Role of calcium metabolism in the pathogenesis of osteoporosis and colorectal cancer

Doctoral dissertation

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INTRODUCTION

In the Hungarian population the average calcium intake is significantly lower with a range of 400-600 mg per day than the suggested 1200-1500 mg per day in adults. Epidemiological studies suggest the preventive role of calcium intake in osteoporosis (OP) and colorectal cancer (CRC) next to other diseases.

Calcium seems to have little effect on bone loss if administered within the first five years of the menopause, when mainly estrogen withdrawal has a dominant role. Among women who were postmenopausal for six years or more, calcium supplementation (400-650 mg/day for two years) reduced bone loss. Moreover, in a heterogeneous elderly population a meta-analysis showed that calcium and vitamin D combined therapy reduce the rate of annual bone loss by 0.54 % at the hip and 1.19 % in the spine and all types of fractures by 12 % over 24-84 months. These effects were better with calcium doses of 1200 mg or more than with lower doses, and with vitamin D doses of 800 IU (international unit) or more than with lower doses. The relative changes in spine bone mineral density (BMD) from baseline is -1.1 % and in total hip BMD from baseline is -0.5 % in late postmenopausal women. Lower calcium intake is a key factor in abnormal bone mineralization which acts not only through reducing available calcium for bone mineralization but through the consequently elevated parathyroid hormone level (PTH) (secondary hyperparathyroidism) which results in bone loss via stimulating osteoclast maturation, proliferation and activity. In Hungary 900,000 people suffer from OP (600,000 women and 300,000 men). The number of fractures associated with OP was 30-40,000 in the lumbar spine, 25-28,000 in the wrist, 15,000 in the hip (femoral neck and pertrochanteric fractures) and 8-10,000 in the proximal humerus during one year.

Even though colorectal tumor genesis is a complex process, epidemiological and experimental data indicate that calcium has a chemopreventive role in the development of CRC. In a heterogeneous population (mean age = 61 years) calcium supplementation (1200 mg/day) for four years was also shown to decreased polyp recurrence by 25 % which is known as the starting point in colorectal carcinogenesis. In another study among male CRC patients (mean age = 60 years), dietary calcium intake (risk reduction was 54 %) was shown to be more beneficial than only total calcium intake (risk reduction was 32 %) with a follow-up of 6 years. Intraluminal calcium precipitates intestinal fatty acids and secondary bile acids, these are known as soluble hydrophobic surfactants with carcinogen effects. Both factors have show activity in colon epithelial cells and they have strong synergistic cytotoxic effects. Moreover, bile acids stimulate cell proliferation directly via an increased generation of 12-hydroxyeicosatetraenoic acid, a significant lipoxygenase product in carcinogenesis. Proliferative activity of colonic mucosa, reactive oxygen production enhanced by protein kinase C activation and apoptosis resistance induction are involved in the effects of bile acids. Consequently, the beneficial effect of calcium on colorectal carcinogenesis may act through the formation of insoluble salts with fatty acids and secondary bile acids which result in altered effects on colon mucosa. In Hungary, with approximately 8800 newly diagnosed cases annually, CRC is the second most common cancer type. The incidence rate with 87 cases (per 100,000) is significantly higher than the same value measured as 45-63 cases (per 100 000 for male-female) in white's people in the USA. The significance of this disease is emphasized by the poor five-year survival, which is approximately 40 %.

Dehydroepiandrosterone sulphate (DHEAS) and dehydroepiandrosterone (DHEA) play an important role in calcium metabolism, and thus they are also related to the development of OP and perhaps CRC. DHEA, a prohormone of androgens and estrogens, can be converted into potent androgens and estrogens in the adrenal gland and in peripheral tissues. DHEAS is desulfated by steroid sulfatase, and converts into bioactive androgens and estrogens as DHEA. DHEA can be converted into DHEAS catalyzed by sulfotransferase

enzyme. 99 % of DHEA is circulating as DHEAS. DHEA(S) output declines by 2 % per year. The use of DHEA cream for 12 months results in increasing BMD in the total hip by 2 % in postmenopausal women through altered interleukin-6 level, an osteoclast stimulating cytokine and through altered insulin-like growth factor-1 concentration, the known factor in the pathogenesis of bone loss. DHEA(S) may act on bone via converting into estrogens. Estrogen supplementation (conjugated equine estrogen) increased vertebral and total hip BMD by 4.6 % and 3 %, respectively, over three years in postmenopausal women. The role of androgens in osteoporosis is minor, patients with androgen insensitivity syndrome develop no OP. DHEA(S) level is not related to the risk of CRC in humans; however, in human adenocarcinoma cell line DHEA treatment resulted in growth inhibition and G1 arrest. Furthermore, DHEA supplementation reduced the rate of 1,2-dimethylhydrazine induced colon and anal tumors. DHEA(S) can also prevent colorectal carcinogenesis via conversation to estrogens and androgens. In postmenopausal women estrogen supplementation reduced the colorectal cancer risk by 19-46 % depending on the duration of therapy (one versus eleven years). In human colorectal cell lines 5-dihydrotestosterone also reduced proliferation.

