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# Interaction Between Clinical and Laboratorys Indicators and Genetic Polymorphysms of the Calcaneal Bone Mineral Density in Children Starting Puberty. Is this a Time for Intervention?

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## Abstract

The aim of this study was to investigate the association of selected genetic polymorphisms and bone mineral density (BMD) in children reaching puberty and atthe age of 18. The study sample consisted of 168 boys residing in Slavonski Brod, Croatia. Calcaneal quantitative ultrasound measurements were undertaken with Sahara device (Hologic). Genetic polymorphisms for CYP19 aromatase, IGF-1,estrogen receptor and androgen receptor were analysed. Each examinee completed a survey in order to estimate dietary habits and other possible behavioural patterns associated with bone mineral density. The results indicated significant association ofCYP19 aromatase polymorphism and estrogen receptor gene with quantitative ultrasound index (P=0.039) and estimated bone mineral density (P=0.049), as well as significant association of calcium intake and physical activity.

Although bone mineral density is a result of very complex and multiple mechanisms, findings of this study give us an insight to which subjects are at increased risk for developing osteoporosis and other related adverse events in later life and suggests means of an interventional program including dietary habits, calcium intake and increased physical activity that could ameliorate bone structure density weakness, detected in pre-pubertal period and connected to mentioned gene polymorphisms. The program should take place during puberty itself, a known period of largest bone mineral density acquirement.

**Keywords:** Bone mineral density; Children; Puberty; Genetics; Physical activity

## Introduction

The purpose of this study was to determine the value of ultrasonographical measures of calcaneal BMD in children entering puberty and after the period of largest BMC gain, to analyse the association of these parameters with microsatellite genetic polymorphisms as well as behavioural and habit differences.

Bone gain in humans is greatest during the intensive growth period, puberty and adolescence [1-3]. At this stage of growth children reach 90% of adult height, but only 57% of total adult bone mineral content (BMC), adding up to 90% of adult BMC around age 18 [3]. This significant increase stops at the end of the third decade [4-6]. Peak bone mass is an important risk factor for the development of osteoporosis in later life [7]. By knowing physiological variations in bone mass accrual during childhood and adolescence we could predict who is at greater risk for osteoporotic fractures and other bone metabolism disorders [8,9]. By optimizing the attainment of peak bone mass better prevention of osteoporosis in later life [9] could be achieved.

Many factors affect bone growth, BMC and BMD, such as birth weight, maternal ultraviolet B exposure during pregnancy [10], and behavioural factors like physical activity [9,11], diet [7], vitamin D [12] and calcium intake [13,14], alcohol consumption [7] and carbonated soft drinks [15]. Hormonal balance and its fine interplay are also very important and necessary for normal bone development, primarily sex hormones, growth hormone (GH) [16] and insulin-like growth factors (IGFs) [17]. Estrogen is important in both genders, [18] and it is well known that girls with amenorrhoea have decreased BMD in lumbar spine compared to girls with regular cycles [19,20]. Serum concentrations of IGF-1 was positively associated with periosteal circumference and total BMC throughout peripuberty, and with tibial length before menarche, suggesting that during puberty, circulating IGF-1 promotes bone periosteal apposition and mass accrual [9].

Genetic factors also affect the speed of maturation and growth. Adult height is considered to be a highly heritable and polygenetic trait. Bone mineral density is also highly heritable trait, with as much as 60-80% of variance attributable to genetic factors [21-23]. In recent years many studies investigated genetic background of BMD and osteoporosis revealing different genetic markers across the chromosomes [24-27].

Previous results indicated the association between genetic markers for IGF-1 gene, gene for estrogen receptor alpha, gene for androgen receptor, aromatase gene [28], vitamin D receptor gene [18], gene for colagen-1 [19] with BMD, bone geometry and osteoporosis. Majority of the research included adults, only recently there is an increased interest in researching genetic determinants of bone metabolism in children [19,20].

There are few approaches in measurement of bone mineral density (BMD), but the easiest and ethically more acceptable approach in children is the use of quantitative ultrasound (QUS), a quick, non-invasive and inexpensive method to measure bone strength [21-23].

