The importance of the \textit{UGT1A1} variants in the development of osteopenia and osteoporosis in postmenopausal women

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The UDP-glucuronosyltransferase 1A1 (\textit{UGT1A1}) is involved in the process of estrogen conjugation and elimination. The aim of the study was to analyze whether the \textit{UGT1A1} genetic variants are associated with the development of osteopenia and osteoporosis in postmenopausal women. The analysis of the rs4148323 (\textit{UGT1A1}*6) and rs3064744 (\textit{UGT1A1}*28) variants in the \textit{UGT1A1} gene was conducted using real-time PCR. A significant correlation was observed between the genotypes of the rs3064744 (\textit{UGT1A1}*28) sequence variant and body mass in women with osteoporosis. The analysis of the Z-score values revealed that women with osteoporosis and carrying the 6/6 variant had the lowest Z-score values as compared to women with the 6/7 and the 7/7 variants ($-1.966 \pm 0.242$ vs. $-1.577 \pm 0.125$ and $-1.839 \pm 0.233$). In addition, the odds ratio for the investigated genotypes (6/6, 6/7, 7/7) indicated an increased risk for osteopenia and osteoporosis in women with the 7/7 homozygous genotype. The analysis of the frequencies of the GG, GA and AA genotypes of the rs4148323 \textit{UGT1A1} gene showed no statistically significant differences between the groups. Our analysis revealed that the \textit{UGT1A1} rs3064744 variant may affect the risk of developing osteoporosis in postmenopausal Polish women. The \textit{UGT1A1} rs4148323 variant is not directly associated with the development of osteopenia and osteoporosis.

Osteoporosis belongs to the group of 'diseases of affluence', with the loss of bone mass and deteriorated bone structure as the dominant symptoms. The pathomechanism of osteoporosis is complex and multifactorial, associated with changes in the concentration profiles of hormones, cytokines, and growth factors. Over 30% of all postmenopausal women are affected by osteoporosis. According to the data from the International Osteoporosis Foundation, 200 million women worldwide are diagnosed with this disease (1/5 at the age of 70 and as many as 2/3 over the age of 90). In addition, 1/3 of the women suffer osteoporosis-related bone fractures, which is a typical occurrence in osteoporosis. Initially, the symptoms of the disease are hardly noticeable by the patient, with low-energy fractures as the first indication of abnormal bone metabolism. For this reason, it is necessary to raise awareness about the risk factors and symptoms of osteoporosis, which in turn will help to minimize the effects of bone mass loss. There are four types of osteoporosis: (1) true osteoporosis, in the course of which normal physical activity causes pain or fractures, mainly of the spine; (2) physiological osteopenia, with lower bone mechanical resistance as the result of low physical activity and decreased muscle strength, and fractures occurring as the consequence of high trauma; (3) combination of true osteoporosis with physiological osteopenia; and (4) transient osteopenia, which is the result of reduced physical activity associated with injury or disease.
A better understanding of the molecular mechanisms underlying osteoporosis is vital for the diagnosis and treatment, not to mention the earliest possible identification of the factors predisposing to the development of the disease. So far, numerous molecular analyses were performed to investigate the possible role of the genetic factors in the etiology of osteoporosis. The risk of osteoporosis and osteopenia has been linked to genetic variants, especially the COL1A1, VDR, BMP2, TLR genes, as well as the LRP5 gene involved in the Wnt/β-catenin signaling pathway.

The search for new genes that play an important role in the regulation of bone mass and the development of osteoporosis continues. Previous GWAS studies demonstrated a link between polymorphisms of the genes related to estrogen metabolism and osteoporosis and the risk of bone fracture. Recently, much attention has been paid to the potential biomarkers, including UDP-glucuronosyltransferases (UGTs).

