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Genetic predispositions and the short- and long-term effects of hormonal therapy on bone mineral density in girls with functional hypothalamic amenorrhoea

Predyspozycje genetyczne oraz krótko-i długoterminowe efekty terapii hormonalnej na gęstość mineralną kości u dziewcząt z brakiem miesiączki typu funkcjonalnego

Elżbieta Sowińska-Przepiera[1,2], Kornel Chełstowski[3], Zbigniew Friebe[2], Anhelli Syrenicz[1]

[1]Department of Endocrinology, Metabolic Diseases and Internal Diseases, Pomeranian Medical University, Szczecin, Poland
[2]Department of Gynaecology, University of Medical Sciences, Poznan, Poland
[3]Department of Laboratory Diagnostics and Molecular Medicine, Pomeranian Medical University, Szczecin, Poland

Abstract

Introduction: The aim of this study was to verify if genetic factors influence the short- and long-term therapeutic responses to oestroprogestagen (OP) therapy, implemented in girls with functional hypothalamic amenorrhoea (FHA) in order to improve their bone mineral density (BMD).

Material and methods: The study included 78 FHA girls who underwent a four-year sequential OP therapy with 17-beta oestradiol and didrogesterone. Changes in the lumbar spine BMD were determined at the end of the therapy and six years after its discontinuation, and analysed in regards to *PvuII* and *XbaI* polymorphisms of oestrogen receptor-alpha gene, *BsmI* polymorphism of vitamin D_3 receptor gene, and *Sp1* polymorphism of the type-1 collagen gene.

Results: After four years of OP therapy, a significant increase in BMD was documented in the studied group. Follow-up densitometry performed six years after completing the therapy revealed a significant decrease in BMD level; nonetheless, the values of this parameter were still significantly higher compared to pretreatment level. Neither the particular polymorphisms nor their combinations influenced the relative change in BMD at the end of the therapy and after a six-year follow-up.

Conclusions: Variability of genes involved in oestrogen, vitamin D_3 and collagen metabolism does not influence the short- and long-term results of OP therapy in girls with FHA. **(Endokrynol Pol 2012; 63 (6): 420–426)**

Key words: *bone mineral density, functional hypothalamic amenorrhoea, hormone replacement therapy, osteoporosis*

Streszczenie

Wstęp: Celem niniejszej pracy było ustalenie, czy czynniki genetyczne wpływają na bliskie i odległe wyniki terapii estrogenowo-progestagenowej, zastosowanej w celu normalizacji gęstości mineralnej kości (BMD, *bone mineral density*) u dziewcząt z brakiem miesiączki typu funkcjonalnego (FHA, *functional hypothalamic amenorrhoea*).

Materiał i metody: Badaniem objęto 78 dziewcząt z FHA poddanych 4-letniej sekwencyjnej terapii estrogenowo-progestagenowej 17-beta estradiolem i didrogesteronem. Wielkość zmian w BMD odcinka lędźwiowego kręgosłupa oceniano po zakończeniu terapii oraz 6 lat później. Uzyskane wyniki analizowano w odniesieniu do występowania u pacjentek: polimorfizmów *PvuII* i *XbaI* genu receptora estrogenowego alfa, polimorfizmu *BsmI* genu receptora witaminy D_3, oraz polimorfizmu *Sp1* genu kolagenu typu 1.

Wyniki: Po 4 latach terapii estrogenowo-progestagenowej w badanej grupie odnotowano znamienny wzrost BMD. Kontrolne badanie densytometryczne wykonane po 6 latach od zakończenia leczenia wykazało znamienny spadek poziomu BMD; tym niemniej, wartości tergo parametru były wciąż istotnie wyższe w porównaniu z poziomem przed rozpoczęciem terapii. Na względną zmianę poziomu BMD bezpośrednio po zakończeniu leczenia oraz 6 lat później nie wpływało w sposób istotny występowanie żadnego z analizowanych polimorfizmów ani ich kombinacji.

