

# The Role of Serum Expression Levels of Microrna-21 on Bone Mineral Density in Hypostrogenic Postmenopausal Women with Osteoporosis: Study on Level of RANKL, OPG, TGF $\beta$ -1, Sclerostin, RANKL/OPG Ratio, and Physical Activity

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## ABSTRAK

**Latar belakang:** MiR-21 telah diketahui memainkan peranan dalam proliferasi dan diferensiasi osteoklas, tetapi peran ekspresi miR-21 dalam serum terhadap osteoporosis masih belum jelas. Penelitian sebelumnya menemukan bahwa ekspresi miR-21 dalam serum berkorelasi positif dengan kepadatan mineral tulang pasien osteoporosis pascamenopausal, tetapi faktor-faktor lain yang terlibat dalam osteoporosis pascamenopause masih belum diketahui. Penelitian ini bertujuan menentukan peran ekspresi miR-21 dalam serum, kadar RANKL, OPG, TGF- $\beta$ 1, sklerostin dan kalsium dalam serum, rasio RANKL/OPG dan aktivitas fisik terhadap kepadatan mineral tulang di tulang belakang pada wanita pascamenopause hipoestrogenik dengan osteoporosis / postmenopausal women with osteoporosis (PMOP) dibandingkan tanpa osteoporosis (PMNOP) dengan titik berat pada ekspresi miR-21 dalam serum. **Metode:** penelitian ini dilaksanakan dengan desain potong lintang komparatif. Subjek penelitian dibagi menjadi dua kelompok, yaitu PMOP dan PMNOP. Metode kuantifikasi absolut dengan real-time PCR digunakan untuk menentukan kadar ekspresi miR-21 dalam serum. **Hasil:** nilai median ekspresi miR-21 dalam serum pada kelompok PMOP lebih tinggi secara bermakna dibandingkan dengan kelompok PMNOP ( $p=0,001$ ). Ekspresi miR-21 dalam serum, RANKL, rasio RANKL/OPG dan aktivitas fisik secara bermakna berkorelasi dengan nilai kepadatan mineral tulang/bone mineral density (BMD) pada kelompok PMOP. Aktivitas fisik sedang berkorelasi negatif secara bermakna dengan ekspresi miR-21 dalam serum. Kami juga mendapatkan persamaan regresi linear  $BMD=1,373-0,085*Ln.miR-21-0,176*Log10.RANKL$  ( $R^2=52,5\%$ ). **Kesimpulan:** Ekspresi miR-21 dalam serum pada PMOP lebih tinggi dibandingkan dengan PMNOP. Ekspresi miR-21 dalam serum terbukti mempunyai efek negatif terhadap nilai kepadatan mineral tulang di tulang belakang (spinal BMD) pada wanita pascamenopause hipoestrogenik dengan tingkat osteoporosis sebesar 8,5%. Persamaan yang didapatkan, yaitu

$BMD = 1,373-0,085*Ln.miR-21-0,176*Log10.RANKL$  dapat menjelaskan nilai spinal BMD sebesar 52,5%.

**Kata kunci:** bone mineral density (BMD), osteoporosis, pascamenopause, miR-21 dalam serum.

## ABSTRACT

**Background:** MiR-21 is known to play a role in osteoclast proliferation and differentiation, but the role of serum miR-21 expression in osteoporosis remains unclear. Previous research found that serum miR-21 expression was positively correlated with bone mineral density in postmenopausal osteoporosis patients, but other factors involved in postmenopausal osteoporosis still unknown. This study aimed to determine the role of serum miR-21 expression, concentration of RANKL, OPG, TGF- $\beta$ 1, sclerostin and serum calcium, RANKL/OPG ratio, and physical activity on bone mineral density of spine in hypoestrogenic postmenopausal women with osteoporosis (PMOP) compared with no osteoporosis (PMNOP), with point of interest on the expression of serum miR-21. **Methods:** this study was conducted by comparative cross-sectional design. The subjects were divided into 2 groups of PMOP and PMNOP. We used an absolute quantification real-time PCR method to determine serum miR-21 expressions level. **Results:** Median of serum miR-21 expression at the PMOP group was significantly higher compared to PMNOP group ( $p = 0.001$ ). Serum miR-21 expression, RANKL, RANKL/OPG ratio, and physical activity were significantly correlated with BMD values in the PMOP group. Moderate physical activity was significantly negatively correlated with serum miR-21 expression. We also obtained a linear regression equation  $BMD = 1.373-0.085*Ln.miR-21-0.176*Log10.RANKL$  ( $R^2 = 52.5\%$ ). **Conclusion:** serum miR-21 expression in PMOP was higher compared with PMNOP. Serum miR-21 expression proved to have a negative effect on spinal BMD values in hypoestrogenic postmenopausal women with osteoporosis of 8.5%. Obtained equation of  $BMD = 1.373-0.085*Ln.miR-21-0.176*Log10.RANKL$  can explain the value of spinal BMD by 52.5%.