UDP-glucuronosyltransferases comprise a superfamily of membrane-bound conjugating enzymes involved in the inactivation and elimination of numerous endogenous and exogenous compounds. UGTs catalyze the glucuronidation reaction, which is associated with the metabolism of bilirubin, bile acids, fatty acids, steroid hormones, thyroid hormones, and fat-soluble vitamins. Glucuronidation is also one of the most important phase II biotransformation reactions. UGTs are expressed in various tissues: brain, liver, kidneys, small intestine, colon, stomach, lungs, epithelium, ovaries, testes, mammary glands, and prostate.

The aim of the study was to investigate whether the UGT1A1 rs3064744 (UGT1A1*28) and the rs4148323 (UGT1A1*2) genetic variants are associated with the development of osteopenia and osteoporosis in postmenopausal women.

Methods

Patients. The study included 675 Polish postmenopausal women (109 with osteopenia, 333 with osteoporosis and 233 healthy controls). BMD measurements were performed at the Laboratory of Densitometry, Clinical Hospital No. 1, Pomeranian Medical University in Szczecin. BMD was measured in the lumbar spine, from L2 to L4 vertebrae, using DEXA (Dual Energy X-ray Absorptiometry). Densitometry was performed using the LUNAR DPX 100 camera (Lunar Corp., Madison, USA). Normal BMD value using DEXA is between one standard deviation from the mean in relation to the age of peak bone mass (−1 < T-score < 1). Based on these measurements, the women were classified into the following groups: osteopenia (−2.5 < T-score < −1), osteoporosis (T-score < −2.5), and normal T-score—controls (T-score > −1). The ratio of mean BMD in relation to mean value for young adults (YA) and in comparison to age (age-matched, AM), was also evaluated. Furthermore, height and weight were measured, and the body mass index (BMI) was calculated. Data on disease manifestation, drug use, age at first and last menstruation, gravidity and parity, and birth weight were collected. The inclusion criteria for the study were as follows: menopause at least 1 year before, no hormone replacement therapy (HRT) or drugs affecting bone mass (selective estrogen receptor modulators (SERMs), calcitonin, bisphosphonates, heparin, steroids, thyroid hormones, antiepileptic drugs, GnRH analogues, tibolone). Patients with endocrine and metabolic disorders, hematological diseases, kidney disease, cancers, autoimmune and connective tissue diseases, and after bilateral ovariectomy were excluded from the analysis. Additionally, women who did not smoke were qualified for the study because tobacco smoking may increase the risk of osteoporosis. Moreover, women were not selected in terms of physical activity. The study procedures were approved by the Bioethics Committee of Poznan University of Medical Sciences, Poland (no. 1415/03 (158/06)). The Ethics statement was approved according to the Helsinki Declaration. Written informed consent was obtained from all participants.

Analysis of the rs4148323 (UGT1A1*6) and the rs3064744 (UGT1A1*28) variants in the UGT1A1 gene. Blood samples were collected at the Department of Orthopedics and Traumatology, Pomeranian Medical University. The analysis of the UGT1A1 gene variants was conducted at the Department of Stem Cell and Regenerative Medicine, Institute of Natural Fibers and Medicinal Plants, Poznan. Genomic DNA was extracted from peripheral blood using QIAamp Blood Kit (Qiagen GmbH, Hilden, Germany), in accordance with the manufacturer's protocol. DNA concentration was measured using DeNovix DS-11 Spectrophotometer (DeNovix Inc., USA). LightCycler FastStart DNA Master HybProbe (Roche Diagnostics) assay and LightCycler®480 instrument for the UGT1A1 gene genotyping were used. Determination of the rs4148323 and the rs3064744 variants of the UGT1A1 gene was performed using LightSNIP (TIB Molbiol), which contained the primers and probes specific for the amplified fragment. PCR was performed in 10 μl reaction mixture according to the manufacturer’s protocol under the following conditions: initial denaturation at 95 °C for 10 min, and 35 cycles as follows: denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, elongation for 15 s at 72 °C, and melting for 30 s at 95 °C and 40 °C for 120 s. The UGT1A1 sequence variants were observed as different melting curves of the PCR products. The UGT1A1 promoter region generally contains six TA repeats, but alleles containing seven repeats lead to reduced gene expression (UGT1A1*28 variant, rs3064744). All genotyping data obtained were double-assessed to minimize error. A duplicate plate was entered to check the quality of genotyp-
ing. No incompatibilities were observed. Additionally, positive controls for heterozygote, wild-type and mutant homozygote were used.

