Bone density and body weight is associated with MTHFR677 polymorphism in girls with anorexia nervosa

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ABSTRACT

Introduction: Anorexia nervosa (AN) is associated with dysfunction of the hypothalamus-pituitary-gonadal axis, and thus with adverse effects on skeletal integrity. We aimed to identify the combination of polymorphisms linked to deterioration of bone health in a sample of adolescent girls with AN.

Patients and Methods: The sample of this cross-sectional study consisted of 40 young girls (12-21 years old), diagnosed with AN according to the American Psychiatry Association criteria. A detailed medical history was recorded for each participant and blood samples were taken for hormonal evaluation and genotyping. Lumbar spine bone density (bone mineral density, BMD) was evaluated using dual energy X-ray absorptiometry.

Results: Lower BMD values were observed in girls with: i) presence of the CTR-AluI polymorphism vs wild type (BMD, CC&CT vs TT genotypes: 0.84±0.21g/cm^2 vs 0.96±0.12g/cm^2, p-value=0.028); ii) presence of the MTHFR677T polymorphism vs wild type (BMD, TT&CT vs CC genotypes: 0.86±0.23g/cm^2 vs 0.94±0.11g/cm^2, p-value=0.047, adjusted for age, BMI, amenorrhoea). BMD measures exhibited a graded stepwise decrease with the addition of the CTR-AluI and/or the MTHFR677 polymorphic variant (BMD, wild type vs one vs two polymorphic variants: 0.97±0.11g/cm^2 vs 0.90±0.11g/cm^2 vs 0.75±0.33g/cm^2, p-value for linear trend 0.011). Girls carrying the MTHFR677 polymorphism had 5.67-times higher odds of having a higher BMI (BMI >16.4kg/m^2 vs ≤16.4kg/m^2, MTHFR677 polymorphism, CT&TT vs CC genotypes: OR=5.667, 95% CI: 1.254—25.606, p-value=0.024).

Conclusion: Combined presence of the MTHFR677 and CTR-AluI polymorphisms is associated with lower bone density in young girls with AN, implying a dose-response effect. The association between MTHFR677 and bone metabolism is likely mediated by body weight.

KEYWORDS

Bone density, body mass index, CTR-AluI genetic polymorphism, MTHFR C677T genetic polymorphism, anorexia nervosa.

Introduction

Even though rarely encountered among the general population, eating disorders are relatively common among teenage girls and young women. Anorexia nervosa (AN) is an eating disorder of unknown aetiology [1]. The incidence rate of AN in adolescent girls has been reported as 270 cases per 100,000 person years, while the lifetime prevalence has been reported as 2.2% [2]. This state of low energy availability results in dysfunction of the hypothalamus-pituitary-gonadal axis and hypothalamus-pituitary-adrenal axis [3]. The hormonal consequences of this are hypoestrogenism, hypercortisolemia and insulin growth factor-1 deficiency [3]. These hormonal alterations have been linked to significant adverse effects on health and well-being, including impaired skeletal integrity [3,4]. The link between AN and bone health has been investigated extensively [4,5]. This chronic state of malnutrition has been linked to osteopenia in up to 51.7% of affected cases and with osteoporosis in up to 34.6% 6. Moreover, females with AN have an approximately 1.6-times higher risk of fracture at all ages [7]. However, the deterioration of bone density is not uniform among affected individuals [6,7], implying that the association between bone health and AN might be influenced by other factors, not as yet explored. Secondary osteoporosis or bone density below the expected range for age is commonly linked to genetic background.

Single-nucleotide polymorphisms (SNPs) have been linked to genes encoding important pathways of bone metabolism. These genes include the calcitonin receptor (CTR) [8], estrogen

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receptor alpha (ESR1) [9,10], collagen type 1 (COL1A1), and methylenetetrahydrofolate reductase (MTHFR) genes [11-14]. As previously shown, presence of the ESR1 polymorphic variants is an important predicting factor of lower bone density in girls with AN [15]. We hypothesized that a combination of SNPs linked to one or more genes that represent important regulators of bone metabolism might predispose girls with AN, already in a hypoestrogenic state, to further loss of bone mass.