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| LOCUS | CHROMOSOMS | SEKVENS | NO ALLELS | 5' PRIMER | 3' PRIMER | PCR CONDITIONS |
|---|---|---|---|---|---|---|
| **CYP19** | 15 | TTTA | 8 | GCA GGT ACT TAG TAG TTA GCT AC | TTACAG TGA GCC AAG GTC GT | 95$^0$C 30"<br>60$^0$C 30" 35X<br>72$^0$C 30"<br>94$^0$C 50" |
| **AR** | X | CAG | 18 | TCC AGA ATC TGT TCC AGA GCG TGC | GCT GTG AAG GTG GCT GTT CCT CAT | 58$^0$C 40" } 35X<br>72$^0$C 1'<br>94$^0$C 45"<br>70$^0$C 30" } 1X<br>72$^0$C 30"<br>94$^0$C 45"<br>70$^0$C<br>(-1$^0$C/ciklus) 30" } 9X |
| **IGF-I** | 12 | CA | 6 | GCT AGC CAG CTG GTG TTA TT | ACC ACT CTG GGA GAA GGG TA | 94$^0$C 35"<br>61$^0$C 30" } 25X<br>72$^0$C 30" |
| **ER** | 6 | TA | 10 | GAC GCA TGA TAT ACT TCA CC | GCA GAA TCA AAT ATC CAG ATG | 94$^0$C 2'<br>58$^0$C 1' } 30X<br>74$^0$C 1' |

**Table 1:** Primer characteristics.

## Materials and Methods

This study included a sample of 168 boys. Age of examinees was limited, only children aged between 11 and 13 years were eligible. All were included from the elementary schools in the Slavonski Brod, Eastern region in Croatia. A total of four schools were enrolled, two from the rural setting and two from urban. In order to participate, parents had to provide a written consent and allow for their child to be involved in the study. They all completed a survey in order to estimate dietary habits and other possible behavioural patterns like a consumption of calcium /mg per day, fizzy drinks /dcl per day, watching TV / hours per day/, sun exposer sumer or winter /hours per day/ Blood samples were taken to these respondents, consisting a total of 30 mL of blood for biochemical and genetic analysis.

### Bone mineral density estimates and anthropometry

Bone densitometry was performed for each participant. An ultrasound-based Sahara Hologic device was used. The device was re-calibrated on a daily basis, and all the measurements were done by two nurses. Additionally, all participants had their weight and height measured and Tanner puberty development stage estimated. All participants were in first Tanner stage. Anthropometry was performed by the two specialists of school medicine. Lastly, parents provided information on the medical record, history of possible fractures, lifestyle and osteoporosis in the family.

### Laboratory measurements and procedures

Three bone-turnover and status parameters were measured from the samples: bone-specific alkaline phosphatase, Sexual-hormone binding protein (SHBG), osteocalcin and β-cross laps (ELISA, Nordic Bioscience Diagnostics). Additionally, we measured serum concentrations of estradiol ($E_2$), free testosterone and 25-OHD3. $E_2$ was measured using Ortho Johnson & Johnson ligands, free testosterone using radio immunochemical methods based on the DPC method, while 25-OHD3 was measured using ELISA by IDS.

### Genotyping

All participants provided 5ml of peripheral blood, which was collected for PCR reactions. Laser-induced fluorescence and Allele Locator were used for analysis (ALF express, Pharmacia-Amersham, Uppsala, Sweden) (Table 1).

### Statistical analysis

The data were presented as means and standard deviations or numbers and percentages, depending on the variable type. In order to achieve normal data distribution several transformations were used: logarithm (QUI, BUA, body mass index and estrogen), inverse (weight, testosterone and vitamin D), square root (crosslaps). Since bone mineral density was estimated on the basis of ultrasound, we denoted this by using an abbreviation of eBMD (estimated bone mineral density). The analysis was performed using t-test. Both linear and quadratic regression models were used in the initial analysis of the association between genetic markers and bone mineral density estimates, in order to allow for both linear and non-linear association assumptions. Furthermore, a multiple linear regression model was used in the final analysis step, in order to assess the importance of a wider range of predictor variables. SPSS (SPSS Inc, Chicago, IL) was used in the analysis, with significance set at $P<0.05$.