**Statistical analysis.** Data analysis was performed using SPSS Statistics 17.0 for Windows. The observed frequencies were compared with the expected frequencies and tested for the Hardy–Weinberg equilibrium. The expected results are presented with 95% confidence intervals (CI). The odds ratio (OR) for the genotypes and the alleles was calculated. Then, the effect of the UGT1A1 genetic variants on T-score, Z-score, L2L4YA (bone mineral density in young adult), L2L4AM (bone mineral density between lumbar vertebrae L2–L4), BMI (body mass index), and other clinical parameters was evaluated. Correlation analysis between the genotypes and the clinical parameters was conducted using one-way ANOVA. The p-value of < 0.05 was considered as statistically significant.

All methods were carried out in accordance with relevant guidelines and regulations.

**Results**

The analysis of the rs4148323 (UGT1A1*6) and the rs3064744 (UGT1A1*28) variants in the UGT1A1 gene was based on different melting curves of the PCR products. Table 1 presents the clinical parameters of postmenopausal women classified into the groups with osteoporosis, osteopenia and controls. The association of the UGT1A1 on the risk of developing osteopenia and osteoporosis was evaluated, which was then correlated with the clinical parameters, including bone parameters. Analyzing the obtained results in the women with osteoporosis, we observed that the body mass was lower in carriers of genotypes 6/6 (60.379 ± 1.265 kg) and 6/7 (60.325 ± 1.204 kg) compared to women with genotypes 7/7 (66.833 ± 2.023 kg, p < 0.005). The inverse relationship was observed in the control group (genotype 6/6: 68.045 ± 2.241 kg and genotype 6/7: 70.212 ± 2.228 kg vs. genotype 7/7: 64.571 ± 2.930 kg, p = 0.142) and women with osteopenia (genotype 6/6: 66.438 ± 1.712 kg and genotype 6/7: 64.954 ± 1.801 vs. genotype 7/7: 63.526 ± 1.714, p = 0.243).

Interestingly, women with osteopenia and osteoporosis had lower birth weight as compared to the control group. Analyzing the Z-score values, we also determined that women with osteoporosis and carrying the 6/6 variant had the lowest Z-score value as compared to women with the 6/7 and 7/7 variants (~ 1.966 ± 0.242 vs. ~ 1.577 ± 0.125 and ~ 1.839 ± 0.233, p = 0.096). For the T-score values in relation to the genotypes for the UGT1A1 variant, no differences were observed between the studied groups. In addition, the effect of the UGT1A1 genetic variants on the duration of a woman's reproductive years was analyzed. No statistically significant differences among the groups were found, because the reproductive years of an average woman were between the ages of 12 and 52 in all groups. The frequency of homozygous 6/6 genotype of the UGT1A1*28 variant (rs3064744) did not differ in the group of women with osteopenia and postmenopausal controls (Table 2). A slightly higher 6/7 genotype frequency was demonstrated in the control group, whereas the frequency of the 7/7 genotype was higher in the group with osteopenia and osteoporosis as compared to controls (15.0% vs. 10.7%, p = 0.146, OR = 1.47, 95% CI 0.86–2.56; 18.3% vs. 10.7%, p = 0.048, OR = 1.87, 95% CI 0.93–3.70, respectively). In addition, the odds ratio for the investigated genotypes (6/6, 6/7, 7/7) indicated a higher risk for osteopenia and osteoporosis in women with the 7/7 homozygous genotype (Tables 2 and 3).

The analysis of the frequencies of the GG, GA and AA genotypes in the rs4148323 polymorphism of the UGT1A1 gene showed no statistically significant differences between the investigated groups (Table 4). The GG genotype was dominant among the women with osteopenia, osteoporosis and controls, while the GA genotype was sporadic in the control group and women with osteopenia. The AA genotype was not found in any of the groups. As far as frequency of the rs4148323 polymorphism and the clinical parameters were concerned, no statistically significant differences were observed (Table 4).