**Keywords:** bone mineral density (BMD), osteoporosis, postmenopausal, serum miR-21.

## INTRODUCTION

Osteoporosis is a systemic bone disease characterized by decreased bone mass and changes in bone tissue microarchitecture, leading to increased bone fragility and susceptibility to fracture.<sup>1</sup> Estrogen plays an important role in bone loss mechanism in a postmenopausal woman. There are 20 to 30 percent postmenopausal women that are presented with osteoporosis.<sup>2-4</sup> Other factors involved in postmenopausal osteoporosis are Osteoprotegerin (OPG), receptor activator of nuclear factor kappa- $\beta$  ligand (RANKL), RANKL/OPG ratio, sclerostin, growth factors (TGF- $\beta$ , IGF-1, BMP, etc.), hormones (thyroid, insulin, glucocorticoid, parathyroid etc.), cytokines (IL-1, IL-2 and TNF- $\alpha$ ), physical activity and calcium intake.<sup>5</sup>

MicroRNA (miRNA) is a short non-coding RNA molecule that has a function in RNA silencing and posttranscriptional regulation of gene expression.<sup>6</sup> The present study showed that microRNA-21 (miR-21) plays a role in osteoblast proliferation and differentiation. miRNA-21

stimulated osteoclastogenesis by increasing c-Fos concentration through down-regulation of programmed cell death 4 (PDCD4) protein and induction of RANKL.<sup>7</sup> Previous study shown that estrogen reduces miR-21 biogenesis, therefore it causes osteoclast apoptosis. It has also been discovered that miR-21 serum was positively correlated with bone mineral density (BMD) in osteoporotic patients.<sup>8,9</sup> However, up until now there has been no study on the effect of low estrogen levels (hypoestrogenic) with miR-21 expression.

This study will assess the role of miR-21 in determining bone mineral density in hypoestrogenic postmenopausal women and analyzing other factors involved in postmenopausal osteoporosis such as OPG, RANKL, RANKL/OPG ratio, sclerostin, growth factor (TGF- $\beta$ 1), serum calcium concentration, physical activity and body mass index, by excluding confounding factor such as insulin, thyroid hormone and use of glucocorticoid.

## METHODS

We conducted a comparative cross-sectional study in 120 women, comprised of 60 postmenopausal hypoestrogenic women with osteoporosis (PMOP) and 60 postmenopausal hypoestrogenic women without osteoporosis (PMNOP), ages 55 to 70 years who had entered the postmenopausal period and had blood estradiol <30 pg/mL. Subject were excluded when they received hormone replacement therapy, suffered from malignant or autoimmune diseases, liver disease, underwent routine hemodialysis, received systemic steroid therapy for more than 3 months, received bisphosphonate therapy, received anticonvulsant therapy, thyroid disease, diabetes mellitus, anemia (Hb <12 g/dl) and active smokers.

Subsequently, subjects were asked to sign an informed consent. Bone mineral density (BMD) was assessed using DXA: Medix 90, the precision for spine and hip was 0.6–1.5% and coefficient of variation was 1.5% for both. One professional operator with a certificate of competence in bone densitometry carried out BMD examination.

This study has been approved by The Ethics Committee of Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia, with a reference number 672/UN2.F1/ETIK/2015.