We hypothesized the potential role of LCT 13910 C/T, CaSR A986S and CYP3A7*1C polimorphisms in OP and colorectal carcinogenesis acted through altered calcium metabolism. The CC genotype of the 13910 C/T polymorphism of LCT gene (encoding lactase phlorizin hydrolase (LPH) enzyme) has been reported to perfectly match with lactose intolerance (LI) and decreased milk consumption. Milk is one of the main calcium source in Caucasian rass. Moreover, in LI subjects the calcium absorption is lower, too, because it is decreased by the higher remaining lactose content in the gut. Calcium-sensing receptor (CaSR) A986S polymorphism results in higher PTH levels. CaSR on cell surface provides a connection between serum calcium level and several different effector mechanisms depending on the cell type. In osteoblasts, higher extracellular calcium stimulated alkaline phosphatase (ALP) activity and bone mineralization. CaSR regulates the proliferation, differentiation and apoptosis of colonic epithelial cells. We hypothesized that the polymorphism altered these beneficial effects. CYP3A7 is responsible for the metabolism of DHEAS, estrogen and androgen hormons in the fetal age. In case of a mutant CYP3A7 variant, - CYP3A*1C - the enzyme expression level stands at a higher level resulting in increased elimination of DHEAS and a consequent decreased DHEAS level.

We examined the role of calcium supplementation on MC3T3-E1 osteoblasts. Higher extracellular calcium related to higher alkaline phosphatase activity and osteocalcin expression via CaSR and mothers against DPP homolog-3 (SMAD-3) as a critical component of the transforming growing factor beta (TGF-β) signaling pathways. We hypothesized that the calcium supplementation next to increasing ALP activity stimulates extracellular structural protein (type I procollagen alpha 1 (COL1A1), type II procollagen alpha 1 (COL2A1), decorin (DCN), bone sialoprotein (BSP), fibronectin-1 (FN-1)) and other protein expressions (CaSR, bone gamma carboxyglutamate protein (osteocalcin) (BGLAP)) with important role in calcium metabolism. Also, we examined the significance of other members of TGF-β family in bone modeling, as bone morphogenetic protein-4 (BMP-4) and SMAD-3, mothers against DPP homolog-6 (SMAD-6).

OBJECTIVES

1. Examining association between LCT 13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms, BMD and bone fracture rate
2. Examining association between LCT 13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms and the incidence and/or progression of CRC

3. Examining the role of serum calcium and DHEAS level in OP and colorectal carcinogenesis
4. Examining the role of calcium supplementation in the proliferation, alkaline phosphatase activity and gene expression pattern (COL1A1, COL2A1, DCN, BSP, FN-1, BMP-4, SMAD-3, SMAD-6, CaSR, BGLAP) of MC3T3-E1 osteoblasts

PATIENTS AND METHODS

Population 1 - OP study

We examined 595 postmenopausal women including 267 subjects with age-related osteoporosis (mean age = 62 ± 10 years), 200 patients with osteopenia (mean age = 62 ± 10 years) and 128 healthy subjects (mean age = 56 ± 10 years) regarding LCT and CaSR genes. Milk consumption, body mass index (BMI), body height and weight, menopausal age, smoking habits, alcohol and caffeine consumption, history of steroid use and previous vertebral or non-vertebral fractures and laboratory parameters (serum calcium, phosporus, 25-OH-vitamin D_3, beta-crosslaps levels and alkaline phosphatase activity) were also recorded. We examined 319 postmenopausal women in the relation to CYP3A7 gene: 217 patients had age-related osteoporosis (mean age = 68 ± 6 years) and there were 102 healthy subjects (mean age = 55 ± 8 years). Body mass index (BMI), body height and weight, menopausal age, smoking habits, alcohol consumption, history of steroid use and previous non-vertebral fractures and laboratory parameters (serum DHEAS, calcium, phosporus, 25-OH-vitamin D_3 levels and alkaline phosphatase activity) were also recorded.

Population 2 - CRC study

We collected 538 participants, 278 subjects (130 female and 148 male) with primary colorectal cancer and 260 healthy blood donors. The age at the time of the diagnosis was 61 ± 11 years. Tumor localization, sex, tumor, node, metastasis stages (TNM) and American Joint Committee on Cancer stages (AJCC), locoregional and/or distant recurrence, time of death and laboratory parameters (serum alpha1-fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and calcium levels) were also obtained. Median follow-up period was 17 months (range 1-20 months). The control group consisted of healthy Caucasian blood donors without clinical data.