Therefore, this study aimed to evaluate the link between the CTR-AluI polymorphism, the COL1A1-Sp1 polymorphism, the ESR1-Pvull polymorphism, the ESR1-Xbal polymorphism, and the MTHFR C677T polymorphism and bone density in a sample of girls with diagnosed AN

Methods

Study sample

This pilot study recruited a total of 40 young girls (14-17 years old) from the Child and Adolescent Gynaecology Outpatient Clinic of the 2nd Department of Obstetrics and Gynecology, Aretaieio Hospital, Athens, Greece. All participants had been diagnosed with AN according to the American Psychiatry Association criteria (proposed DSM-V, 2012) [16]. The inclusion criteria included body mass index (BMI) values of 12.5-18.5kg/m^2, corresponding to values below the 5th percentile for age, as well as physical exercise amounting to no more than 2 hours per week. The exclusion criteria included the presence of current or previously diagnosed metabolic bone disease, use of hormone supplements or contraceptives, malnutrition or current smoking. All participants as well as their legal guardians signed an informed consent document and the study was approved by the Ethics Committee of Aretaieio Hospital.

Protocol study procedures

A detailed medical history was obtained from all the participants at the time of their first visit to the clinic. Moreover, all the girls completed questionnaires collecting demographic and socioeconomic characteristics, which included questions regarding self-esteem in relation to their body. Weight and height were measured using an electronic scale and a wall height meter, in the early morning with the participants dressed in light clothing. BMI was calculated using the algorithm BMI = body weight (kg) / height^2 (m).

The age-related BMI z-score was calculated using the online calculator of the Baylor College of Medicine, Children's Nutrition Research Center [17]. Fasting blood samples were collected in the morning, between 8:30 a.m. and 9:30 a.m. Blood specimens used to assess polymorphisms were collected in K3EDTA (3mL) and stored at -20°C, while those used to assess oestradiol (E2) levels were centrifuged, and the serum was stored at -80°C until assessment. In participants with normal menstruation, blood samples were collected during the 3rd–5th day of the cycle, while in participants with amenorrhoea they were collected randomly. Serum levels of E2 were determined using standard laboratory techniques.

Bone densitometry

Lumbar spine bone mass density (LBMD) was measured on the same day by means of dual energy X-ray absorptiometry (anteroposterior projection at L2-L4) performed using a Norland Excell Plus densitometer (Cooper Surgical Inc., Fort Atkinson, WI, USA). Age- and gender-matched LBMD z-scores (corrected for age and gender) were calculated using reference data from the study by Zanchetta et al. [18]. Bone density lower than expected for age was defined as BMD z-score values less than -2 [19].

DNA preparation and pyrosequencing

DNA was extracted using the Nucleospin Blood QuickPure kit (Macherey-Nagel GmbH & Co, Düren, Germany), and SNPs were detected by pyrosequencing. The primers used are shown in the supplementary table. The polymerase chain reaction (PCR) technique was applied, in 50mL reaction volumes with 2mL DNA, 1mL of each primer and 25mL Hot Start Master Mix (GE Healthcare Biosciences, Pittsburgh, PA, USA). After the initial denaturation at 95°C for 5min, amplification consisted of 30 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s and elongation at 72°C for 30s, followed by a final elongation step at 72°C for 4min. Reactions were carried out in microplates using the PyroMark Q24 system (Qiagen GmbH Hilden, Germany), and results were analysed using the PyroMark Q24 software. The following polymorphisms were analysed: the AluI polymorphism in the CTR gene (CTR-AluI, rs1801197), the Sp1 binding site polymorphism in the COL1A1 gene (COL1A1-Sp1, rs1800012,), the PvuII and XbaI polymorphisms in the ESR1 gene (ESR1-PvuII, rs2234693; ESR1-XbaI, rs934079), and the C677T polymorphism in the MTHFR gene (MTHFR C677T, rs1801133).