### Serum miR-21 Examination

Total RNA extraction was done using MirVana PARIS KIT (Ambion, Vilnius, Lithuania) and RNA concentrations were measured using NanoDrop™ 2000CC (Thermo Scientific, Pittsburgh, USA). cDNA synthesized using TaqMan miRNA reverse transcription kit and miRNA stem-loop primers which is for miR-21 (Applied Biosystems, Vilnius, Lithuania). The instrument used in cDNA synthesis

was Veriti Thermo Cycle (Applied Biosystems, California, USA). miR-21 amplification using real-time Polymerase Chain Reaction (RT-PCR) was done using TaqMan 2x Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Warrington, United Kingdom) and TaqMan miRNA Assay (Applied Biosystems, Vilnius, Lithuania) consist of specific primer and probe for miR-21. The instrument used for RT-PCR was Exicycler 96 (Bioneer Corp., Daejeon, South Korea).

ELISA method was used to examine the serum concentration of sclerostin, OPG, RANKL, TGF- $\beta$ 1 dan estradiol. Total serum calcium examination using Arzenazo-III dye and we ran it in the spectrophotometer with 660 nm wavelength. International Physical Activity Questionnaire was used to measure the subject's physical activity.

### Statistical Analysis

We used Mann-Whitney U non-parametric statistical test to analyze the difference of median value between two groups. Multiple regression multivariate analysis was used to assess the correlation between nine independent variables with one dependent variable. A p value of < 0.05 was considered significant. Spearman correlation test was used to analyze the correlation between variables that were not meet a normal distribution criterion.

## RESULTS

The clinical features of a postmenopausal patient with and without osteoporosis are shown in **Table 1**.

**Table 1.** Clinical characteristic of patient

Characteristics	PMOP (n=60)	PMNOP (n=60)	p-Value
Age (year)	62 (67)	58.5 (65)	0.107
Weight (kg)	53.5(60)	60 (65)	0.014
Height (cm)	151.5 (155)	152.5 (155)	0.367
Menopausal period (year)	10.5 (15)	9 (15)	0.299
BMI (kg/m <sup>2</sup> )	23.38 (26.2)	24.72 (27.3)	0.083
BMD (g/m <sup>2</sup> )	0.647 (0.707)	0.867 (0.947)	< 0.001
T-score (L1-4)	-2.9 (-2.7)	- 0.9 (-0.3)	< 0.001
Estradiol (pg/mL)	< 11.8 – 26.0	< 11.8 – 29.0	

The value is the median (75th percentile) except estradiol only in the minimum - maximum form because estradiol value of less than 11.8 pg/mL is immeasurable, the p-value was measured using Mann-Whitney U test.

### MiR-21 Expression and Other Variables in Both Groups

We found out that miR-21 expression in a hypoestrogenic postmenopausal woman with osteoporosis group was higher than without osteoporosis, this difference was statistically significant with a p-value <0.05.

Other variables that have statistically significant differences between the two groups are sclerostin, TGF- $\beta$ 1, RANKL, OPG, RANKL/OPG ratio and moderate physical activity (**Table 2**). We didn't analyze heavy physical activity because not all the subject had it.

**Table 2.** Serum MiR-21 expression and other variables in PMOP and PMNOP group

	PMOP (n=60)	PMNOP (n=60)	p-value
Serum miR-21 expression (copies/ $\mu$ L x 1010)	35.30 (45.97)	24.85 (40.97)	0.006
Sclerostin (pmol/L)	26.65 (37.77)	22.8 (34.10)	0.023
TGF- $\beta$ 1 (pg/mL)	42571.5 (50541.50)	50706.0 (63920.0)	< 0.001
RANKL (pmol/L)	198.55 (415.75)	132.95 (315.50)	0.001
OPG (pmol/L)	5.15 (6.40)	7.8 (10.12)	< 0.001
RANKL/OPG ratio	41.11 (83.94)	16.81 (37.47)	< 0.001
Calcium (mg/dL)	9.7 (10.20)	9.8 (10.17)	0.707
Physical Activity (score)			
- Total	2891.0 (6052.0)	3030.0 (7224.0)	0.407
PMOP : n = 60			
PMNOP : n = 60			
- Walking	1366.0 (2772.0)	1378.0 (2772.0)	0.198
PMOP : n = 60			
PMNOP : n = 60			
- Moderate	500.0 (1260.0)	840.0 (3360.0)	0.019
PMOP : n = 51			
PMNOP : n = 50			

