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ORIGINAL ARTICLE

Non-synonymous polymorphisms in the *P2RX$_4$* are related to bone mineral density and osteoporosis risk in a cohort of Dutch fracture patients

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Abstract In the present study we investigated whether single nucleotide polymorphisms (SNPs) in the *P2RX$_4$*, which alter the *P2X$_4$R* function, are associated with the development of osteoporosis and whether an interaction between the *P2X$_4$R* and *P2X$_7$R* confer a synergistic effect of these two receptors on osteoporosis risk. Patients with fracture (690 females and 231 males, aged ≥50 years) were genotyped for three non-synonymous *P2X$_4$R SNPs*. Bone mineral density (BMD) was measured at the total hip, lumbar spine, and femoral neck. Subject carrying the variant allele of the Tyr315Cys polymorphism showed a 2.68-fold (95 % CI, 1.20–6.02) higher risk of osteoporosis compared with wild-type subject. Furthermore, significant lower lumbar spine BMD values were observed in subjects carrying the Cys315 allele as compared with wild-type (0.85±0.17 and 0.93± 0.17 g/cm^2, respectively; $p<0.001$). Assuming a recessive model, carriers of the variant allele of the Ser242Gly polymorphism showed increased BMD values at the lumbar spine compare to wild-type subject (1.11±0.35 and 0.92± 0.17 g/cm^2, respectively; $p=0.0045$). This is the first study demonstrating an association of non-synonymous polymorphisms in the *P2RX$_4$* and the risk of osteoporosis, suggesting a role of the *P2X$_4$R* in the regulation of bone mass.

Keywords *P2X$_4$* receptor · Osteoporosis · Bone mineral density · Polymorphisms

Introduction

Bone remodeling is the process that maintains the bone quality during life. Mechanical loading is one of the factors

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that controls the local bone remodeling process via regulating bone formation by osteoblasts and bone resorption by osteoclasts [1]. As a result of mechanical loading, ATP is released from mechanically stimulated cells [2–4]. ATP is known as one of the main mediators of the response of bone cells to mechanical loading. ATP acts on bone cells through its binding to specific P2 purinergic receptors. The P2 receptor family includes seven members of *P2X* receptors ($P2X_{1-7}$), and eight members of *P2Y* receptors ($P2Y_{1,2,4,6,11,12,13,14}$) [5]. Several *P2X* and *P2Y* receptors have been shown to be expressed in bone cells (reviewed in [6]), including the $P2X_4$ receptor ($P2X_4R$) and $P2X_7$ receptor ($P2X_7R$).

Expression of the $P2X_4R$ on osteoblasts was first described by Nakamura et al. [7], who demonstrated the presence of mRNA coding for the $P2X_4R$ in human osteoblast-like MG-63 cells. This result was confirmed in human osteoblastic SaM-1 cells and in cells from human osteosarcoma HOS, MG63, and SaOs cell lines [8–10], as well as from a rat osteoblast primary cell line Orriss (abstract) [27]. In osteoclasts, mRNA transcripts for $P2X_4$ were first identified in rabbit osteoclasts purified by micromanipulation [8]. Expression of the $P2X_4R$ was later confirmed in isolated rat osteoclasts and human osteoclastic cells [9, 10].

The fact that $P2X_4Rs$ are expressed in osteoblasts and osteoclasts would suggest a potential role in bone physiology for this receptor subtype. Binding of ATP to the $P2X_4R$ causes a conformational change of this receptor, which results in channel formation and membrane depolarization. This leads to an increase of intracellular [Ca^{2+}] both by direct Ca^{2+} ion influx and by an inward Na^{+} current that depolarizes the cell and opens voltage-operated Ca^{2+} channels [11–13].