Wnioski: Zmienność genów zaangażowanych w przemiany estrogenów, witaminy D_3 i kolagenu nie wpływa na bliskie i odległe wyniki terapii estrogenowo-progestagenowej u dziewcząt z FHA. **(Endokrynol Pol 2012; 63 (6): 420–426)**

Słowa kluczowe: *gęstość mineralna kości, brak miesiączki typu funkcjonalnego, terapia hormonalna, osteoporoza*

Introduction

Functional hypothalamic amenorrhoea (FHA) is a psychosomatic disorder causing impairment in the ovarian oestrogen synthesis. According to the few available reports, the prevalence of FHA in developed countries is estimated at between 2.6% and 8.5% [1].

Besides disrupting sexual maturation, oestrogen deficiency affects negatively osteogenesis in FHA girls. This unfavourable effect results from antiresorp-

Elżbieta Sowińska-Przepiera M.D., Department of Endocrinology, Metabolic Diseases and Internal Diseases, Pomeranian Medical University, Unii Lubelskiej St. 1, 71–252 Szczecin, Poland, tel.: +48 91 425 35 40, fax: +48 91 425 35 42, e-mail: elasowprzep@wp.pl

tive and anabolic influences of oestrogens on skeletal tissue [2, 3].

Consequently, hormonal substitution constitutes the accepted direction in the management of FHA patients; this treatment is aimed at the gradual normalisation of the hypothalamic-pituitary-ovarian axis function [4, 5]. The effectiveness of this approach, in terms of bone mass levels in FHA girls, has been the subject of several studies [6–11] including research conducted by our group [12–14]. In these studies, implementation of long-term oestroprogestagen (OP) therapy was reflected by an increase in bone mineral density (BMD) of replacement patients. Although our previous experiments revealed a significant variability in the degree of therapeutic responses to OP [13], we failed to confirm the influence of genetic factors on the therapeutic outcomes [12, 14].

One should remember however, that both of the abovementioned studies examined the effects of genetic factors on bone mineral density determined at the end of hormonal treatment: consequently, we were not able to analyse long-term changes in BMD that occurred with time after discontinuation of the therapy. Thus, when data from long-term follow-up of the same patients became available, we decided to verify if genetic factors influence the long-term outcomes of hormonal treatment with regard to bone mineral density. In order to analyse the entire genetic background of osteogenesis, aside from testing the previously studied polymorphisms of ER-alpha and VDR genes, the variability of the type-1 collagen (*COLIA1*) gene was also considered. Moreover, genetic influences on BMD were tested with univariate and multivariate analysis.

Material and methods

Patients

The study included 78 girls with FHA, aged 16 and 17, who were treated at the Department of Gynaecology, University of Medical Sciences in Poznan (Poland) between 2004 and 2009, and followed-up for six years after completing the therapy. Detailed characteristics of the study participants are summarised in Table I. All procedures were approved by the Local Ethics Committee of the University of Medical Sciences in Poznan. Both the subjects and their parents gave their informed consent before the start of any procedure.

Qualification criteria for this study included: 1) at least six months of amenorrhoea preceded by at least three years of oligomenorrhoea; and 2) psychological problems confirmed by a clinical psychologist. The exclusion criteria, based on medical history and standardised questionnaire survey [13], included the following: 1) polycystic ovary syndrome, congenital adrenal hyperplasia or premature ovarian failure; 2) low birth weight or preterm birth; 3) at least one confirmed episode of eating disorders; 4) malnutrition during childhood or puberty; 5) history of impaired growth and body mass gain; 6) extensive participation in sports that may have influenced bone mineralisation; 7) metabolic disorders that may be associated with decreased bone mineralisation; 8) prolonged use of stimulants or drugs that may affect bone metabolism; 9) familial history of osteoporosis; and 10) incomplete 10-year follow-up (cessation of treatment due to medical indications or because of other reasons, failure to return for the follow-up visit).

Intervention

The patients underwent a four-year sequential OP therapy with a preparation which included the natural female sex hormone 17-beta oestradiol (2 mg from the 2^{nd} to 25^{th} day of the menstrual cycle) and didrogesterone (10 mg from the 16^{th} to the 25^{th} day of the menstrual cycle). The goal of the treatment was the resumption of regular menstrual bleeding and, therefore, the therapy was continued for a period of four years. In addition to hormonal treatment, patients were encouraged to modify their lifestyles and dietary habits. They were prescribed calcium and vitamin D_3 preparations at individual doses adjusted for their dietary content and season of the year. Moreover, regular physical activity (15 minutes of recreational gymnastics twice a day) was recommended. During the follow-up visits, all the patients declared that they had adhered to these recommendations.