Statistical analysis

Statistical analysis was performed using SPSS v.25 (SPSS Inc., Chicago, IL, USA). The Chi-square test was utilized to test for the Hardy-Weinberg equilibrium, in each of the evaluated polymorphisms. All polymorphisms were evaluated comparing individuals carrying at least one mutated variant (homozygous or heterozygous) vs girls homozygous for the wild-type genotype. Qualitative variables were expressed as absolute frequencies (%) and quantitative variables were expressed as mean values and standard deviation (mean±SD) or median values and interquartile range. Differences between qualitative variables were assessed using the Chi-square test, whereas quantitative variables were compared using Student's t-test for independent variables.

Linear regression analysis was performed to determine the ability of genetic polymorphisms to predict change in BMD, which was set as the primary outcome measure of this study. The multivariate models were adjusted for age and BMI. Partial correlation coefficients were evaluated to identify, as secondary outcome measures, potential correlations between variables assessed in the multivariate analysis (i.e. genetic polymorphisms, age, BMI). In the event of significant partial correlation, we continued the investigation for a potential mediation effect of the primary hypothesis, by using simple correlation analysis

between parameters of interest (Spearman's correlation coefficient) and by evaluating differences between quantitative variables. BMI values were treated both as continuous variables and as dichotomous variables, with the median of 16.4 kg/m^2 taken as the cut-off value. Statistical significance was set at the level of p-value<0.05.

Results

This sample consisted of 40 adolescent girls, previously diagnosed with AN. Table 1a presents the descriptive analysis of the anthropometric and demographic parameters as well as BMD mean values. The genotypes of the CTR-AluI genetic polymorphism showed the following frequencies: CC vs CT vs TT: 5% (1/40) vs 30% (12/40) vs 67.5% (27/40). The genotype frequencies of the assessed genetic polymorphisms were as follows: in ESR1-Pvull, CC vs CT vs TT (wild type) was estimated as 10% (4/40) vs 55% (22/40) vs 35% (14/40); in COL1A1, TT vs GT vs GG (wild type) was estimated as 5% (2/40) vs 17.5% (7/40) vs 77.5% (31/40); in ESR1-Xbal, GG vs AG vs AA (wild type) corresponded to 7.5% (3/30) vs 47.5% (19/40) vs 45% (18/40); in MTHFR C677T, TT vs CT vs CC (wild type) was estimated as 7.5% (3/40) vs 25% (10/40) vs 67.5% (27/40).

The participants had a mean age of 15.3±1.64 years and a mean BMI of 16.3±1.41kg/m^2. In total, 30% (12/40) of the girls had bone density values lower than expected for age (BMD z-score of less than -2). Using the Chi-square test, we observed that the assessed genotypes for all polymorphisms were in Hardy-Weinberg equilibrium (p-value >0.05).

Table 1b shows mean BMD values according to the presence or absence of the evaluated polymorphic variants. Lower BMD values were observed in girls carrying the polymorphic C allele of the CTR-AluI gene when compared with those carrying the wild type (CTR-AluI polymorphic variants, CC&CT genotype vs TT genotype: 0.84±0.21g/cm^2 vs 0.96±0.12g/cm^2, p-value=0.028). Similarly, lower mean values of BMD were observed in girls carrying the mutated T allele of the MTHFR C677T polymorphism vs the wild type (MTHFR677 polymorphism, TT&CT genotype vs CC genotype: 0.86±0.23g/cm^2 vs 0.94±0.11g/cm^2, p-value=0.047, adjusted for age, BMI, presence of amenorrhoea). Aiming to further evaluate the impact of the genetic polymorphisms on bone density, we compared BMD values between girls carrying one polymorphic variant (either the CTR-AluI or the MTHFR677 polymorphism) and those carrying two polymorphic variants (both the CTR-AluI and the MTHFR677 polymorphism). As presented in Figure 1, BMD values showed a graded stepwise decrease with the addition of each polymorphic variant (BMD values, wild type vs carriers of the CTR-AluI or the MTHFR677 polymorphism vs carriers of both the

Table 1a Mean demographic/anthropometric parameters and mean values of bone density measures of the 40 adolescent girls comprising our sample.