To date only a few studies have investigated the possible role of the $P2X_4R$ in bone physiology. Naemsch and colleagues [8] reported that the activation of rabbit osteoclasts by ATP resulted in a rapid inward current which showed several characteristics consistent with those of $P2X_4$-mediated currents. The authors suggested that nucleotides released as a result of mechanical stimulation may act through binding to $P2X_4Rs$ to enhance the activity of osteoclasts and, consequently, the resorption of bone [8]. Possible involvement of the $P2X_4R$ in bone resorption was also shown by the group of Hoebertz et al. [10]. More recently further evidence for a role of the $P2X_4R$ in the activation of osteoclasts was reported by Binderman et al. [14], who showed that the expression of $P2X_4R$ in marginal gingival cells was significantly up-regulated after surgical separation of the marginal gingiva in an in vivo experiment. Moreover, these studies showed that a single local application of apyrase (an enzyme that degrades ATP) during surgery reduced alveolar bone loss, suggesting that the $P2X_4R$ was directly involved in activation of osteoclasts. In addition to its apparent effects on osteoclast function, a functional effect of the $P2X_4R$ on osteoblast-like cells has been suggested by Liu and Chen [15]. They showed that both the $P2X_7R$ and $P2X_4R$ may be involved in an ATP-induced cell proliferation in human osteosarcoma HOS cells.

Given the above observations, single nucleotide polymorphisms (SNPs) in the *P2X4* receptor gene ($P2RX_4$) that have putative effects on the function of this receptor subtype may affect bone mass and quality in humans. Therefore, the $P2RX_4$ may be a good candidate gene to predict osteoporosis risk as well as the associated fracture risk.

The $P2RX_4$ is located on the long arm of chromosome 12 (12q24.32), spans over 50 kbp with 12 exons. To date, four non-synonymous SNPs have been found in the $P2RX_4$. Stokes et al. [16] showed a loss of receptor function for one of these non-synonymous SNPs (Tyr315Cys), but no major effect on receptor function could be shown for the other three non-synonymous SNPs (Ala6Ser, Ile119Val, and Ser242Gly). Because the $P2RX_4$ lies only 23 kbp away from $P2RX_7$ to which it is closely related, these separate receptor subtypes may have evolved by a process of gene duplication [17]. The $P2RX_7$ is highly polymorphic with at least eight non-synonymous SNPs leading to a functional effect on the receptor. Several of these non-synonymous $P2X_7$ SNPs were shown to be significantly associated with bone mineral density (BMD) [18–21]. It is conceivable that the significant associations found between SNPs within the $P2RX_7$ gene and BMD arise through a genetically linked effect of the $P2X_7R$ and $P2X_4R$ on bone remodeling processes. Therefore, it is possible that an interaction of SNPs at the $P2RX_7$/$P2RX_4$ locus exerts a maximal effect on BMD and thus leads to high susceptibility for osteoporosis.

We here report on the association between $P2X_4R$ *SNPs* and osteoporosis in a Dutch cohort of fracture patients. Furthermore, we investigated whether an interaction between SNPs in the $P2X_4R$ and $P2X_7R$ confers the maximal effect on osteoporosis risk. We hypothesized that non-synonymous SNPs within the $P2RX_4$ causing a functional defect of the $P2X_4R$ would be related with the risk of osteoporosis and that an interaction between SNPs at the $P2X_7$/$P2X_4$ locus would have a synergistic effect in affecting BMD. To our knowledge this is the first study focusing on the association between SNPs within the $P2RX_4$ and osteoporosis.

Methods

Study population and design

The study population consisted of men and women aged ≥50 years, recruited at the osteoporosis outpatient clinic at the Maastricht University Medical Centre (MUMC^{+}),

The Netherlands, from patients receiving regular medical follow-up for a recent fracture. The regular medical follow-up procedure for fracture patients as well as the recruitment of the patients for the present study has been fully described previously [22].

Patients were divided in either cases [i.e., fracture patients suffering from osteoporosis (defined by *T*-score≤−2.5)] or controls [i.e., non-osteoporotic fracture patients (*T*-score>−2.5)]. The study was approved by the ethical committee of the University Hospital Maastricht and Maastricht University.