## Results

The study encompassed 168 children. All anthropometric, ultrasound and laboratory parameters were shown (Table 2). Correlation of various indicators of ultrasound BMD measurements showed a great

| Characteristic | Mean ± SD |
|---|---|
| T-score | -0,30 ± 0,73 |
| QUI | 97,6 ± 11,9 |
| eBMD | 0,54 ± 0,07 |
| BUA | 69,7 ± 12,3 |
| SOS | 1560,7 ± 19,8 |
| Height (cm) | 148,3 ± 6,10 |
| Weigth (kg) | 41,7 ± 8,00 |
| Body mass index [BMI] (kg/m$^2$) | 18,9 ± 2,70 |
| Testosteron (nmol/L) | 3,02 ± 3,00 |
| Vitamin D (µmol/L) | 26,4 ± 9,50 |
| Osteocalcin (nmol/L) | 143,2 ± 50,3 |
| Crosslaps (nmol/L) | 1309,8 ± 460,4 |
| Sexual-hormone binding protein (SHBG) | 71,6 ± 35,2 |
| Albumin (g/L) | 44,5 ± 2,60 |
| Estrogen (E2) (nmol/L) | 37,7 ± 31,1 |
| IGF1 (nmol/L) | 65,0 ± 28,9 |

*t-test was used in data analysis; SD – standard deviation

**Table 2:** Basic desciptive, boys.

dependence between explored indicators which was for all comparisons at level P<0.001, and correlation coefficients were in range from 0.58 (for BUA-SOS couplet) until almost complete correlation for couplet BMD-OUI (Table 3).

Comparison of allele frequency is without significant results for CYP19gene (P=0.285), IGF (P=0.602) and AR (P=0.150). For ER gene a value of borderline significance is measured (P=0.030) In CYP 19 gene only one variant allele was noted at IGF gene two alleles, and at ER gene three variant alleles (Table 4).

Overview of genotype frequencies implied a great deal of variability. Genotype variability (H) of CYP19 gene was 0.84, IGF-1 gene 0.76, and variability of genes AR and ER were even higher – 0.96 for both.

| | QUI/Stiffness | BMD | BUA | SOS |
|---|---|---|---|---|
| Total | | | | |
| T-Score | 0,97 | 0,97 | 0,81 | 0,84 |
| QUI | | 0,99 | 0,84 | 0,86 |
| BMD | | | 0,84 | 0,86 |
| BUA | | | | 0,58 |

*QUI – Quantitative ultrasound index
*BMD – Bone mineral density
*BUA - broadband ultrasound attenuation
*SOS - speed of sound

**Table 3:** Correlation between ultrasounds parametars of bone mineral density.

| | | All | |
|---|---|---|---|
| Gen | Alel (engl. *peaks*) | N | % |
| CYP19 | 7 | 62 | 18,5 |
| | 7del3 | 116 | 34,5 |
| | 8 | 46 | 13,7 |
| | 9 | 5 | 1,5 |
| | 10 | 8 | 2,4 |
| | 11 | 72 | 21,4 |
| | 12 | 16 | 4,8 |
| | 13 | 1 | 0,3 |
| | 19.0 | 1 | 0,3 |
| | Unknown | 9 | 2,7 |
| IGF | 16 | 7 | 2,1 |
| | 17 | 21 | 6,3 |
| | 18 | 218 | 64,9 |
| | 19 | 47 | 14,0 |
| | 20 | 19 | 5,7 |
| | 21 | 4 | 1,2 |
| | 16.8 | 1 | 0,3 |
| | 17.0 | 1 | 0,3 |
| | Unknown | 18 | 5,4 |
| AR | 15 | 4 | 1,2 |
| | 16 | 9 | 2,7 |
| | 17 | 26 | 7,7 |
| | 18 | 32 | 9,5 |
| | 20 | 40 | 11,9 |
| | 21 | 28 | 8,3 |
| | 22 | 32 | 9,5 |
| | 23 | 34 | 10,1 |
| | 24 | 19 | 5,7 |
| | 25 | 9 | 2,7 |
| | 26 | 7 | 2,1 |
| | 27 | 3 | 0,9 |
| | 28 | 1 | 0,3 |
| | Unknown | 92 | 27,4 |
| ER | 11 | 1 | 0,3 |
| | 12 | 4 | 1,2 |
| | 13 | 26 | 7,7 |
| | 14 | 75 | 22,3 |
| | 15 | 28 | 8,3 |
| | 16 | 6 | 1,8 |
| | 17 | 4 | 1,2 |
| | 19 | 21 | 6,3 |
| | 20 | 10 | 3,0 |
| | 21 | 24 | 7,1 |
| | 22 | 22 | 6,5 |
| | 23 | 45 | 13,4 |
| | 24 | 17 | 5,1 |
| | 25 | 13 | 3,9 |
| | 26 | 5 | 1,5 |
| | 27 | 1 | 0,3 |
| | 15.6 | 14 | 4,2 |
| | 15.8 | 2 | 0,6 |
| | 16.0 | 2 | 0,6 |
| | Unknown | 16 | 4,8 |