DEMOGRAPHIC/ANTHROPOMETRIC	MEAN±SD OR FREQUENCY (%)	MEDIAN	IQR
Age (years)	15.3±1.64	15	14 – 17
BMI (kg/m^2)	16.3±1.41	16.4	15.4 – 17.4
BMI z-score	7.71±9.29	4.85	0.38 – 12.68
Prevalence of amenorrhoea	80%		
Bone density measures			
BMD (g/cm^2)	-0.92±0.16	0.93	0.84 to 0.99
z-score	-1.58±0.92	-1.54	-2.4 to -1.06
Bone density less than expected for age	30%		

BMD=bone mass density; SD=standard deviation; IQR=interquartile range.

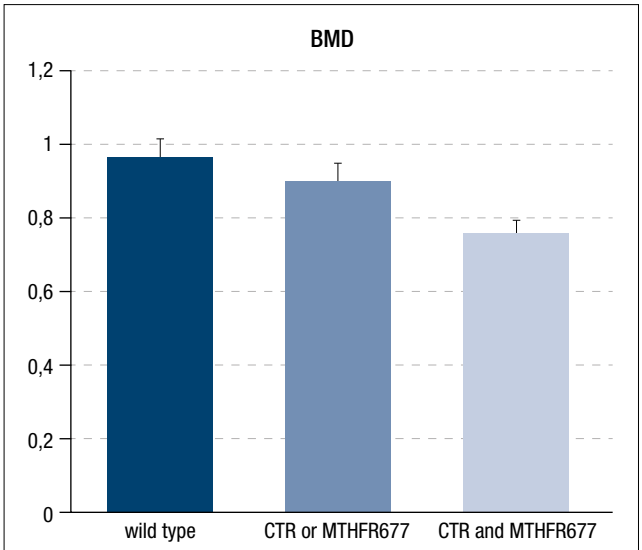
Table 1b Mean values of bone density according to the presence of genetic polymorphisms in the 40 girls with anorexia nervosa.

	BONE MASS DENSITY (g/cm^2)	MEAN	SD	P-VALUE ANOVA OR ANCOVA
CTR AluI	Presence of T allele (wt)	**0.96**	**0.12**	**0.028**
	Presence of C allele	**0.84**	**0.21**	
COL1A1-Sp1	Presence of G allele (wt)	0.91	0.17	0.657
	Presence of T allele	0.94	0.12	
ESR1-Pvull	Presence of T allele (wt)	0.92	0.12	0.885
	Presence of C allele	0.91	0.18	
ESR1-Xbal	Presence of A allele (wt)	0.90	0.07	0.674
	Presence of G allele	0.93	0.21	
MTHFR677	Presence of C allele (wt)	**0.94**	**0.11**	**0.047***
	Presence of T allele	**0.86**	**0.23**	

**Adjusted for age, BMI and presence of amenorrhoea; Bold indicates statistical significance; Statistical significance was set at the level of p-value<0.05.*