Bone density measurements

As part of the standard medical follow-up of fracture patients, bone mineral density (in grams/square centimeters) of the lumbar spine (L2–L4), femoral neck, and total hip (trochanter and neck) was assessed by dual X-ray absorptiometry, using the cross-calibrated Hologic QDR 4500 Elite densitometer (Waltham, MA, USA).

DNA extraction

Blood samples

DNA was extracted from blood using the Maxwell DNA purification system. EDTA anticoagulated blood (400 μL) was used, and the isolation procedure was performed according to the manufacturer's instructions.

Saliva samples

A plain cotton swab collection device (Salivette™, Sarstedt AG & Co., Numbrecht, Germany) was used to collect small amount of saliva for DNA extraction. Upon return the Salivette™ containing the saliva swab was stored in a refrigerator at 4 °C until DNA extraction. First, the swab which was kept in the collection tube was centrifuged at 4,000 rpm for 10 min, and the saliva was transferred to a 15-mL Nunc tube which was kept at 5 °C overnight. Using a pair of sterile tweezers, the swab was then transferred from the collection tube to a 50-mL Nunc tube; 4 mL sterile water was added, and the tube was kept at room temperature overnight. The next day the swab plus water was transferred back into the collection tube and again centrifuged at 4,000 rpm for 10 min; the saliva yield was again transferred to the 15-mL Nunc tube already containing the saliva yield from the day before. Next, cells were isolated from the saliva by centrifuging the saliva-containing 15 mL Nunc tube at 4,000 rpm for 10 min. Subsequently, the supernatant was carefully removed, leaving a cell pellet of 600–800 μl cells over the pellet. DNA extraction was carried out using Maxwell 16 DNA Purification Kits on the Maxwell 16 Instrument (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Genotyping

Genotyping was done by Sequenom (Sequenom, Hamburg, Germany) using the Sequenom MassARRAY® iPLEX Gold assay. The mass difference between SNP alleles was analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Internal validation study

To assess the accuracy of the genotyping assay, an internal validation study was performed in which a randomly selected number of samples (N=45) was genotyped for the second time, using restriction enzyme digestion of appropriate PCR products or Taqman assay. This was done according to a previously published protocol Hansen et al. [28]. When the results were compared with the original genotyping, we observed a discrepancy of ~4.2 % between the two different genotyping methods. The discrepancy appeared to be smaller (~2.7 %) if the original genotyping with the Sequenom MassARRAY® iPLEX Gold assay had failed for the maximum one SNP. Therefore, all subjects who had failed more than two SNPs in original genotyping were excluded from the statistical analysis.

Statistical analysis

Descriptive statistics were used to determine the prevalence of osteoporosis in the cohort of fracture patients, as well as to assess distributions of possible risk factors, including sex, age (in years), and body mass index (BMI, in kg/cm2), and to describe the occurrence of different fracture types. These analyses were performed using SAS, version 9.1.

Deviation of genotype frequencies from those expected under Hardy–Weinberg equilibrium was tested in the non-osteoporotic control subjects by the χ^2 test. The software package PLINK [23] was used to test for association between genetic variations and BMD and osteoporosis. All analyses were adjusted for age, sex, and BMI. Furthermore, we performed analyses stratified by sex. Both single SNPs and haplotypes were tested for association and different models of inheritance—additive, recessive, and dominant—were used.

Haplotype construction and linkage disequilibrium analysis

To test whether the *P2X4* SNPs had an independent effect on osteoporosis risk and whether an interaction between *P2X4* and *P2X7 SNPs* conferred a synergistic effect of these two receptors on osteoporosis risk, haplotypes were

constructed by means of Haploview v4.1, using 17 marker SNPs that change receptor function (i.e., two $P2X_4$ and 15 previously genotypes $P2X_7$ non-synonymous SNPs [19]. The nonrandom distributions of the haplotype were assessed by calculating pairwise linkage disequilibrium (LD) coefficient, D'. The value of $D'=1$ indicates complete dependency, i.e., no evidence for recombination between the SNP pairs; while $D'=0$ indicates no LD, i.e., linkage equilibrium. Strong LD was defined as having pairwise $D'>0.85$. Patterns of LD are visualized using Haploview v4.1. Haplotype block structure was examined if the LD color scheme indicated bright red or shade of pink/red.