Densitometry

BMD of the lumbar spine (L_2–L_4) was measured prior to the therapy, after each 12 months of treatment, and six years after its discontinuation. BMD measurements were performed based on the DXA method (GE Lunar Prodigy Advance, Madison, WI, USA; software enCORE version 8.8), using an automatic scan mode. Results were presented as absolute values (g/cm^2) and relative changes (%).

Testing for genetic polymorphisms

Samples of venous blood (10 mL) were collected from every patient and stored in EDTA anticoagulant in order to extract genomic DNA from peripheral blood mononuclear cells using a non-enzymatic inorganic method [15]. *PvuII* and *XbaI* polymorphisms of oestrogen receptor-alpha gene [16], *BsmI* polymorphism of vitamin D_3 receptor gene [17], and *Sp1* polymorphism of the collagen type-1 gene [18] were analysed in this material.

Table I. *Detailed characteristics of the study participants prior to OP therapy (year 0), at its completion (year 4), and six years thereafter (year 10); all values expressed as mean ± SD*

Tabela I. *Szczegółowe charakterystyki uczestniczek badania przed rozpoczęciem terapii OP (0 lat), po jej zakończeniu (4 lata) oraz sześć lat później (10 lat); wszystkie wartości przedstawione jako średnie ± SD*

Parameter	Year 0	Year 4	Year 10
Age (years)	16.70 ± 1.16	20.70 ± 1.16	26.67 ± 1.14
Height [cm]	162.27 ± 6.63	164.18 ± 6.38	165.27 ± 7.06
Weight [kg]	49.21 ± 6.23	54.31 ± 4.52	57.36 ± 6.45
BMI [kg/m^2]	18.68 ± 2.05	20.19 ± 1.80	21.33 ± 2.40

BMI — body mass index

Table II. *BMD levels prior to OP therapy (year 0), at its completion (year 4), and six years thereafter (year 10)*

Tabela II. *Poziom BMD przed rozpoczęciem terapii OP (0), po jej zakończeniu (4 lata) oraz sześć lat później (10 lat)*

Year	BMD			Change in BMD (%)		
	Mean	SD	Range	Mean	SD	range
0	0.816	0.088	0.548–0.987	–	–	–
4	0.998*	0.097	0.799–1.172	23.3	14.3	(–) 1.5–63.3
10	0.944**	0.109	0.646–1.155	–5.3	7.8	(–) 34.1–16.7

*,**significantly different compared to other periods ($p < 0.001$, Friedman ANOVA); BMD — bone mineral density

Statistical evaluation of the results

Continuous variables were presented as arithmetic means and their standard deviations (SD); their normal distribution was tested using the Shapiro-Wilk test. Arithmetic means of continuous variables between groups of patients determined based on certain genotypes and their combinations were compared with ANOVA and the Tukey post-hoc test. The significance of time changes in analysed variables was tested with the Friedman ANOVA. Additionally, the effects of studied gene polymorphisms on therapeutic responses were verified in a logistic regression model. All calculations were performed using Statistica 8 (StatSoft®, Poland) software, and statistical significance was defined as $p \leq 0.05$.

Results

All patients resumed spontaneous menstrual bleeding as a result of hormonal treatment. After four years of OP therapy, a significant increase in BMD was documented in the studied group. However, follow-up densitometry performed six years after completing the therapy revealed a significant decrease in BMD level; nonetheless, the values of this parameter were still significantly higher compared to the pretreatment level (Table II).

None of the single gene polymorphisms was revealed to influence significantly the relative change in BMD level after four years of the therapy. Furthermore, the effects of polymorphisms on treatment outcomes were also not observed in multivariate analysis (Table III) and subanalysis of ER-alpha gene haplotypes.