CTR-AluI and the MTHFR677 polymorphism: 0.97±0.11g/cm^2 vs 0.90±0.11g/cm^2 vs 0.75±0.33g/cm^2, p-value for linear trend 0.011, eta squared = 17.2). Multivariable linear regression analysis models were fitted to evaluate the effect of each of the significant genetic polymorphisms on bone density, adjusted for age and BMI (Table 2). We observed that presence of the CTR-AluI polymorphism associated significantly with BMD measures (CTR-AluI, CC&CT vs TT genotype, b-coefficient= -0.399, p-value=0.012), an effect also mediated by age and BMI. Similarly, the MTHFR677 polymorphism showed a borderline association with BMD measures (MTHFR677, TT&CT vs CC genotype, b-coefficient= -0.321, p-value=0.050), an association also mediated by BMI. Finally, the girls with both the CTR variant and the MTHFR677 polymorphic variant showed significantly lower BMD values than those carrying wild-type polymorphisms (CTR-AluI and MTHFR677 polymorphisms vs wild type: b-coefficient = -0.514, p-value=0.001), an effect also associated with BMI but independent of age. Partial correlation coefficients were indicative of a direct association between BMI values and the evaluated genetic polymorphisms. Aiming to further explore the direction of these associations between BMI and genetic polymorphisms in a univariate approach, we performed a simple correlation analysis using Spearman's correlation coefficient. The results showed an almost significant correlation between presence of the MTHFR677 polymorphism and BMI values (r-coefficient = 0.286, p-value = 0.074). The presence of the CTR-AluI polymorphic variant did not correlate with BMI values (r-coefficient = 0.171, p-value = 0.293). Comparing girls with the TT genotype vs the CC genotype of the MTHFR C677T polymorphism, we observed significantly higher BMI values in carriers of the T allele (TT vs CC genotype, BMI: 17.2±0.6kg/m^2 vs 16.0±1.4kg/m^2, p-value=0.045, t-test for independent samples), as well as higher values of the BMI z-score adjusted for age (TT vs CC genotype, BMI z-score adjusted for age: -1.2±0.26 vs -2.16±1.35, p-value=0.005, t-test for independent samples). We then compared the prevalence of genetic polymorphisms according to BMI, taking the median BMI value of 16.7kg/m^2 as the cut-off value. No association was observed between the presence of the CTR genetic polymorphism and BMI values. However, the prevalence of the MTHFR677 genetic polymorphism was found to be associated with higher BMI values (BMI >16.4kg/m^2 vs ≤16.4kg/m^2, prevalence of MTHFR677 genetic polymorphism: 50% vs 15%, p-value=0.018 for Chi-square test, Figure 2). Multivariable regression analysis showed that presence of the T allele of the MTHFR677 genetic polymorphism as opposed to the wild type was associated with 5.7 times higher odds of "healthier" BMI (BMI >16.4kg/m^2 vs ≤16.4kg/m^2, MTHFR677 genetic polymorphism, TT&CT vs CC genotype: OR 5.667, 95% CI: 1.254 to 25.606, p-value=0.024, data not shown).

Figure 1 Mean values of bone mineral density between girls carrying either two polymorphic variants or one polymorphic variant, or only wild type polymorphisms. Genetic polymorphisms evaluated include CTR-AluI and MTHFR C677T.



Bone mineral density values, wild type vs carriers of the CTR-AluI or the MTHFR677 polymorphism vs carriers of both the CTR-AluI and the MTHFR677 polymorphism: 0.97+0.11g/cm^2 vs 0.90+0.11g/cm^2 vs 0.75+0.33g/cm^2, p-value for linear trend 0.11, eta squared = 17.2. Statistical significance was set at the level of p-value<0.05.

Discussion

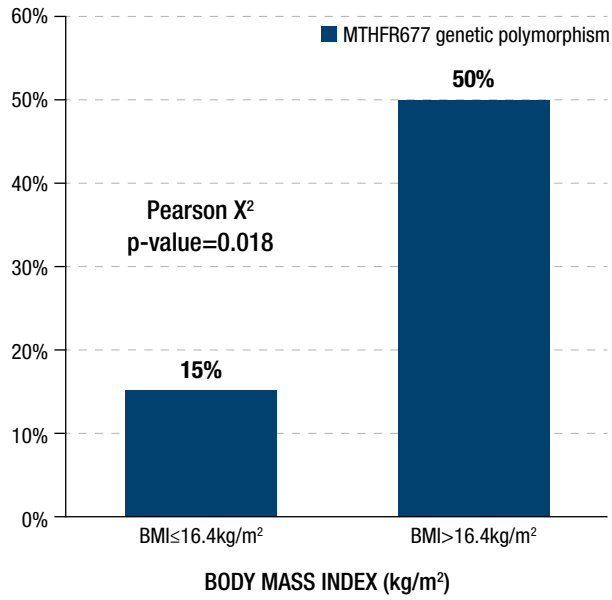
The results of this study indicate that presence of two polymorphic variants, namely CTR-AluI and MTHFR677, is associated with lower bone density in girls with AN. Carriers of at least

Table 2 Linear multivariable regression analysis including genetic polymorphisms as independent variables and bone mineral density levels as dependent variables in 40 girls with anorexia nervosa. The models were adjusted for age and body mass index.