Results

Study population

Results of the recruitment procedures have been published previously [19]. Briefly, we collected blood from a total of 381 fracture patients and saliva from a total of 1,064 patients with recent fracture. In blood samples genotyping failed in five (1.3 %) patients. In saliva samples DNA extraction failed in 27 (2.5 %) saliva samples and genotyping in 492 (46.2 %) samples. In total 921 samples were successfully genotyped and used for subsequent analyses.

We identified 283 (31 %) cases, of which 61 (22 %) are men and 222 (78 %) are women, and 638(69 %) controls, of which 170 are men (27 %) and 468 (73 %) are women. Characteristics of the case and control groups are shown in Table 1.

$P2X_4$ polymorphisms and linkage disequilibrium

Subjects were genotyped for three non-synonymous SNPs within the $P2RX_4$ (Fig. 1). We found no subjects carrying the minor allele of the Ile119Val (**rs28360470**) polymorphism. The Ser242Gly (**rs25644**) polymorphism was found to have a minor allele frequency of 0.105 ($n=182$), and the loss-of-function Tyr315Cys (**rs28360472**) polymorphism was found to have a minor allele frequency of 0.013 ($n=24$) (Table 2). The genotype distributions for the polymorphisms Ser242Gly and Tyr315Cys polymorphisms were in Hardy–Weinberg equilibrium (Table 2). Pairwise LD calculations showed that the Tyr315Cys polymorphism was in weak LD with loss-of-function polymorphisms Gly150Arg ($P2X_7\_474G\_A$) ($D'=0.35$) and the Glu496Ala ($P2X_7\_1513A\_C$) ($D'=0.62$) within the $P2RX_7$ (Fig. 2). The Ser242Gly polymorphism was found to be in LD with a haplotype block within the $P2RX_7$, containing four functional SNPs, including two loss-of-function SNPs, Thr357Ser ($P2X_7\_1096C\_G$),and Glu496Ala ($P2X_7\_1513A\_C$); one gain-of-function SNP, Ala348Thr ($P2X_7\_1068G\_A$); and one marker gain-of-function SNP (i.e., the clinical effect of this polymorphism is caused by another polymorphism in linkage disequilibrium with this polymorphism), Gln460Arg ($P2X_7\_1405A\_G$).

Table 1 Characteristics of the study population

Characteristics	Total ($N=921$)	Cases[a] ($N=283$)	Controls ($N=638$)
Age (years)	65.0 (9.8)	67.3 (9.6)	64.0 (9.7)
Weight (kg)	72.5 (13.8)	67.7 (12.8)	74.6 (13.7)
Height (cm)	165.8 (9.1)	163.8 (9.3)	166.7 (8.8)
BMI (kg/m2)	26.3 (4.2)	25.2 (3.9)	26.8 (4.3)
Femoral neck BMD (g/cm2)	0.69 (0.13)	0.60 (0.10)	0.72 (0.12)
Total hip BMD (g/cm2)	0.84 (0.15)	0.72 (0.13)	0.88 (0.14)
Lumbar spine BMD (g/cm2)	0.93 (0.17)	0.77 (0.12)	0.99 (0.14)

BMD bone mineral density

[a] Cases of osteoporosis as defined by BMD T-score values of $T\leq -2.5$

Associations of $P2X_4$ polymorphisms with osteoporosis

Table 2 shows the genotype distribution in cases and controls. No difference in genotype frequency between cases and controls was found for the Ser242Gly polymorphism. The frequency of carrying the G allele of the Tyr315Cys polymorphism was significantly higher among cases (5 %) compared to controls (2 %) ($p=0.013$). The additive risk model showed a 2.68-fold higher odds ratio (95 % CI, 1.20–6.02) on the risk of osteoporosis for carriers of the 315Cys mutation.