Based on the median relative change in BMD after four years of the therapy (21.9%), two subgroups of patients with a different therapeutic response were distinguished (relative increase in BMD ≤ 22%, n = 40 *v.* > 22%, n = 38). The single polymorphisms that were analysed were not found to determine a poorer therapeutic response (VDR: OR = 1.09, 95% CI 0.66–1.81, p = 0.718; *XbaI*: OR = 1.07, 95% CI 0.65–1.77, p = 0.782; *PvuII*: OR = 1.34, 95% CI 0.75–2.37, p = 0.320; *COLIA1*: OR = 0.83, 95% CI 0.28–2.46, p = 0.728). Furthermore, the significant effects of variability in studied genes were not observed in multivariate analysis (VDR: OR = 1.09, 95% CI 0.66–1.83, p = 0.727; *XbaI*: OR = 0.77, 95% CI 0.35–1.71, p = 0.519; *PvuII*: OR = 1.69, 95% CI 0.69–4.14, p = 0.251; *COLIA1*: OR = 0.83, 95% CI 0.28–2.51, p = = 0.739) and in subanalysis of ER-alpha gene haplotypes (*XbaI*: OR = 0.75, 95% CI 0.34–1.65, p = 0.470; *PvuII*: OR = 1.71, 95% CI 0.70–4.21, p = 0.237).

Moreover, variability of any single gene did not modulate significantly the level of relative decrease in BMD observed six years after the discontinuation of the therapy. Significant effects of variability in studied genes on long-term therapeutic outcomes were also not documented in multivariate analysis (Table IV), or in subanalysis of the influences of ER-alpha gene haplotypes.

Based on median decrease in BMD six years after the completion of the therapy (5.8%), two groups of patients

Table III. *Effects of VDR, XbaI, PvuII and COLIA1 gene polymorphisms on relative change in BMD observed after four years of OP therapy (ANOVA, p = 0.162)*

Tabela III. *Wpływ polimorfizmów VDR, XbaI, PvuII i COLIA1 na względną zmianę poziomu BMD po czterech latach terapii OP (ANOVA, p = 0,162)*

Genotype				n	Change in BMD (%)	
VDR	*XbaI*	*PvuII*	*COLIA1*		Mean	SD
bb	*xx*	*Pp*	*SS*	4	30.3	8.9
bb	*xx*	*Pp*	*Ss*	1	29.1	–
bb	*xx*	*PP*	*SS*	3	22.1	8.6
bb	*xx*	*Pp*	*SS*	2	19.3	2.0
bb	*XX*	*PP*	*SS*	8	18.5	9.2
bb	*Xx*	*Pp*	*SS*	1	39.7	–
bb	*Xx*	*PP*	*SS*	5	12.9	12.7
bb	*Xx*	*Pp*	*SS*	3	41.2	20.3
bb	*Xx*	*Pp*	*Ss*	2	22.8	7.3
BB	*xx*	*Pp*	*Ss*	1	31.2	–
BB	*xx*	*Pp*	*SS*	3	22.1	5.8
BB	*xx*	*Pp*	*Ss*	2	33.1	2.5
BB	*xx*	*PP*	*SS*	3	19.5	16.8
BB	*XX*	*PP*	*Ss*	1	0.5	–
BB	*Xx*	*Pp*	*SS*	4	25.2	22.1
BB	*Xx*	*Pp*	*Ss*	1	45.1	–
Bb	*xx*	*Pp*	*Ss*	1	25.6	–
Bb	*xx*	*Pp*	*SS*	9	12.4	9.0
Bb	*xx*	*Pp*	*Ss*	3	25.4	17.4
Bb	*xx*	*PP*	*SS*	4	31.2	22.9
Bb	*XX*	*PP*	*SS*	4	18.4	3.2
Bb	*XX*	*Pp*	*SS*	1	8.5	–
Bb	*Xx*	*PP*	*Ss*	1	45.8	–
Bb	*Xx*	*Pp*	*SS*	9	29.3	16.4
Bb	*Xx*	*Pp*	*Ss*	2	20.9	1.3