\*Value is median (75th Percentile), p-value result: Mann-Whitney U test

**Table 3.** Correlation of MiR-21 expression with other variables in PMOP and PMNOP groups

	miR-21 Serum			
	PMOP (n=60)		PMNOP (n=60)	
	r*	p Value	r*	p Value
Sclerostin (pmol/L)	-0,001	0,995	0,171	0,192
TGF- $\beta$ 1 (pg/mL)	-0,246	0,058	-0,047	0,720
RANKL (pmol/L)	0,693	< 0,001	0,032	0,807
OPG (pmol/L)	-0,021	0,876	0,052	0,692
RANKL/OPG ratio	0,661	< 0,001	0,022	0,692
Calcium (mg/dL)	0,138	0,293	0,150	0,251
Physical Activity (score)				
Total	0.079	0.547	0.202	0.122
Walking	0.029	0.825	-0.182	0.165
Moderate				
PMOP: n = 51 PMNOP: n = 50	-0.405	0.003	-0.453	0.001
Weight (kg)	0.163	0.213	-0.066	0.617
BMI (kg/m <sup>2</sup> )	0.138	0.293	0.023	0.862
BMD (g/m <sup>2</sup> )	-0.669	< 0.001	0.231	0.078

r\*: Spearman correlation test

**Table 4.** Correlation of MiR-21 expression with other variables in low-level estrogen patient

	miR-21 Serum			
	PMOP (n=40)		PMNOP (n=39)	
	r*	p Value	r*	p Value
Sclerostin (pmol/L)	0.032	0.844	0.133	0.421
TGF- $\beta$ 1 (pg/mL)	-0.398	0.011	-0.109	0.508
RANKL (pmol/L)	0.618	< 0.001	0.015	0.928
OPG (pmol/L)	-0.461	0.003	-0.036	0.828
RANKL/OPG ratio	0.615	< 0.001	0.015	0.927
Calcium (mg/dL)	0.079	0.629	0.098	0.555
Total Physical Activity (score)	0.002	0.990	0.217	0.185
Weight (kg)	0.215	0.183	0.001	0.997
BMI (kg/m <sup>2</sup> )	0.204	0.206	0.034	0.836
BMD (g/m <sup>2</sup> )	-0.467	0.002	0.128	0.438

r\*: Spearman correlation test

**Table 5.** Correlation between vertebral BMD levels with other variables

	BMD (L1-L4)			
	PMOP		PMNOP	
	r*	p Value	r*	p Value
Serum miR-21 expression (copies/ $\mu$ L x 1010)	-0.669	< 0.001	0.231	0.078
Sclerostin (pmol/L)	0.086	0.516	0.038	0.776
TGF- $\beta$ 1 (pg/mL)	-0.083	0.531	-0.085	0.521
RANKL (pmol/L)	-0.726	< 0.001	-0.063	0.633
OPG (pmol/L)	0.241	0.065	0.144	0.274
RANKL/OPG Ratio	-0.723	< 0.001	-0.078	0.553
Calcium (mg/dL)	0.023	0.861	0.151	0.251
Total physical activity (score)	0.326	0.011	-0.091	0.495
IMT (kg/m <sup>2</sup> )	-0.096	0.467	-0.061	0.643

r\*: Spearman correlation test

### Correlation Between Serum MiR-21 Expression with Variables Measured

The correlation of serum miR-21 expression with other variables in PMOP and PMNOP groups is shown in **Table 3**. In the PMOP group, expression of miR-21 has a significantly positive correlation with both RANKL and RANKL/OPG ratio and a significantly negative correlation with moderate activity and BMD. In PMNOP group, the miR-21 expression has a negative correlation with moderate physical activity.

We also analyzed the correlation between miR-21 with other variables in the subject with a low estrogen level (<11.8 pg/mL) shown in **Table 4**. We found that in the PMOP group with low

estrogen level, miR-21 has a positive correlation with RANKL level and RANKL/OPG ratio, and a negative correlation with TGF- $\beta$ 1, OPG, and BMD. Both findings are statistically significant. On the other hand, there is no statistically significant correlation between the entire variable in PMNOP group with low-level estrogen.

Moderate physical activities have a negative correlation with serum miR-21 expression in both groups. In PMNOP group, moderate physical activities also have a positive correlation with OPG levels ( $r=0.404$ ,  $p=0.004$ ) We also found out that, OPG levels also have a positive correlation with body weight ( $r=0.420$ ;  $p=0.002$ ).