Association between *P2X4* polymorphisms and BMD

The Tyr315Cys polymorphism, which showed an association with osteoporosis, was also associated with lumbar spine BMD (Table 3). Subjects carrying the Cys315 allele had lower BMD at the lumbar spine 0.85±0.17 g/cm^2 (mean±SD) as compared with wild-type subjects (0.93±0.17 g/cm^2) ($p<0.001$) (Table 3). Similar results were found in the sex-stratified analyses. Both men and women harboring the Cys315 SNP showed significantly decreased lumbar spine BMD values compared to wild-type subjects (men, 0.96±0.17 g/cm^2 versus 0.98±0.17 g/cm^2, $p=0.015$; women, 0.82±0.16 g/cm^2 versus 0.91±0.17 g/cm^2, $p=0.012$).

For the Ser242Gly polymorphism, we observed increased BMD values at all sites for homozygous subjects. Using a recessive model, we found a significant increase in BMD values at the lumbar spine in subjects carrying the Gly242 mutation (1.11±0.35 g/cm^2 versus 0.92±0.17 g/cm^2, $p=0.005$). Again, similar results were shown in gender-stratified analyses (Table 3).

Fig. 1 non-synonymous SNPs in the *P2X₄* receptor gene which were assessed in the study population. *Inverted triangle* indicates polymorphisms with reduced receptor function

Associations of haplotypes with osteoporosis

Although LD was shown between the *P2X₇* Glu496Ala (**rs3751143**) polymorphism and the Tyr315Cys polymorphism (Fig. 2), it was not included in the haplotype analysis because it did not affect the BMD values. Furthermore, by including the Glu496Ala, the statistical power would be reduced since it would subdivide haplotype H11 in two haplotypes.

Subjects who carries either of Gly150Arg (**rs28360447**) and Tyr315Cys SNPs [i.e., H12, H13, and H14 (H12, Arg150-Tyr315; H13, Gly150-Cys315; H14, Arg150-Cys315)] showed a significantly increased risk of osteoporosis compared to wild-types [OR=2.27 (95 % CI 1.23–4.17), p=0.008].

Haplotypes H12, H13, and H14 showed an increased risk of osteoporosis when compared to the other haplotypes [H12 versus H11, H13, and H14, OR=2.08 (95 % CI, 0.89–4.85); H13 versus H11 and H12, H14, OR=2.44 (95 % CI, 0.84–7.13); H14 versus H11, H12, and H13, OR=2.60 (95 % CI, 0.64–10.50)] (Table 4). However these increased risks did not reach statistical significance (p=0.009, p=0.103, and p=0.180, respectively) (Table 4).

Associations of haplotypes with BMD

Haplotypes containing at least one mutation of the Gly150Arg and Tyr315Cys polymorphisms (i.e., H12–H14) showed significantly decreased lumbar spine BMD values when compared to wild-types [i.e., haplotype H11) (0.87±0.16 versus 0.93±0.17, respectively (p=<0.001)] (data not shown).

Discussion

This is the first study investigating the association between non-synonymous SNPs in the *P2RX₄* with osteoporosis. Results showed that the Tyr315Cys polymorphism was significantly associated with decreased BMD values and increased risk of osteoporosis. Furthermore, the Ser242Gly polymorphism showed an association with increased lumbar spine BMD values. These results provide supportive evidence to our hypothesis that the *P2X₄R*, together with the *P2X₇R*, plays a role in bone physiology, as it has been shown that both receptors involve in activation of both osteoclasts and osteoblasts [8, 10, 14]. However, the overall effect of the *P2X₄R* and *P2X₇R* in bone metabolism is unclear.

The allele frequencies for the Tyr315Cys and Ser242Gly polymorphism in our population were almost identical to the previously published data Stokes et al. [26]. Both SNPs were shown to be in HWE, indicating a valid population for current study. However, the minor allele of Ile119Val SNP was not found in this population.