**Discussion**

In this study, the UGT1A1 genetic variant (UGT1A1*28) was used as a complementary marker of bone mass loss. Early identification and detection of the factors predisposing to the development of osteopenia or osteoporosis allow to implement appropriate prophylaxis and, if necessary, initiate pharmacotherapy. Changes in the parameters such as bone density and bone mass affect predominantly postmenopausal women, which is the consequence of the changes in their hormonal profile. In postmenopausal women, estrogens are synthesized almost exclusively from the androstenedione formed in the adrenal glands, which is converted into estrone in extraglandular tissues. With the increase in body weight and fat content, which is observed in postmenopausal women, the number of estrogen sources increases, while bone turnover decreases, with simultaneous increase in bone mass loss, which seems to be the dominant mechanism of bone tissue changes.

**UGT1A1** is involved in the process of estrogen conjugation and elimination. In the present study, the frequency of the UGT1A1*28 variant among Caucasian women was assessed. The search for a connection and a possible correlation between the variants of the analyzed gene and various diseases has so far been reported in the literature for neonatal jaundice, and tumors, among others. In this study, a comparison of the homozygous 6/6 genotype frequency of the UGT1A1*28 variant (rs3064744) between the women with osteopenia and postmenopausal controls revealed no differences. However, the frequency of the 6/7 genotype was higher in the control group, while the 7/7 genotype seemed to be more common in people with osteopenia and osteoporosis as compared to controls. In addition, the heterozygous 6/7 genotype was found to be slightly less common in women with osteoporosis. The frequencies of the GG, GA and AA genotypes were also analyzed, but no statistically
significant differences between the groups were found. Nevertheless, it can be concluded that among the three analyzed genotypes, the GG genotype was dominant, and the AA genotype was not found.
It seems that the UGT1A1*28 metabolism of anticancer drugs, but also, bearing in mind the hormonal associations with osteoporosis, because UGT1A1 variants is not only important in the context of the heterogeneity of the study population is a vital issue in the analysis of genetic variants in terms of their frequency27. Since the UGT1A1*28 allele occurs mainly in Caucasian and African American populations, while the UGT1A1*6 allele is widely described in Asian populations, taking into account the race parameter seems to be well-justified. The study of the UGT1A1 alleles is not only important in the context of the metabolism of anticancer drugs, but also, bearing in mind the hormonal associations with osteoporosis, because it seems that the UGT1A1*28 genetic variant may affect the rate of estrogen metabolism32. Thus, changes in the nucleotide sequence of the UGT1A1 gene might affect the severity and progression rate of osteoporosis. It is also possible that the described genetic variants may be related to the rate of bone mass loss, thereby affecting the rate of symptom onset. Our results showed the UGT1A1 rs3064744 (UGT1A1*28) genetic variant may affect the risk of developing osteopenia and osteoporosis in postmenopausal women, especially in the presence of homozygous genotypes containing two mutated alleles. Studies by Trontelj et al. showed that patients with the UGT1A1*28 genetic variant may affect bone mineral density in women with osteoporosis taking raloxifene. They indicated that women with the *28/*28 (7/7) genotype had an increased BMD compared to patients with the *1/*1 (6/6) and *1/*28 (6/7) genotypes33.

When comparing the frequency of the analyzed genetic variants and the clinical parameters, a correlation between the genotypes of UGT1A1*28 and body mass was observed in the group of women with osteoporosis. No statistically significant differences were found for other clinical parameters. It seems, therefore, that the limited number of parameters between which correlation was found is a favorable phenomenon in the context of the diagnostic process and the use of research on the genetic variants on the development of osteoporosis. It eliminates the need to search for connections with other clinical parameters and, consequently, allows for a more accurate prediction of the actual impact of the polymorphisms on the development of osteoporosis.