with different long-term outcomes were distinguished (relative decrease in BMD ≤ 6%, n = 41 *v.* > 6%, n = 37). We did not observe negative effects of any single polymorphism on long-term therapeutic response (VDR: OR = 1.16, 95% CI 0.70–1.93, p = 0.551; *XbaI*: OR = 1.01, 95% CI 0.61–1.67, p = 0.959; *PvuII*: OR = 0.96, 95% CI 0.54–1.70, p = 0.893; *COLIA1*: OR = 1.44, 95% CI 0.48–4.37, p = 0.510). Significant effects of studied genes' variability were also not confirmed in multivariate analysis (VDR: OR = 1.16, 95% CI 0.70–1.93, p = 0.560; *XbaI*: OR = 1.09, 95% CI 0.50–2.36, p = 0.829; *PvuII*: OR = 0.89, 95% CI 0.37–2.14, p = 0.796; *COLIA1*: OR = 1.41, 95% CI 0.46–4.32, p = 0.540) and in subanalysis of ER-alpha gene haplotypes (*XbaI*: OR = 1.09, 95% CI 0.51–2.35, p = 0.816; *PvuII*: OR = 0.89, 95% CI 0.37–2.12, p = 0.792).

Discussion

The results of this study revealed that the administration of four-year hormonal therapy is reflected by an improvement in BMD of FHA girls. While the control densitometry performed six years after the completion of the treatment documented a significant decrease in bone mineral density, this parameter still remained significantly higher compared to its pretreatment values.

We have previously revealed that directly after completing the hormonal treatment, BMD of FHA patients is still lower than in healthy peers from the control group; furthermore, we have documented inhomogeneous therapeutic response of FHA girls to hormonal therapy [13]. Consequently, we have proposed that the degree

Table IV. *Effects of VDR, XbaI, PvuII and COLIA1 gene polymorphisms on relative change in BMD observed six years after completing OP therapy (ANOVA, p = 0.083)*

Tabela IV. *Wpływ polimorfizmów VDR, XbaI, PvuII i COLIA1 na względną zmianę poziomu BMD po sześciu latach od zakończenia terapii OP (ANOVA, p = 0,083)*

Genotype				n	Change in BMD (%)	
VDR	*XbaI*	*PvuII*	*COLIA1*		Mean	SD
bb	*xx*	*Pp*	*SS*	4	–9.3	6.1
bb	*xx*	*Pp*	*Ss*	1	1.5	–
bb	*xx*	*PP*	*SS*	3	–5.3	3.5
bb	*xx*	*Pp*	*SS*	2	-8.8	8.2
bb	*XX*	*PP*	*SS*	8	–6.7	6.2
bb	*Xx*	*Pp*	*SS*	1	–9.0	–
bb	*Xx*	*PP*	*SS*	5	–7.5	5.3
bb	*Xx*	*Pp*	*SS*	3	–10.3	9.2
bb	*Xx*	*Pp*	*Ss*	2	–13.1	5.5
BB	*xx*	*Pp*	*Ss*	1	0.0	–
BB	*xx*	*Pp*	*SS*	3	0.5	12.0
BB	*xx*	*Pp*	*Ss*	2	–7.5	8.5
BB	*xx*	*PP*	*SS*	3	–8.0	6.0
BB	*XX*	*PP*	*Ss*	1	10.0	–
BB	*Xx*	*Pp*	*SS*	4	–1.4	9.5
BB	*Xx*	*Pp*	*Ss*	1	–5.2	–
Bb	*xx*	*Pp*	*Ss*	1	–34.1	–
Bb	*xx*	*Pp*	*SS*	9	–0.4	10.4
Bb	*xx*	*Pp*	*Ss*	3	–5.1	2.4
Bb	*xx*	*PP*	*SS*	4	–6.3	4.6
Bb	*XX*	*PP*	*SS*	4	–3.9	3.8
Bb	*XX*	*Pp*	*SS*	1	–9.6	–
Bb	*Xx*	*PP*	*Ss*	1	–1.4	–
Bb	*Xx*	*Pp*	*SS*	9	–5.0	5.4
Bb	*Xx*	*Pp*	*Ss*	2	–0.8	7.7

of therapeutic response is at least partially determined by polymorphism of genes involved in the control of osteogenesis. However, our further studies have not unequivocally confirmed the role of oestrogen receptor-alpha gene polymorphism in this setting [14], and have excluded the involvement of vitamin D_3 receptor gene variability in this process [12]. This study has provided further evidence that genetic factors have a minor impact on the normalisation of bone mineral density impaired due to psycho-emotional and secondary hormonal disorders. The level of long-term therapeutic response to hormonal treatment was not modulated either by polymorphisms in oestrogen receptor-alpha gene or by the variability of genes encoding vitamin D_3 receptor and collagen type-1.