Table 2 HWE in the study population and genotype distribution in cases (i.e., patients suffering from osteoporosis as defined by BMD *T*-score≤−2.5) and controls (i.e., patients having a BMD *T*-score>2.5) for each single *P2X₄* SNP

	Cases	Controls	MAF	HWE p value
Ser242Gly (**rs25644**)	–	–	0.105	1
AA (%)	224 (80)	500 (80)	–	–
AG (%)	53 (19)	119 (19)	–	–
GG (%)	3 (1)	1	–	–
χ^2	OR=0.99 (95 % CI, 0.7165–1.37), χ^2=0.003, p=0.956	–	–	
Tyr315Cys (**rs28360472**)	–	–	0.013	1
AA (%)	270 (95)	622 (98)	–	–
AG (%)	13 (5)	11 (2)	–	–
GG	–	–	–	–
χ^2	OR=2.68 (95 % CI, 1.194–6.024), χ^2=6.169, p=0.013	–	–	

OR odds ratio; *CI* confidence interval; *MAF* minor allele frequency; *HWE* Hardy–Weinberg equilibrium

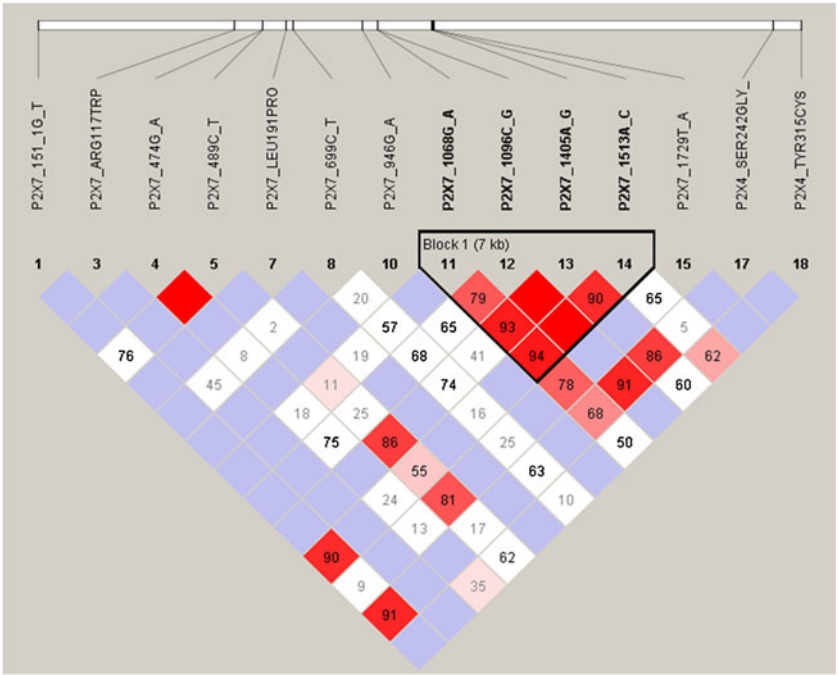

Fig. 2 Haploview analysis of pairwise linkage disequilibrium between $P2X_4$ and $P2X_7$ SNPs using 14 marker SNPs that change receptor function. Plot of relative D'/LOD scores between the $P2X_4$ and $P2X_7$ SNPs from Dutch Caucasian subjects produced by the Haploview program. Standard color scheme is displayed as follows: *bright red* ($D'=1$; LOD≥2), *blue* ($D'=1$; LOD<2), shade of *pink*/*red* ($D'<1$; LOD≤2), and *white* ($D'<1$; LOD<2). Numbers represent D' scores for pairwise linkage disequilibrium

Previous in vitro studies showed two functional effects for the Tyr315Cys SNP. First, the Cys315 mutation showed a reduction in agonist (i.e., ATP) potency, suggesting that the Tyr315 locus may be involved in ATP binding [16, 24, 25]. Second, it reduced the maximum response of the $P2X_4R$ caused by disruption in ion channel function [16]. Therefore, the Tyr315Cys polymorphism is considered to be a loss-of-function polymorphism of the *P2X4R*. Combining these in vitro results with the results found in the present study, which shows decreased lumbar spine BMD values and increased risk of osteoporosis in subjects harboring the minor allele of the Tyr315Cys SNP, the overall effect of the *P2X4R* in bone appears to be mainly pro-osteogenic.