Genetic analysis of CaSR A986S G/T, LCT 13910 C/T and CYP3A7*1C polymorphisms

Genomic DNA was isolated from EDTA blood using a commercially available kit (Magnesil KF Genomic System, Promega, Madison, WI). We genotyped the LCT 13910 C/T (rs4988235) and CaSR A986S G/T (rs1801725) polimorphisms by Taqman RT-PCR method (Applied Biosystems, Foster City, CA). Genetic analysis for CYP3A7*1C T167G variant (rs11568825) was carried out by restriction fragment length polymorphism (RFLP) analysis (Sigma-Aldrich, St. Louis, MO; Sigma-Genosys, Woodlands, Texas; Promega, Madison, WI, USA). Results were quantitated by electrophoresis using standard horizontal Tris-Acetate-EDTA (TAE)-agarose gel-electrophoresis equipment and Fluorchem 8900 imaging system (Alpha Innotech, San Leandro, CA, USA).

Bone densitometry

Bone mineral density (BMD) at the lumbar spine (L1-4), total hip, femoral neck, Ward's triangle and radius were measured by dual energy X-ray absorptiometry (Lunar Prodigy, GE Medical Systems, Diegem, Belgium). For CYP3A7*1C polymorphism we used BMD at lumbar spine (L2-4) and femoral neck. Osteoporosis or osteopenia were defined by WHO criteria as a T-score < -2.5 standard deviation (SD) or -1 < T-score < -2.5 SD at any site.

Laboratory parameters

Serum calcium (2.25-2.61 mmol/L), serum phosphorus (0.8-1.45 mmol/L), albumin (35-50 g/L) levels and alkaline phosphatase activity (35-123 U/L) were measured by colorimetric assays (Roche Diagnostics, Indianapolis, IN). Serum beta-crosslaps (0-320 pg/mL), DHEAS (between the ages of 45-74 year: 0.3-7.0 µmol/l), CEA (< 4.3 ng/mL), AFP (< 13.6 ng/mL) and CA19-9 (< 39 U/mL) levels were measured by an automated immuno-chemiluminescence assay (Roche Diagnostics). Serum 25-OH-vitamin D_3 (75-160 nmol/L) measurement was carried out by high performance liquid chromatography (Bio-Rad Laboratories, Hercules, CA). (reference range for the measured parameters)

Cell cultures

The mouse osteoblast-like MC3T3-E1 cell line deriving from newborn mouse calvaria was cultured in α-MEM containing 1.8 mmol/L of calcium and 25 µg/ml ascorbic acid (Sigma-Aldrich) supplemented with 10 % fetal calf serum and antibiotics (Sigma-Aldrich).

Determination of viable cells

CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to determine the number of viable cells in culture with non-supplemented α-MEM and medium supplemented with 25.5 mM $CaCl_2$ after 24 hours. The method based on quantitation of ATP content which signals the presence of metabolically active cells. Luminescence was recorded by Fluoroskan Ascent FL (Thermo Fisher Scientific Inc, MA, USA).

Measurement of alkaline phosphatase activity on culture plates

ALP activity was determined by measuring p-nitrophenol generated from p-nitrophenil-phosphate due to enzymatic activity using an Olympus AU2700 analyzer (Olympus Corporation, Tokyo, Japan) after 15 days of incubation with non-supplemented α-MEM and medium supplemented with 25.5 mM $CaCl_2$. ALP levels were normalized against cellular protein levels: cells were digested with CellLytic TM buffer (Sigma-Aldrich) then protein concentrations were measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 280 nm by using Bradford assay (Sigma-Aldrich).

Gene expression study

To determine the effect of calcium concentration on gene expression, MC3T3-E1 cells were cultured with non-supplemented α-MEM and medium supplemented with 25.5 mM CaCl2. Total RNA was extracted from cells after 15 days by using High Pure RNA Isolation Kit (Roche Diagnostics). RNA quantity and purity was assessed by NanoDrop ND-1000

spectrophotometer (NanoDrop Technologies) at 260 nm. cDNA was synthesized by reverse transcription (Promega). Amplification was carried out by real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems). GAPDH as housekeeping gene was used to normalize gene expression.