In the PMOP group, serum miR-21 expression

has a positive correlation with RANKL levels and RANKL/OPG ratio, and it has a negative correlation with OPG and TGF- $\beta$ 1. OPG level is positively correlated with TGF- $\beta$ 1 level ( $r = 0.449$ ,  $p = 0.004$ ). In PMNOP group, OPG level has a significantly negative correlation with RANKL/OPG ratio ( $r = -0.533$ ,  $p = <0.001$ ).

### Correlation Between BMD with Other Variables

Correlation between BMD lumbar (L1-L4) as a dependent variable with other variables measured are shown in **Table 5**. In PMOP group BMD has a negative correlation with serum miR-21 expression, RANKL level, and RANKL/OPG ratio, and has a positive correlation with total physical activity. On the other hand, there is no significant correlation between BMD with other variables in PMNOP group.

### Multiple Linear Regression Analysis of MiR-21 with Bone Mineral Density

In order to see the role of miR-21 to vertebral BMD, we did a multiple multivariate linear regression analysis with vertebral BMD as a dependent variable and serum miR-21 expression, RANKL, OPG, and total physical activity as independent variables. By using a backward stepwise method, we acquired linear regression equation for BMD based on serum miR-21 expression levels and RANKL levels,  $BMD = 1.373 - 0.085 \times \text{Ln. miR-21} - 0.176 \times \text{Log}_{10}\text{RANKL}$ , ( $R^2 = 52.5\%$ ).

We tried to find the cutoff point of serum miR-21 expression between PMOP and PMNOP group, in order to make basic data that will be useable to develop further research in the future. Based on that data, we acquired the cutoff point for miR-21 in both groups is 30.1-copies/ $\mu\text{L} \times 1010$ . Based on this cutoff point, we found that there are 42 patients (72%) in PMOP group and 19 patients (31%) in PMNOP group who had a higher result of cutoff point miR-21 expression.

## DISCUSSION

This study revealed that serum miR-21 expression in the PMOP group is significantly higher than PMNOP group. It also significantly negatively correlated with vertebral BMD. Sun et al.<sup>10</sup> also discovered an up-regulation of miR-

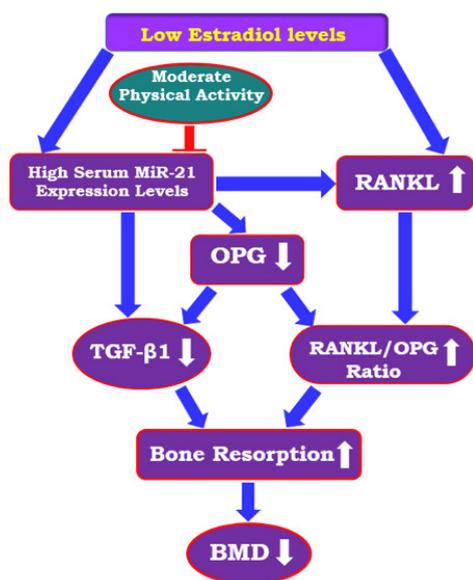
21 expression in postmenopausal osteoporotic patients than without osteoporosis. Lian et al.<sup>11</sup> also discovered an increase of serum miR-21 expression in 15 postmenopausal women with pelvic fracture than 12 post-menopausal women with severe osteoarthritis which need a prosthetic implant. However, Sugatani et al.<sup>12-13</sup> should a positive correlation of miR-21 expression with vertebral BMD value. Yavropoulo et al.<sup>14</sup> also found lower expression of serum miR-21 expression in 35 postmenopausal women with low BMD levels accompanies with at least one vertebral fracture than 35 low BMD levels postmenopausal woman without any vertebral fracture.<sup>15-17</sup> These different outcomes compared to our study can be due to the different number of patient involvement in the study. In addition, this study also did not measure the estrogen level of the study subject.

