF	22 (33%)	17 (59%)
Ff	29 (43%)	6 (21%)
ff	16 (24%)	6 (21%)

**Table 6a:** Distribution of Vitamin D receptor genotypes among patients with and without osteoporosis/osteopenia.

Genotypes	Osteoporosis/osteopenia	
	Yes (No. pts/%)	No (No. pts/%)
Vit D receptor FokI		
FF	22 (33%)	17 (59%)
Ff+ff	45 (67%)	12 (41%)

**Table 6b:** Vitamin D receptor genotypes related to osteoporosis/osteopenia in PWS patients.

The active metabolites of vitamin D play an important role in regulating bone cell function and maintenance of serum calcium homeostasis by binding to the VDR and regulating the expression of other response genes. The FokI polymorphism of the VDR gene has been associated with BMD in many studies [15]. A similar study looking for genetic modifiers regarding osteoporosis/osteopenia was performed by Arnheim et al. [17] in patients with Gaucher Disease. In Gaucher patients FokI polymorphisms were strongly associated with osteoporosis. The same correlation was found for PWS patients: the F allele was protective for osteoporosis/osteopenia while the f was a risk factor for developing osteoporosis.

Our PWS cohort was differed from the Gaucher group, however, since it included a wider age range of participants from the age of 3-6/12 years through age 47 years. We found that PWS patients developed osteoporosis /osteopenia starting at a very young age. The VDR polymorphisms showed similar correlations as was found in the Gaucher study and in the general population. These findings suggest that other genetic and environmental factors need to be considered in order to explain the increased prevalence of low BMD in PWS patients compared to other populations.

The role of genes in the critical PWS region as contributing to the pathogenesis of osteoporosis was addressed recently by Khor et al. [10]. They found that micro deletion of small non-translated nucleolar RNAs of the Snord 116 may lead to reduced bone mass in Prader-Willi Critical Region (PWCR) knock-out mice, thereby demonstrating a link between genetic factors inside the PWCR and bone involvement. Future research might find more genetic factors in the PWCR influencing bone mineral density. Other modifiers in different regions of the genome, such as FOK VDR polymorphism, might also mitigate the degree of bone involvement and help predict the need for early surveillance and treatment in specific high-risk PWS individuals.

## Conclusion

In conclusion, we found that individuals with PWS have low BMD/osteoporosis at a much younger age than the general population. We analysed 11 polymorphisms in genes related to bone mineral density in a large cohort of patients with PWS. The significant correlation between VDR genotypes and BMD is not specific for PWS. Other genetic and non-genetic factors might also increase the risk of osteoporosis in PWS. Treatment with vitamin D, calcium, exercise, specific drugs which slow bone loss or build new bone and hormone replacement should be considered in PWS patients with the high-risk genetic polymorphisms.

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