As shown by LD calculations, the Tyr315Cys mutation is in weak LD with the functional Gly150Arg SNP in the *P2RX7*. We previously showed that this *P2X7* SNP is associated with decreased BMD values (Supplementary Table 2) [19]. Furthermore, the frequency of carrying the G allele of the Gly150Arg polymorphism was significantly higher among cases (5 %) compared to controls (2 %) ($p=0.021$) (Supplementary Table 3). It could therefore be possible that the association of the Tyr315Cys polymorphism with BMD and osteoporosis found in the present study is in fact due to functional effects of the Gly150Arg polymorphism in the *P2RX7*. However, haplotype analyses showed that both haplotype H12, containing Arg150-Tyr315, and H13, containing Gly150-Cys315, were associated with decreased lumbar spine BMD values and increased risk of osteoporosis, suggesting independent effects of both the Tyr315Cys and Gln150Arg polymorphisms. An independent effect was confirmed by the similar values for D' ($D'=0.35$) found in cases and controls (data not shown). No conclusions could be drawn whether these SNPs confer a synergistic effect on BMD values and on the risk of osteoporosis, since the numbers of subjects harboring both mutated alleles was too low ($N=9$). Replication in larger cohort studies is therefore warranted.

Although in vitro studies showed no functional effect of the Ser242Gly polymorphism [16], we found significantly increased lumbar spine BMD values in homozygous subjects. Haploview showed strong LD for this SNP with a haplotype block containing four functional SNPs within the *P2RX7*, of which two (Ala348Thr and Gln460Ala) are known to be associated with increased BMD values [19–21]. However, the LD between the SNPs was shown to be caused by the co-inheritance of the Gly242 minor allele with the major alleles (i.e., wild-type) of Ala348Thr and Gln460Ala polymorphisms (Supplementary Table 1). Therefore, the protective effect on osteoporosis found for the Ser242Gly polymorphism in the present study is unlikely due to the genetic linkage with either the Ala348Thr or Gln460Ala gain-of-function SNP. However, it remains possible that other yet-to-be-identified functional polymorphisms in LD with the Ser242Gly SNP are responsible for the effect of this SNP on BMD found in the present study.

The present study has several limitations. First, the low number of subjects may have influenced the precision of the results, especially when performing stratified and haplotype analyses. Replication in large cohort studies is therefore necessary. Another limitation that could have influenced the precision of the results is the lack of access to the risk factors influencing the development of osteoporosis. However, genetic association studies are not likely to be