Serum miR-21 has a positive correlation with RANKL levels, this indicates that miR-21 overexpression led to an increase in RANKL levels which will affect bone mineral density. Serum miR-21 expression has a negative correlation with OPG level. High expression of miR-21 will cause a decrease in OPG concentration. This is similar to a study done by Pitari et al.<sup>19</sup>, up-regulation of miR-21 will cause down-regulation of OPG. In PMOP group, OPG level is lower than PMNOP group. Osteoprotegerin (OPG) act as a decoy in RANK-RANKL signal which will inhibit the formation and suppress the life span of the osteoclast.<sup>19-21</sup>

In this study, the RANKL/OPG ratio in the PMOP group is higher than PMNOP and statistically significant. This is similar to a study conducted by Xu et al.<sup>22</sup>, which also found an increase of RANKL/OPG ratio in the PMOP group. High RANKL/OPG ratio will also increase the number of osteoclasts and resorptive activity of the bone. In addition to miR-21, we found out that serum miR-21 expression has a positive correlation with the RANKL/OPG ratio. This also indicates that overexpression of miR-21 will lead to an increase of RANKL/OPG ratio which later will a decrease of BMD.

Serum TGF- $\beta$ 1 levels are statistically significantly lowered in the PMOP group. Several previous studies reported that TGF- $\beta$ 1

has a protective role against osteoporosis and assist bone recovery. Wu and colleagues found that TGF- $\beta$ 1 levels in postmenopausal women are lower than premenopausal women.<sup>22</sup> TGF- $\beta$ 1 regulates osteoblast and osteoclast differentiation, producing a balance in bone formation and resorption.<sup>23</sup> We discovered that serum miR-21 expression has a negative correlation with the TGF- $\beta$ 1 level in PMOP group. Overexpression of serum miR-21 will decrease serum TGF- $\beta$ 1 concentration.



**Figure 1.** Proposed mechanism of miR-21 role on bone mineral density in hypoestrogenic postmenopausal women with osteoporosis.

We also measure physical activity score between the two groups. We found that moderate physical activity has a negative correlation with serum miR-21 expression and have a positive correlation with OPG levels in both groups. These suggest that moderate physical activity can suppress miR-21 expression and promote OPG production that will lead to reduce of bone resorption and normalize BMD.

Based on the above result, we proposed a mechanism scheme of miR-21 role with bone mineral density in hypoestrogenic postmenopausal women with osteoporosis. Low estrogen levels in postmenopausal women cause an increase of serum miR-21 expression. High expression of mir-21 increases RANKL production and decreases OPG and TGF- $\beta$ 1

levels. Increasing of RANKL levels followed by the reduction of OPG levels led to an increase of RANKL/OPG ratio. All of that will eventually increase bone resorption and reduce BMD, causing osteoporosis. Moderate physical activity inhibits serum miR-21 expression; this lower the effect of miR-21 expression to BMD compare to RANKL. **(Figure 1)**

We acquired a linear progression formula  $BMD = 1,373 - 0,085 * \text{Ln.miR-21} - 0,176 * \text{Log } 10.\text{RANKL}$ . Based on the formula, every unit of miR-21 expression will decrease BMD for about 8.5%. Meanwhile, RANKL will decrease BD for about 17.6%. The formula able to predict the BMD value for 52.5% ( $R^2 = 52.5\%$ ). This finding may able to allow a potential combined therapy of miR-21 and RANKL in Osteoporosis.

We also measured other variables such as sclerostin and calcium levels. We found that sclerostin levels are higher in the PMOP group than PMNOP. Calcium levels are higher in PMNOP group than PMOP. Both variables don't have a significant correlation with serum miR-21 expression.

This study has several limitations. The first limitation is we did not measure other parameters that also play an important role in bone remodeling, which may affect BMD values such as IL-1, IL-6, TNF- $\alpha$ , and nutritional intake. Another limitation, we did not perform serum miR-21 test in subjects who are still in menstrual age or premenopausal so that the term of overexpression cannot be determined. The last limitation, it is still unknown whether the association between miR-21 expression and BMD values also reflects bone metabolic activity because we did not evaluate bone resorption and bone formation markers.

## CONCLUSION

Serum miR-21 expression in hypoestrogenic postmenopausal women with osteoporosis has a higher expression compared with no osteoporosis. Serum miR-21 expression proved to have a negative effect on spinal BMD values in hypoestrogenic postmenopausal women with osteoporosis of 8.5%, with linear regression equation  $BMD = 1.373 - 0.085 * \text{Ln.miR-21} - 0.176 * \text{Log } 10.\text{RANKL}$ . This equation can explain the value of spinal BMD by 52.5%.

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