Statistical analysis

The normal distribution of variables was analyzed by Kolmogorov-Smirnov-Liliefors test. The effect of characteristic variables on continuous variables with normal distribution were measured by analysis of variance (ANOVA) and/or analysis of covariance (ANCOVA). Without normal distribution Mann-Whitney unpaired t-test or Kruskal-Wallis tests were calculated. The relation between characteristic variables was examined by Chi-square test or binary logistics regression; we calculated Odds Ratio (OR) and 95 % Confidence Intervals (95% CI). Linear regression was used to measure relation between the continuous variables. The overall (OS) and disease free survival (DFS) were analyzed by Kaplan-Meier method or Cox regression. Hazard ratio (HR) and 95 % confidence intervals (95% CI) were also calculated. The survival factor levels were compared with log-rank test. We examined the effect of polimorphism on variables in recessive model, unless noted otherwise. The results were given as mean ± standard deviation (SD) or standard error of mean (SEM) for parameters with normal distribution or median (range, minimum - maximum) for parameters without normal distribution, unless noted otherwise. Significance was defined as $p < 0.05$. The data were analyzed using the SPSS statistical package (SPSS Inc., Chicago, IL; version 15.0 for Windows).

RESULTS

Effects of LCT 13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms in postmenopausal osteoporosis

There was no difference in the mutant allele frequencies between subjects with decreased BMD and healthy women.

Frequency of aversion to milk consumption was significantly higher for LCT 13910 CC ($p = 0.03$) and CaSR 986 SS ($p = 0.026$) genotypes and for CCSS genotype combinations ($p = 0.0002$). Albumin-adjusted serum calcium levels ($p = 0.031$), body height ($p = 0.002$) and BMD (Z-score) at radius ($p = 0.038$), Ward's triangle ($p = 0.044$) and total hip (CC vs. TT, $p = 0.041$) were significantly decreased in women with CC genotype. By excluding osteopenic subjects and analyzing only a group consisting of osteoporotic and healthy women ($n = 395$), a more marked influence of CC genotype on BMD (Z-score) could be demonstrated at lumbar spine ($p = 0.029$). In multiple regression analysis for Z-score using BMI as covariant, the percentage variance of BMD defined by LCT 13910 C/T genotype was 1.1 % at the radius ($p = 0.048$) and 1.7 % at total hip ($p = 0.018$). Serum calcium level did not correlate with BMD at any sites.

CaSR A986S polymorphism was found to be associated with OP only in the unified group of osteopenic and healthy subjects ($n = 328$): total hip Z-score was significantly lower for SS than other genotypes ($p = 0.036$). In multiple regression analysis for Z-score using BMI as covariant, the percentage variance of BMD defined by CaSR genotype was 1.3 % at total hip ($p = 0.04$).

We did not observe any association between CYP3A7*1C polymorphism and DHEAS concentration in our 319 subjects after adjusting for age. No correlation was found between genotypes and other laboratory parameters. Age negatively correlated with serum DHEAS

levels ($r = -0.52$, $p < 0.005$). The homozygous mutant GG genotype associated with significantly reduced Z-score ($p = 0.048$) at lumbar spine as compared to wild type in the whole study population. This correlation remained significant after adjusting for menopausal age, alcohol consumption, steroid intake, smoking habits and previous fractures ($p = 0.047$). We found significant regression between serum DHEAS levels and BMD expressed as Z-score at both lumbar spine ($r = 0.23$, $p < 0.005$) and femoral neck ($r = 0.17$, $p = 0.003$). The vertebral or non-vertebral bone fracture incidence was not different according to genotypes of polimorphisms.

Effects of LCT13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms in the pathogenesis of colorectal cancer

Frequency of the S allele of CaSR 986 was found to be significantly higher in CRC patients than in the control population ($p = 0.026$). The CCSS ($n = 5$) genotype combination was seen only in the patient group ($p = 0.033$). The distribution of C allele of the LCT gene and G allele of CYP3A7*1C gene was not significantly different between groups.

In patients with distant metastasis significantly higher CC frequency was seen during the follow-up period ($p = 0.012$). In men the DFS was also shorter for CC genotype ($p = 0.024$). There was no difference between the mean age of patients with CC genotype and other genotypes. In women we detected shorter survival by Kaplan-Meyer method only for genotype combination and not for single genotypes: DFS was worse for TCSS ($n = 3$) (log rank test $p < 0.005$). In methods examining the effect of polimorphism on survival parameters we used the type of therapy as an adjusting factor. In patients with lower serum calcium levels the T stages was higher ($p = 0.009$). Female patients with LCT 13910 CC genotypes had lower serum calcium concentrations ($p = 0.038$), no similar relationship could be seen in men. We could not find any associations between overall survival (OS), AJCC stages, histological grade or localization and LCT genotypes.

In female patients with CaSR 986 SS genotypes, serum calcium levels were higher as compared to other genotypes ($p = 0.033$). There was no correlation between locoregional or distant metastases, disease-free or overall survival, TNM, AJCC stages, histological grade, localization and CaSR A986S genotypes.

There was no correlation between locoregional or distant metastases, disease-free or overall survival, TNM, AJCC stages, histological grade, localization and CYP3A7*1C genotypes.