A thorough search through the literature did not yield any published research analysing the relationship between the genetic factors and the degree of changes in bone mineral density induced by hormonal treatment. Furthermore, a review of literature dealing with the genetic determinants of bone mineral density in women revealed marked discrepancies between the results of previous studies. In one study, pre-pubertal girls with the *PP PvuII* genotype of ER-alpha gene had a nearly 5-fold higher relative risk of fractures and a significantly lower BMD of the spine compared to their peers with the *pp* genotype [19]. However, studies of postmenopausal women using hormone replacement therapy did not distinguish which of the genetic variants of ER-alpha is a predictor of worse therapeutic response

in the context of bone mineral density normalisation. Depending on the study, either the *xxPP* haplotype [20, 21] or *XX Xbal* [22, 23] and *PP Pvull* genotypes [23] were found to be the negative predictive factors. Furthermore, no relationships between ER-alpha genotype and the response to hormonal treatment were observed by several authors [24–26].

Similar discrepancies pertain to the effects of the VDR receptor gene polymorphism on BMD level. The variability of the different sites at intron 8/exon 9 of this gene, *BsmI*, *ApaI* and *TaqI*, and the *FokI* site at exon 2.3, was analysed during previous experiments with various restriction enzymes. The most unambiguous data regarding the involvement of the VDR gene in the determination of bone mineral density pertains to the polymorphism of the *FokI* region. Several authors have observed that the *FF* genotype is associated with better calcium absorption, while the *ff* genotype frequently coexists with bone mass deficiencies. However, the existing evidence of the role of *BsmI* polymorphism, analysed in this study, is conflicting. Some authors have observed that the highest BMD levels are found among carriers of homozygous genotypes of this polymorphism, *BB* or *bb*, while others have shown the *Bb* genotype to be predictive for high bone mineral density [17, 27–32].

The results of previous studies of the role of *Sp1* polymorphism of *COLIA1* suggest that variability in this region of collagen type-1 gene can influence bone mass, particularly in the context of osteoporotic risk in old age. However, the results of the multicentre GENOMOS study suggest that such a relationship is not directly linked to BMD [33].

Interpreting the results of our research, one should consider the potential limitations related to the interventional character of this study. The first potential flaw pertains to the relatively small sample size related to the fact that some participants did not complete the four-year course of the therapy and some patients failed to attend follow-up visits. Another potential limitation is the lack of a randomly selected control group consisting of FHA girls who did not receive hormonal treatment and/or were subjected to a different type of intervention. Due to this limitation, we were unable to distinguish to what extent the observed improvement in bone mineral density resulted from spontaneous normalisation of osteogenesis.

Nonetheless, these aforementioned flaws did not constitute an obstacle to attaining the principal objective of this study. Both our findings and the literature evidence suggest limited clinical application of genetic tests in determining potential predictors of bone mass response to hormonal treatment. In view of the current evidence, we are not able to determine whether genetic factors *per se* do not affect osteogenesis in adolescent girls, or whether the lack of such dependence can be attributed to the shortcomings of the currently used tests or incorrect interpretation of their results. Although this distinction is vital from the scientific point of view, it is of lesser importance for clinical practitioners. From the clinical viewpoint, the principal implication of the current and previous studies is the negligible usefulness of genetic predisposition analysis in planning activities aimed at normalisation of bone mass in FHA girls. In view of the current evidence, the optimal approach to this group of patients includes the administration of hormonal therapy, along with modification of external factors that can modulate the level of bone mass — particularly supplementation with vitamin D_3 and calcium preparations, and properly adjusted levels of regular physical activity [34].

Conclusions

This study revealed that the variability of genes involved in oestrogen, vitamin D_3 and collagen metabolism does not influence the short- and long-term results of OP therapy in girls with FHA.

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