The frequency of the specific alleles and genotypes of the UGT1A1*28 variant in the group of women with osteopenia and controls. The expected value was calculated in accordance with the Hardy–Weinberg equilibrium (HWE). HWE equilibrium test was used to obtain the exact p value. OR odds ratio.

| Genotypes | Osteopenia | Control |
|-----------|-----------|---------|
|           | Observed value n (%) | Expected value (%) | Observed value n (%) | Expected value (%) | OR 95% CI | p  |
| 6/6       | 43 (39.5) | 36.7 | 88 (37.8) | 40.3 | 1.07 | 0.65–1.75 | 0.049 |
| 6/7       | 46 (42.2) | 47.8 | 120 (51.5) | 46.4 | 0.69 | 0.42–1.12 |  
| 7/7       | 20 (18.3) | 15.5 | 25 (10.7) | 13.2 | 1.87 | 0.99–3.70 |  
| Total     | 109 (100.0%) | 100.00 | 233 (100%) | 100.00 | – | – |  

Table 2. The frequency of the specific alleles and genotypes of the UGT1A1*28 variant in the group of women with osteopenia and controls. The expected value was calculated in accordance with the Hardy–Weinberg equilibrium (HWE). HWE equilibrium test was used to obtain the exact p value. OR odds ratio.

| Genotypes | Osteoporosis | Control |
|-----------|--------------|---------|
|           | Observed value n (%) | Expected value (%) | Observed value n (%) | Expected value (%) | OR 95% CI | p  |
| 6/6       | 129 (38.7) | 38.3 | 88 (37.8) | 40.3 | 1.04 | 0.73–1.49 |  
| 6/7       | 154 (46.2) | 47.2 | 120 (51.5) | 46.4 | 0.81 | 0.57–1.15 | 0.146 |
| 7/7       | 50 (15.0) | 14.5 | 25 (10.7) | 13.2 | 1.47 | 0.86–2.56 |  
| Total     | 333 (100.0%) | 100.00 | 233 (100%) | 100.00 | – | – |  

Table 3. The frequency of specific alleles and genotypes of the UGT1A1*28 variant in the group of women with osteoporosis and controls. The expected value was calculated in accordance with the Hardy–Weinberg equilibrium (HWE). HWE equilibrium test was used to obtain the exact p value. OR odds ratio.

No statistically significant differences were found for other clinical parameters. It seems, therefore, that the limited number of parameters between which correlation was found is a favorable phenomenon in the context of the diagnostic process and the use of research on the genetic variants on the development of osteoporosis. It eliminates the need to search for connections with other clinical parameters and, consequently, allows for a more accurate prediction of the actual impact of the polymorphisms on the development of osteoporosis.
into enhanced bioavailability of the estrogen pool, which in turn increase bone mass\textsuperscript{36,37}. Therefore, it can be assumed that the UGT1A1\textsuperscript{*28} genetic variant may be related to the transcriptional activity of the gene followed by enhanced expression of the estrogen receptor.

### Table 4. Characteristics of the postmenopausal women with osteopenia, osteoporosis and normal T-score taking part in the study of the rs4148323 genetic variant of UGT1A1 gene.