Effects of calcium supplementation on the proliferation, ALP activity and protein expression of MC3T3-E1 osteoblast cells

Calcium supplementation (25.5 mmol/L) reduced the proliferation of MC3T3-E1 osteoblast cells ($p < 0.001$), increased ALP activity ($p < 0.001$) and stimulated gene expressions of COL2A1 ($p < 0.01$), SMAD-3 ($p < 0.01$) and SMAD-6 ($p < 0.01$). Also, calcium supplementation reduced the COL1A1 ($p < 0.001$), DCN ($p < 0.01$), BSP ($p < 0.001$) and BMP-4 ($p < 0.01$) expression. CaSR and BGLAP (osteocalcin) genes were not expressed in detectable amount after supplementation. Change in FN-1 expression was not significant.

DISCUSSION

Effects of LCT 13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms in postmenopausal osteoporosis

We focused our investigation on polymorphic variations in LCT, CaSR and CYP3A7 genes to evaluate their potential association with BMD through altered calcium metabolism in postmenopausal women. We could demonstrate a significant correlation of LCT 13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms with BMD. Also, LCT and CaSR genes altered milk consumption. LCT genes related to albumin-adjusted serum calcium levels.

Decreased serum calcium seen in our subjects with LCT 13910 CC genotype could be - at least partly - a result of reduced milk consumption observed in this group. The CC genotype has been reported to perfectly match with lactose intolerance through low lactase phlorizin hydrolase - specific mRNA expression, decreased milk consumption and reduced calcium absorption from gut. Thus, it is not surprising that we observed decreased BMD in subjects carrying CC genotype. All these subjects were lactose intolerant, consuming less calcium and exhibiting lower serum calcium, as well. The contribution of LCT 13910 C/T mutation to BMD was between 1.1 - 1.7 % at different skeletal sites.

We could demonstrate significant association between CYP3A7 gene polymorphism and BMD with homozygous mutants having lower bone mass independently of serum DHEAS concentrations. This finding and the lack of association between CYP3A7*1C polymorphism and serum DHEAS level in women support the hypothesis that this genetic variation might influence bone mass via other CYP3A7 hormonal substrates (estrogen). Lower level of CYP3A7 mRNA expression was detected in the female than in male suggesting that the impact of CYP3A7 mutation on serum DHEAS level is stronger in male than in female. In line with these results, other study could detect a significant correlation between CYP3A7 polymorphism and DHEAS levels in men or in a heterozygous population, and failed to detect this relation in women. Estrogens are known to protect bone and estrogen supplementation increased BMD in postmenopausal women.

In conclusion, we could demonstrate an association between LCT 13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms and BMD that may be mediated through altered calcium metabolism. Lactose-free calcium supplementation could be an important factor in women with CC genotype in order to improve calcium absorption and prevent early reduction in bone mineral content. Emphasizing the significance of this, the incidence of lactose intolerance is high with 37 % in Hungary.

Effects of LCT13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms in the pathogenesis of colorectal cancer

The association between LCT 13910 C/T genotype and CRC incidence is known, but the impact of this polymorphism on CRC progression has not studied yet. Our study had a follow-up design. We found that distant recurrences were more common in patient with CC genotype. Also in men we have seen shorter disease free survival for CC genotype. Patients with LCT 13910 CC genotype and consequent lactose intolerance consume less milk products, which is the main source of calcium. Milk consumption could reduce risk for CRC because its calcium and lactose content. Calcium could act via intraluminal binding to bile and fatty acids causing their precipitation which is known as a pathogenetic factor in this disease. Lactose after converting to galactose could bind to lectins, the known factors of stimulated colon epithelial proliferation through the Thomsen-Friedenreich blood group antigen. In addition, calcium itself may inhibit hyperproliferation of colonic epithelial cells. It

was also shown that extracellular calcium regulates the differentiation of colonic epithelial cells via the CaSR. CaSR expression is the highest in well-differentiated regions of colon cancer and is nearly absent in the poorly differentiated sites which suggest the role of CaSR in colorectal carcinogenesis. The loss function of CaSR led to an increased, uncontrolled proliferation. In our study CaSR 986 SS genotype was accompanied by increased risk for CRC. We hypothesized that the filamin-A, as a significant adapter molecule to apoptotic cascade could not bind to the CaSR in the case of 986 SS genotype resulting in colorectal carcinogenesis. However, this polimorphism did not relate to the progression of CRC.