BONE MASS DENSITY (g/cm^2)	R^2	B-COEFFICIENT	95% CI	P-VALUE	PARTIAL CORRELATION
CTR-AluI		**-0.399**	**-0.242 to -0.032**	**0.012**	**-0.404**
Age (years)	15.9%	0.027	-0.027 to 0.033	0.856	0.030
BMI (kg/m^2)		**0.325**	**0.003 to 0.472**	**0.036**	**0.342**
MTHFR677		**-0.321**	**-0.220 to 0.000**	**0.050**	**-0.321**
Age (years)	9.8%	0.006	-0.030 to 0.032	0.971	0.006
BMI (kg/m^2)		**0.339**	**0.002 to 0.576**	**0.037**	**0.339**
CTR-AluI and MTHFR677		**-0.514**	**-0.602 to -0.050**	**0.001**	**-0.504**
Age (years)	25.1%	0.068	-0.023 to 0.095	0.635	0.080
BMI (kg/m^2)		**0.401**	**0.009 to 0.676**	**0.009**	**0.420**

Bold indicates statistical significance, which was set at the level of p-value<0.05.

Figure 2 Frequency of MTHFR677 genetic polymorphism (T polymorphic variant) according to body mass index values, using the median value of 16.4kg/m^2 as cut-off.



MTHFR677 genetic polymorphism in girls with BMI higher vs lower than the median value: BMI >16.4kg/m^2 vs <16.4kg/m^2, prevalence of the MTHFR677 genetic polymorphism: 50% vs 15%, p-value=0.018 for Chi-square test.

one polymorphic variant have higher BMD values compared with carriers of both genetic polymorphisms, implying a dose-response effect. In addition, the underlying genetic background and, mainly, the presence of the MTHFR677 polymorphism are related to higher BMI among girls with AN. Significantly lower measures of bone density were observed in girls with AN carrying the CTR-AluI polymorphic variant when compared with girls carrying the wild type. The link between the CTR-AluI polymorphism and BMD has been described in different populations; interestingly, a difference in the prevalence of CTR genotypes according to the ethnic background has been noted [20], implying an effect of demographics in the association between CTR polymorphisms and BMD. Evaluating pathophysiological implications of genetic polymorphisms with aging, an Italian study of 663 postmenopausal and 52 perimenopausal women described an impact of the CTR genotypes on the process of acquiring peak bone mass, which as expected was more pronounced in younger women, rather than on the process of age-related bone loss [8]. Studies in postmenopausal populations described conflicting results, with evidence supporting lower BMD values in postmenopausal carriers of the unfavourable T allele [21,22], while other authors were unable to identify any association in young perimenopausal women [23]. Finally, no association between the CTR genotype frequencies and indices of bone metabolism, such as BMD and osteocalcin levels, was observed in a sample of healthy Japanese premenopausal women [20], a result that might be related to the different ethnic background and thus different frequency of the evaluated polymorphism compared with our sample. The results in our sample of girls with AN indicate a borderline association between the presence of the MTHFR C677T polymorphic variant and BMD values. In this sample, the effect of this polymorphism on bone metabolism seems to be mediated by other confounders, such as BMI. A possible contribution of the MTHFR C677T polymorphism on bone metabolism has been described by Li et al. [24], in a meta-analysis of 5,833 postmenopausal women. Their study highlighted that women with the mutated MTHFR gene had lower BMD at the femoral neck, but not at the lumbar spine, compared with women carrying the wild-type MTHFR gene [24]. A meta-analysis of 3,525 cases and 17,909 controls 12 identified a modestly elevated fracture-risk for individuals with the TT genotype of the C677T polymorphism (TT vs CT and CC genotypes, age<60 years: OR 1.51, 95% CI: 1.10 to 2.07). Similarly, the rare TT genotype has been found to be more common in women with vertebral fractures vs controls [12,13]. A study of 346 postmenopausal Thai women found that heterozygous carriers of the unfavourable T allele of the MTHFR C677T polymorphism had a 5.66-times higher risk of osteopenia at all sites combined compared with carriers of the wild type 11. Furthermore, McLean et al. [14] highlighted that the link found between the MTHFR C677T polymorphism and lower hip BMD levels is prevalent only in individuals with folate deficiency, following evaluation of 1,632 male and female participants of the Framingham Offspring Study.