*CYP19 – Aromatase receptor
*IGF – Insulin like factor receptor
*AR- Androgen receptor
*ER – Estrogen receptor

**Table 4:** Allel frequency of researched genes.

The analysis of genetic effects on bone mineral density parameters using a simple linear or quadratic models suggested lack of significant linear effects and presence of two significant quadratic effects, one for the CYP19 gene and the other, which was somewhat weaker for ER (Table 5).

A comparison of the behavioral predictors of bone mineral density

| | | Linear model | | Quadratic model | |
|---|---|---|---|---|---|
| Gene | Indicator | β | P | β | P |
| CYP-19 | T-score | -0.07 | 0.226 | 3.32 | 0.001 |
| | QUI | -0.07 | 0.253 | 4.15 | <0.001 |
| | BMD | -0.07 | 0.225 | 4.10 | <0.001 |
| | BUA | -0.01 | 0.867 | 3.62 | 0.001 |
| | SOS | -0.12 | 0.034 | 3.01 | 0.008 |
| IGF1 | T-score | 0.03 | 0.957 | 0.13 | 0.862 |
| | QUI | <0.01 | 0.938 | 0.16 | 0.833 |
| | BMD | <0.01 | 0.934 | 0.15 | 0.851 |
| | BUA | 0.02 | 0.692 | -0.43 | 0.565 |
| | SOS | -0.01 | 0.927 | 0.72 | 0.349 |
| AR | T-score | 0.06 | 0.376 | -1.97 | 0.020 |
| | QUI | 0.07 | 0.271 | -2.23 | 0.026 |
| | BMD | 0.07 | 0.268 | -1.93 | 0.022 |
| | BUA | 0.05 | 0.454 | -1.14 | 0.179 |
| | SOS | 0.02 | 0.754 | -1.79 | 0.034 |
| ER | T-score | -0.02 | 0.711 | 0.17 | 0.825 |
| | QUI | -0.03 | 0.556 | 0.27 | 0.723 |
| | BMD | -0.04 | 0.541 | 0.33 | 0.669 |
| | BUA | 0.01 | 0.890 | 0.26 | 0.730 |
| | SOS | -0.02 | 0.703 | 0.52 | 0.496 |

*QUI – Quantitative ultrasound index
*BMD – Bone mineral density
*BUA - broadband ultrasound attenuation
*SOS - speed of sound
*β – regresy coefficient

**Table 5:** Linear and quadratic regression model showing the association of selected genetic markers and bone mineral density parameters.

| | Physical activity/hours per week | Fizzy drinks/ dcl per day | TV watching/hours per week | Use of computer/hours per week | Sun exposure / summer/hours per week | Sun exposure / winter/ hours per week |
|---|---|---|---|---|---|---|
| T-score | <0.001 | <0.001 | 0.023 | 0.260 | 0.221 | 0.320 |
| QUI | <0.001 | <0.001 | 0.038 | 0.216 | 0.117 | 0.350 |
| BMD | <0.001 | <0.001 | 0.027 | 0.190 | 0.100 | 0.296 |
| BUA | 0.001 | 0.007 | 0.043 | 0.255 | 0.052 | 0.164 |
| SOS | <0.001 | <0.001 | 0.213 | 0.676 | 0.243 | 0.758 |

*QUI – Quantitative ultrasound index
*BMD – Bone mineral density
*BUA - broadband ultrasound attenuation
*SOS - speed of sound