CYP3A7*1C polymorphism encoding a steroid metabolizing enzyme though all life has been shown to cause decreased DHEAS concentration in heterogenic population. The role of DHEAS in colorectal carcinogenesis is contradictory: no difference in DHEAS level was found between patients with colorectal cancer and healthy controls, however in human colon cancer cell line DHEAS supplementation reduced proliferation rate. We could not detect any associations between CYP3A7*1C polymorphism and colorectal cancer development or progression, emphasizing that DHEAS metabolism may have a minor role in colorectal carcinogenesis. In summary, LCT 13910 C/T and CaSR A986S polymorphisms appear to have an impact on colorectal carcinogenesis via calcium metabolism. The calcium prevention seems to be more important in our country because of the high incidence of CRC and lactose intolerance. CYP3A7*1C polymorphism did not relate to colorecral carcinogenesis.

Effects of calcium supplementation on the proliferation, ALP activity and protein expression of MC3T3-E1 osteoblast cells

Calcium has been shown to be essential for bone mineralization, osteoblast chemotaxis and differentiation. During bone remodeling, substantial amount of calcium (40 mmol/L) was found to be released from mineralized bone matrix, stimulating bone formation by osteoblasts. Higher calcium level acted on osteoblasts via CaSR stimulating SMAD-3 gene expression as a critical component of the TGF-β signaling pathways and associated with higher ALP activity providing bone mineralization. In lining with these results, we found higher SMAD-3 expression and ALP activity in calcium supplementation, emphasizing the role of SMAD-3 in the effect of calcium on osteoblasts. SMAD-6, the inhibitor of osteoblast differentiation, was elevated, too. Expression of bone structural extracellular protein COL2A1, which is characteristic for enchondral ossification was also found to be stimulated by calcium supplementation. BGLAP (osteocalcin) as an osteoblast inhibitor, and CaSR gene expression were not expressed in detectable amount after supplementation. In conclusion, calcium supplementation stimulated bone formation of MC3T3-E1 osteoblast cell through TGF-β signaling pathway resulting in new bone.

CONCLUSIONS

1. The LCT 13910 C/T polymorphism was associated with calcium intake, serum calcium level and bone mineral density
2. The CaSR A986S polymorphism was related to calcium intake and bone mineral density
3. The CYP3A7*1C polymorphism was associated with bone mineral density independently from DHEAS level
4. The LCT 13910 C/T, CaSR A986S and CYP3A7*1C polymorphisms were not associated with bone fracture rate
5. The LCT 13910 C/T polymorphism was related to the progression of colorectal cancer, supposedly acting through the decreased calcium intake

6. The CaSR A986S polymorphism was associated with the incidence of colorectal cancer probably through the altered apoptotic signal transduction
7. The CYP3A7*1C polymorphism did not affect the incidence or the progression of colorectal cancer
8. The beneficial effects of calcium supplementation on MC3T3-E1 osteoblast is linked to TGF-β signaling pathway

SUMMARY

In the Hungarian population, the average calcium intake with a range of 400-600 mg per day is significantly lower than the recommended value for adults (1200-1500 mg). Appropriate calcium intake appears to reduce the risk of bone loss and the development of colorectal cancer (CRC). Approximately, one-third of the Hungarian population is lactose intolerant, a condition caused by LCT gene mutation, that further reduces calcium intake. Our results suggested that LCT 13910 CC genotype with a consequent lactose intolerance leads to lower milk consumption and decreased serum calcium levels. LCT 13910 CC genotype caused decreased supply of calcium for bone metabolism resulting in lower bone mineral density (BMD). LCT 13910 CC genotype seemed to be associated with higher CRC recurrence. The CaSR protein, forwarding the signal of extracellular calcium into the cells, affects osteoblast activity and proliferation as well as colon cell metabolism. The CaSR 986 SS genotype was accompanied with decreased BMD and increased colorectal carcinogenesis. Next to calcium, steroid prohormone (DHEAS) is also important for bone and perhaps colon health. The CYP3A7*1C allele eliminating DHEAS with higher activity may contributed to the development of osteoporosis but not to the pathogenesis of CRC. Based on our results, the supplementation of calcium intake, especially in lactose intolerant people, may be beneficial in the prevention of osteoporosis and colorectal cancer.

Publications related to the dissertation

Bácsi, K., Kósa, JP., Borgulya, G., Balla, B., Lazáry, Á., Nagy, Z., Horváth, C., Speer, G., Lakatos, P. 2007. CYP3A7*1C polymorphism, serum dehydroepiandrosterone sulfate level, and bone mineral density in postmenopausal women. *Calcif Tissue Int* **80**(3): 154-9. **IF: 2.483**

Bácsi, K., Kósa, J., Lazáry, Á., Horváth, H., Balla, B., Lakatos, P., Speer, G. 2007. Importance of dehydroepiandrosterone and dehydroepiandrosterone sulfate in different diseases. *Orv Hetil* **148**(14): 651-7.

Bácsi. K., Kósa, J., Lazáry, Á., Balla, B., Horváth, H., Takács, I., Nagy, Z., Speer, G., Lakatos, P. 2007. Impact of CYP3A7*1C polymorphism on serum dehidroepiandrosteron sulphate level and bone mineral density in postmenopausal women. *Orv Hetil* **148**(27): 1273-80.