Table 3 BMD values for the individual genotypes for each single SNP

SNP	WT	HET	HOMO	*p* value[a]	*p* value[b]	*p* value[c]
Tyr315Cys	AA	AG	GG	–	–	–
N	885	24	–	–	–	–
BMD TH (g/cm^2)	0.84 (0.15)	0.79 (0.17)	–	–	–	0.5467
BMD LS (g/cm^2)	0.93 (0.17)	0.85 (0.17)	–	–	–	<0.001
BMD FN (g/cm^2)	0.69 (0.13)	0.66 (0.15)	–	–	–	0.9356
Female	AA	AG	GG	–	–	–
N	667	19	–	–	–	–
BMD TH (g/cm^2)	0.80 (0.13)	0.76 (0.17)	–	–	–	0.8819
BMD LS (g/cm^2)	0.91 (0.17)	0.82 (0.16)	–	–	–	0.0124
–	BMD FN (g/cm^2)	0.63 (0.16)	–	–	–	0.6703
Male	AA	AG	GG	–	–	–
N	225	5	–	–	–	–
BMD TH (g/cm^2)	0.95 (0.15)	0.92 (0.09)	–	–	–	0.9546
BMD LS (g/cm^2)	0.98 (0.17)	0.96 (0.17)	–	–	–	0.0145
BMD FN (g/cm^2)	0.76 (0.13)	0.73 (0.06)	–	–	–	0.9404
Ser242Gly	AA	AG	GG	–	–	–
N	718	169	10	–	–	–
BMD TH (g/cm^2)	0.84 (0.15)	0.82 (0.15)	0.85 (0.17)	0.8075	0.5728	0.6127
BMD LS (g/cm^2)	0.92 (0.17)	0.92 (0.15)	1.11 (0.35)	0.3448	0.0045	0.4189
BMD FN (g/cm^2)	0.69 (0.13)	0.68 (0.13	0.70 (0.11)	0.7087	0.8949	0.6131
Female	AA	AG	GG	–	–	–
N	549	125	6	–	–	–
BMD TH (g/cm^2)	0.80 (0.13)	0.79 (0.13)	0.78 (0.17)	0.6206	0.5859	0.6883
BMD LS (g/cm^2)	0.91 (0.16)	0.91 (0.15)	1.09 (0.36)	0.5507	0.0202	0.9283
BMD FN (g/cm^2)	0.67 (0.12)	0.65 (0.11)	0.65 (0.10)	0.5907	0.8804	0.5889
Male	AA	AG	GG	–	–	–
N	175	47	4	–	–	–
BMD TH (g/cm^2)	0.95 (0.15)	0.93 (0.17)	0.97 (0.12)	0.8178	0.8931	0.8280
BMD LS (g/cm^2)	0.98 (0.16)	0.98 (0.16)	1.14 (0.38)	0.6539	0.0510	0.9131
BMD FN (g/cm^2)	0.76 (0.13)	0.74 (0.14)	0.77 (0.10)	0.8568	0.9493	0.8231

p values are shown for PLINK association analysis for the bone mineral density (BMD) parameters adjusted for age, BMI and sex. Numbers are mean (SD)

WT wild-type; *HET* heterozygote; *HOMO* homozygote; *TH* total hip; *LS* lumbar spine; *FN* femoral neck

[a]Adjusted for age, BMI, and sex: additive model

[b]Adjusted for age, BMI, and sex: recessive model

[c]Adjusted for age, BMI, and sex: dominant model

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Table 4 Haplotype frequency in the study population in both affected and unaffected subjects

	P2X$_7$, Gly150Arg	*P2X$_4$*, Tyr315Cys	Population frequency	Affected frequency	Unaffected frequency	OR	95 % CI	*p*-Value	Number
H11[a]	G	A	97.4	95.5	98.2	0.44	(0.24–0.81)	0.008	867
H12[b]	A	A	1.3	2.1	0.88	2.08	(0.89–4.85)	0.090	22
H13[c]	G	G	0.8	1.4	0.56	2.44	(0.84–7.13)	0.103	15
H14[d]	A	G	0.5	0.88	0.31	2.60	(0.64–10.50)	0.18	9

Furthermore, the table shows the osteoporosis risk for each haplotype compared to all other haplotypes and their osteoporosis risk

OR odds ratio; *CI* confidence interval

[a] Wild-type

[b] H12: Arg150-Tyr315

[c] H13: Gly150-Cys315

[d] H14: Arg150-Cys315

confounded by behavioral and environmental factors, as these factors are very unlikely to show an association with the genotype (i.e., a necessary condition for confounding).

In conclusion this is the first study demonstrating an association of non-synonymous polymorphisms in the *P2RX4* and the risk of osteoporosis, suggesting a role of the *P2X4R* in the regulation of bone mass. However, to elucidate the exact role of *P2X4R* SNPs in bone physiology and to evaluate the effect of the interaction between *P2RX4* and *P2RX7* on the risk of osteoporosis, future study in larger cohorts is necessary.

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