| Genotype | Osteopenia | | Osteoporosis | | Controls |
|----------|------------|------------|--------------|------------|------------|
|          | Mean ± SD  | Mean ± SD  | Mean ± SD   | Mean ± SD  | Mean ± SD  |
| n = 108  | n = 1      | n = 0      | n = 333     | n = 0      | n = 231    |
| Age (years) | 53.332 ± 8.17 | 52 | 54.447 ± 4.17 | - | 53.617 ± 8.271 | 55.037 ± 2.831 |
| T-score   | -1.803 ± 0.439 | -1.900 | -3.164 ± 0.056 | - | 0.110 ± 0.120 | -0.795 ± 0.095 |
| Z-score   | -0.929 ± 0.112 | -0.487 | -3.569 ± 1.946 | - | 0.689 ± 0.205 | -0.055 ± 0.475 |
| Body mass (kg) | 65.338 ± 1.071 | 74.000 | 61.208 ± 0.938 | - | 68.633 ± 1.571 | 74.052 ± 10.211 |
| BMI (kg/m\(^2\)) | 24.647 ± 0.384 | 27.180 | 23.787 ± 0.318 | - | 26.031 ± 0.591 | 26.345 ± 1.65 |
| Birth weight (g) | 3346.543 ± 100.622 | 3200.000 | 3141.250 ± 134.079 | - | 3630.556 ± 116.258 | 3652.500 ± 164.026 |
| Years of reproduction | 35.643 ± 4.043 | 36 | 35.993 ± 4.023 | - | 36.373 ± 5.523 | 37.563 ± 2.433 |
| Age of first menstruation | 12.432 ± 4.321 | 11 | 13.092 ± 4.317 | - | 13.522 ± 1.812 | 11.542 ± 0.717 |
| Age of last menstruation | 48.435 ± 4.133 | 47 | 48.981 ± 4.032 | - | 50.441 ± 4.332 | 49.000 ± 1.412 |
| Years after menopause | 6.732 ± 4.221 | 5 | 8.572 ± 4.208 | - | 7.112 ± 5.728 | 6.040 ± 1.412 |
| BMD L2–L4 (g/cm\(^2\)) | 0.963 ± 0.022 | 0.964 | 0.975 ± 0.014 | - | 0.973 ± 0.023 | 0.955 ± 0.025 |
| BMD L2–L4 YA (%) | 80.582 ± 1.932 | 80.000 | 81.278 ± 1.240 | - | 81.333 ± 1.291 | 80.703 ± 1.104 |
| BMD L2–L4 AM (%) | 88.531 ± 2.014 | 95.000 | 89.504 ± 1.123 | - | 87.420 ± 2.072 | 88.325 ± 2.475 |
| | | | | | | |

Table 4. Characteristics of the postmenopausal women with osteopenia, osteoporosis and normal T-score taking part in the study of the rs4148323 genetic variant of UGT1A1 gene. BMI body mass index, BMD L2–L4 YA bone mineral density in young adult, BMD L2–L4 AM bone mineral density compared with an age-matched. The AA genotype for the rs4148323 variant in the UGT1A1 gene was not identified. The GA genotype for women with osteoporosis was also not identified.
by the level of protein expression. People with the 6/7 genotype are characterized by a 1/3 reduction in UDP-glucuronosyltransferase activity38. Molecular analysis performed in this study also showed that the heterozygous 6/7 genotype of the UGT1A1*28 variant was slightly less common in women with osteoporosis (46.2%) as compared to healthy controls (51.5%). It was also observed that the 7/7 genotype was more common in women with osteoporosis and osteopenia as compared to the control group (18.3% vs. 10.7%, p = 0.049, OR = 1.87, 95% CI 0.93–3.70, respectively). Hence, it seems safe to conclude osteopenia and osteoporosis in postmenopausal Polish women.

The *UGT1A1* gene showed no statistically significant differences between the variant may affect the risk of developing osteopenia and osteoporosis in postmenopausal women, especially in the presence of homozygous genotypes containing two mutated alleles. The analysis of the frequencies of the GG, GA and AA genotypes of the rs1418323 UGT1A1 gene showed no statistically significant differences between the groups. The UGT1A1 rs4148323 (UGT1A1*6) genetic variant is not directly associated with the development of osteopenia and osteoporosis in postmenopausal Polish women.

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Conceptualization: A.B., A.K. and B.Cz.; methodology: A.K and A.B.; formal analysis: I.U.; investigation A.B. and I.U.; data curation: A.B. and A.S.M.; writing—original draft preparation: A.K., A.B. and I.U.; writing—review and editing: J.G., D.K. and A.S.M.; visualization: A.B.; supervision: B.Cz.; project administration: A.K., A.B. and B.Cz.; funding acquisition: A.K., D.K. and B.Cz. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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