Lazáry, Á., Balla, B., Kósa, JP., **Bácsi, K.**, Nagy, Z., Takács, I., Varga, PP., Speer, G., Lakatos, P. 2007. Effect of gypsium on proliferation and differentation of MC3T3-E1 mouse osteoblast cells. *Biomaterials* **28**(3): 393-9. **IF: 5.196**

Bácsi, K., Hitre, E., Kósa, JP., Horváth, H., Lazáry, Á., Lakatos, LP., Balla, B., Nagy, Z., Lakatos, P., Speer, G. Effects of the lactose-phlorizin hydrolase 13910 C/T and calcium-sensor receptor A986S G/T polymorphisms on the incidence and recurrence of colorectal cancer (submitted for *Int J Cancer*, Manuscript number: IJC-08-0175)

Bácsi, K., Kósa, JP., Lazáry, Á., Balla, B., Nagy, Z., Lakatos, P., Speer, G. Effect of LCT 13910 C/T polymorphism on serum calcium level and bone mineral density in postmenopausal women (submitted for *Osteoporosis Int*, Manuscript number: OI-2008-01-0049)

Publications not related to the dissertation

Lazáry, Á., Speer, G., Varga, PP., Balla, B., **Bácsi, K.**, Kósa, JP., Nagy, Z., Takács, I., Lakatos, P. Effect of vertebroplasty filler materials on viability and gene expression of human nucleus pulposus cells. *J Orthop Res,* 2008. Jan. 4. (article in press, DOI 10.1002/jor.20532) **IF: 2.784**

Lazáry, Á., Balla, B., Kósa, JP., **Bácsi, K.**, Nagy, Z., Takács, I., Varga, PP., Speer, G., Lakatos, P. 2007. Synthetic bone grafts, the role of the gypsum in bone substitution; molecular biological approach. *Orv Hetil*, **148** (51): 2427-33.

Balla, B., Kósa, JP., Kiss, J., Borsy, A., Podani, J., Takács, I., Lazáry, Á., Nagy, Z., **Bácsi, K.**, Speer, G., Orosz, L., Lakatos, P. 2008. Different gene expression patterns in the bone tissue of aging postmenopausal osteoporotic and non-osteoporotic women. *Calcif Tissue Int*, **82**(1): 12-26 **IF: 2.483**

Kósa, JP., Kis, A., **Bácsi, K.**, Nagy, Z., Balla, B., Lazáry, Á., Takács, I., Speer, G., Lakatos, P. 2007. Új, a glükokortikoidok csontsejtekre gyakorolt hatásának közvetítésében szerepet játszó gének azonosítása izolált és immortalizált egér osteoblast sejteken. *Magyar Belorvosi Archívum,* **60**(5): 443-450.

Balla, B., Kósa, JP., Takács, I., Kiss, J., Podani, J., Borsy, A., Lazáry, Á., **Bácsi, K.**, Nagy, Z., Speer, G., Orosz, L., Lakatos, P. Menopauza hatása a csontszöveti génkifejeződésre posztmenopauzás és premenopauzás korú nem oszteoprotikus nőkben *Magyar Belorvosi Archívum* (atricle in press)

Kiss, J., Balla, B., Kósa, JP., Borsy, A., Podani, J., Takács, I., Lazáry, Á., Nagy, Z., **Bácsi, K.**, Szlávy, E., Szendrői, M., Speer, G., Orosz, L., Lakatos, P. Gene expression patterns in the bone tissue of women with fibrous dysplasia (submitted for *J Bone Miner Res*, Manuscript number: J0802072)

Lazáry, Á., Kósa, JP., Tóbiás, B., Balla, B., **Bácsi, K.**, Takács, I., Nagy, Z., Speer, G., Mező, T., Lakatos, P. Single nucleotide polymorphisms in new candidate genes are associated with bone mineral density in Hungarian postmenopausal women (submitted for *Eur J Endocrinol*, Manuscript number: EJE-08-0021)

Abstracts

Bácsi, K., Kósa, JP., Balla, B., Lazáry, Á., Nagy, Z., Takács, I., Speer, G., Lakatos, P. Decreased activity of lactase phlorizin hydrolase and bone mineral density in postmenopausal women. *American Society for Bone and Mineral Research (ASBMR) 29th Annual Meeting* Honolulu, HI, USA September 16-19, 2007. *J Bone Miner Res* 2007, **22**(S1): S186