Furthermore, our results highlight a possible interplay between genetic background and BMI values, mainly prevalent for carriers of the MTHFR C677T polymorphic variant. To our knowledge, ours is the first study to show an association between genetic background and both BMI and bone density values in young girls with AN. The impact of the MTHFR C677T polymorphism on weight gain remains conflicting, with studies of overweight and obese individuals both supporting [25] and rejecting this association [26]. Interestingly, individuals with a higher prevalence of metabolic abnormalities but normal body weight showed, in comparison with obese individuals, a higher prevalence of the favorable CC genotype of the MTHFR C677T polymorphism [27]. In contrast with our findings, a regulatory effect of the CTR-AluI polymorphic variant on weight gain has been described in a study of a sample of healthy Japanese pre-menopausal women, where presence of the unfavourable T allele was linked with a higher tendency to gain weight compared to women who were carrying the wild type genotype [20].

According to our findings, bone disease in girls with AN is particularly evident in those carrying both the CTR-AluI and the MTHFR C677T polymorphism, implying a possible interaction and dose-response association between these polymorphisms. The association between the MTHFR C677T polymorphism and worsening bone density is not surprising considering that the ensuing hyperhomocysteinemia has been shown to be linked to reduced collagen synthesis as well as disruption of collagen cross-linking within bone tissue [28]. The latter is likely due to a direct mechanistic effect of homocysteine on the enzyme lysyl oxidase, which mediates the process of collagen cross-linking [29]. Interestingly, in the present sample of girls with AN, the association between the MTHFR C677T genetic polymorphism and BMD values became non-significant when adjusting for BMI, suggesting an interaction between genetic background and weight gain. The link between presence of the MTHFR C677T polymorphism and body weight is difficult to explain. However, as suggested by others, the MTHFR C677T polymorphism is associated with higher levels of homocysteine, which has been pro-

posed to exert an epigenetic effect on the expression of genes regulating body fat storage [26]. Interestingly, according to data from in vitro, in vivo and genetic studies, homocysteine metabolism is closely related to the methylation process, hence affecting the methylation of DNA and amino acid residues on histones [25-27]. Limitations of our study should be acknowledged. First, the sample size is relatively small. Second, the cross-sectional design does not permit detection of casuality. Third, we did not evaluate z-score values. However, as suggested by the international guidelines on clinical densitometry, z-score values are not indicative of fracture risk, which is instead determined by clinical evidence of fracture, in this young subset of women [19]. Fourth, we did not evaluate folate levels, a possible regulator of the function of the MTHFR gene. Finally, the association between the evaluated MTHFR677 genetic polymorphism and BMI was only a secondary outcome measure. Therefore, our results should not be generalized to all women with AN but should rather be regarded as indicative of a possible mediation effect of BMI in the link observed between MTHFR677 and bone metabolism.

In conclusion, we observed that bone disease in young girls with AN is associated with presence of the CTR-AluI and MTHFR C677T genetic polymorphisms. Moreover, the combined presence of these polymorphisms is linked to further deterioration of bone density, implying a possible underlying genetic interaction and dose-response relationship between the identified polymorphisms. Finally, presence of the association between MTHFR C677T and bone density seems to be mediated by BMI values. Larger cohort studies are required to estimate the significance of our findings.

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