**Table 6:** Significance levels of the correlations between bone mineral estimates and some behavioural risks.

| Characteristic | T-score | QUI | BMD | BUA | SOS |
|---|---|---|---|---|---|
| Age | 0.508 | 0.343 | 0.413 | 0.454 | 0.580 |
| Gender | 0.694 | 0.666 | 0.674 | 0.750 | 0.599 |
| CYP19 | 0.078 | 0.039 | 0.049 | 0.162 | 0.096 |
| IGFI | 0.970 | 0.924 | 0.923 | 0.985 | 0.821 |
| AR | 0.482 | 0.540 | 0.462 | 0.587 | 0.623 |
| ER | 0.679 | 0.598 | 0.600 | 0.450 | 0.718 |
| Testosterone | 0.355 | 0.428 | 0.380 | 0.108 | 0.793 |
| Vitamin D | 0.742 | 0.974 | 0.938 | 0.905 | 0.928 |
| Osteocalcin | 0.381 | 0.365 | 0.348 | 0.025 | 0.728 |
| Cross laps | 0.347 | 0.378 | 0.339 | 0.056 | 0.955 |
| SHBG | 0.151 | 0.105 | 0.140 | 0.092 | 0.395 |
| Albumin | 0.823 | 0.865 | 0.807 | 0.760 | 0.703 |
| E2 | 0.873 | 0.752 | 0.820 | 0.977 | 0.529 |
| IGF1 | 0.347 | 0.325 | 0.350 | 0.069 | 0.856 |
| Body mass index | 0.959 | 0.855 | 0.870 | 0.139 | 0.234 |
| Calcium intake | **<0.001** | 0.002 | 0.001 | 0.002 | 0.010 |
| Fizzy drinks | 0.419 | 0.512 | 0.570 | 0.625 | 0.126 |
| Physical activity | 0.004 | 0.009 | 0.007 | 0.101 | 0.013 |
| TV watching | 0.220 | 0.195 | 0.142 | 0.234 | 0.622 |
| Computer use | 0.761 | 0.491 | 0.423 | 0.264 | 0.744 |
| Sun exposure - summer | 0.788 | 0.907 | 0.919 | 0.951 | 0.598 |
| Sun exposure - winter | 0.208 | 0.277 | 0.267 | 0.531 | 0.358 |
| Sleep duration | 0.291 | 0.312 | 0.299 | 0.230 | 0.468 |

*QUI – Quantitative ultrasound index
*BMD – Bone mineral density
*BUA - broadband ultrasound attenuation
*SOS - speed of sound

**Table 7:** Multiple linear regression results with a number of environmental and genetic predictors.

indicated that physical activity and fizzy drinks use were significantly associated with most analyzed indices (Table 6).

Lastly, an analysis of the wide spectrum of confounding effects on bone mineral density suggested that three predictors were significantly associated with bone mineral density – physical activity, calcium intake and CYP19 gene (Table 7).

## Discussion

Statistically significant results were noted in correlation of gene markers with bone mineral density only with gene for aromatase and AR. Examinees with alels CYP19 7del3 had better values of ultrasound calcaneal bone mineral density. QUS was better 3,4%, BUA 3,7%, and BMD 4,0%. At genes for AR boys with alels 15,25,27 had lower bone mineral density values. Impact of other analysed genes: IGF-1, gene for AR and for ER was not significant (Table 4). Serum concentration of vitamin D in all children was low, under reference limits. (Table 2). Calcium intake and amount of physical activity have a positive effect on children bone structure (Table 7).

The results of this study show that genetic factors seem to have strong and significant effects on bone mineral density in children. While a number of studies have resonated such results in the elderly, studies in children are more scarse and often not focused on a trait that is believed to be important mainly in elderly 4,6]. This study shows that some of the genes involved in bone metabolism also maintained their effect in the regression model that involved a number of behavioural patterns and other indicators, supporting some previous claims that genetic effects on bone could be strongly expressed in younger age [28-41].

Furthermore, from our survey we found that 34% boys take 5 dcl of fizzed drinks daily, 48% ingest daily 600 mg of calcium and 43% sits 4h during computer use. These results also resonate an important message in the sphere of public health, that physical activity and calcium intake

about 850 mg /per day [40,41] are important even in the pubertal life and that commonly heard proposals that impaired BMD is a problem of elderly might not be completely true. Focus of the preventive activities related to osteoporosis should be more systematic and transferred to even younger age, when improvements might be more feasible and easier to achieve [29-35].

Children who took 850 mg calcium pre day has better bone mineral density. Emerges a need for strategy of measures targeting individuals at risk which would optimize their final peak bone mass and fortify their bones for years to come [40,41].

Study proves that genetics and behaviour alike are important predictors of bone mineral density which differs significally even in pre-pubertal period placing people at uneven starting positions at the period of largest bone density accrual [36-41].

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