Bácsi, K., Kósa, JP., Balla, B., Lazáry, Á., Nagy, Z., Takács, I., Speer, G., Lakatos, P. A laktáz phlorizin hidroláz és a csontdenzitás kapcsolatának vizsgálata posztmenopauzális nőkben. *Magyar Osteoporosis és Osteoarthrologiai Társaság XVI. Kongresszusa* Balatonfüred, 2007. május 23-26. *Ca és Csont* 2007, **10**(2): 50

Lazáry, Á., Varga, P., Speer, G., **Bácsi, K.,** Balla, B., Kósa, JP., Nagy, Z., Takács, I., Lakatos, P. Effect of Vertebroplasty Filler Materials on Viability and Gene Expression of Mouse and Human Nucleus Pulposus Cells. *American Society for Bone and Mineral Research (ASBMR) 29th Annual Meeting* Honolulu, HI, USA September 16-19, 2007. *J Bone Miner Res* 2007, **22**(S1): S160

Balla, B., Kósa, JP., Kiss, J., Borsy, A., Podani, J., Takács, I., Lazáry, Á., Nagy, Z., **Bácsi, K.,** Speer, G., Orosz, L., Lakatos, P. Effect of Estrogen Deficiency on Gene Expression Pattern in the Bone Tissue of Postmenopausal Versus Premenopausal Healthy Women. *American Society for Bone and Mineral Research (ASBMR) 29th Annual Meeting* Honolulu, HI, USA September 16-19, 2007. *J Bone Miner Res* 2007, **22**(S1): S261

Speer, G., Lazáry, Á., Kósa, JP., Tóbiás, B., Mező, T., **Bácsi, K.,** Balla, B., Takács, I., Nagy, Z., Lakatos, P. Multiplex SNP Genotyping and Data Analysis on 360 Hungarian Postmenopausal Women. *American Society for Bone and Mineral Research (ASBMR) 29th Annual Meeting* Honolulu, HI, USA September 16-19, 2007. *J Bone Miner Res* 2007, **22**(S1): S286

Balla, B., Kósa, JP., Takács, I., Kiss, J., Podani, J., Borsy, A., Lazáry, Á., **Bácsi, K.,** Speer, G., Orosz, L., Lakatos, P. Ösztrogén hiányában fellépő génexpressziós változás vizsgálata posztmenopauzás és premenopauzás nem osteoporosisos nők csontszövetében. *Magyar Osteoporosis és Osteoarthrologiai Társaság XVI. Kongresszusa* Balatonfüred, 2007. május 23-26. *Ca és Csont* 2007, **10**(2): 51

Lazáry, Á., Speer, G., Kósa, JP., Tóbiás, B., Balla, B., **Bácsi, K.,** Nagy, Z., Takács, I., Mező, T., Lakatos, P. Multiplex SNP genotipizálás és adatfeldolgozás 360 posztmenopauzás nő vérmintájából. *Magyar Osteoporosis és Osteoarthrologiai Társaság XVI. Kongresszusa* Balatonfüred, 2007. május 23-26. *Ca és Csont* 2007, **10**(2): 59

Kis, A., Balla, B., Lazáry, Á., Takács, I., Nagy, Z., Speer, G., Mező, T., **Bácsi, K.,** Lakatos, P. A tartós glükokortikoid terápia okozta osteoporosis létrejöttében résztvevő gének kimutatása osteoblastsejteken. *Magyar Osteoporosis és Osteoarthrologiai Társaság XV. Kongresszusa* Balatonfüred, 2006. május 24-27. *Ca és Csont* 2006, **9**(1): 19

Balla, B., Borsy, A., Kósa, JP., Kiss, J., Lazáry, Á., Takács, I., Nagy, Z., Speer, G., **Bácsi, K.,** Orosz, L., Lakatos, P. Génexpressziós mintázat vizsgálata posztmenopauzában levő egészséges és osteoporotikus nők csontszövetében. *Magyar Osteoporosis és Osteoarthrologiai Társaság XV. Kongresszusa* Balatonfüred, 2006. május 24-27. *Ca és Csont* 2006, **9**(1): 11

Lazáry, Á., Balla, B., Kósa, JP., Takács, I., Nagy, Z., Speer, G., **Bácsi, K.,** Koppány, V., Lakatos, P. A gipsz in vitro hatása az osteoblastok osztódására és differenciálódására. *Magyar Osteoporosis és Osteoarthrologiai Társaság XV. Kongresszusa* Balatonfüred, 2006. május 24-27. *Ca és Csont* 2006, **9**(1): 22

Balla, B., Kósa, JP., Takács, I., Lazáry, Á., **Bácsi, K.,** Lakatos, P. Génexpressziós mintázat vizsgálata prae- és postmenopausás egészséges nők csontszövetében. *A Magyar Endokrinológiai és Anyagcsere Társaság XXI. Kongresszusa* Debrecen, 2006. május 18-20. Magyar Belorvosi Archívum 2006, **1**: 24

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