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ORIGINAL INVESTIGATION

The cannabinoid receptor type 2 (*CNR2*) gene is associated with hand bone strength phenotypes in an ethnically homogeneous family sample

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Abstract Genetic variants within the *CNR2* gene encoding the cannabinoid receptor CB2 have been shown to be associated with osteoporosis and low bone mineral density (BMD) in case-control studies. We now examined the association of polymorphisms in *CNR2* with hand bone strength in an ethnically homogeneous healthy family sample of European origin (Chuvashians) living in Russia. We show that non-synonymous *CNR2* SNPs are significantly associated with radiographic hand BMD and breaking bending resistance index (BBRI) by two different transmission disequilibrium tests. For both tests highly significant *p* values (ranging from 0.007 to 0.008 for hand BMD, and from 0.001 to 0.003 for BBRI) were also obtained with additional SNPs at the *CNR2* locus. The associations remained significant after correction for multiple testing. In conclusion, in addition to the association of *CNR2* polymorphisms with low BMD at selected clinically relevant skeletal sites, we now report their significant association with hand bone strength phenotypes using a family-based study design implying an even broader impact of genetic variation at the *CNR2* locus on bone structure and function.

Introduction

Bone remodelling is a continuous process, which is regulated by various hormones and metabolic factors, most of which have a significant genetic basis. It has been shown that endocannabinoid signalling is an important component involved in the modulation of bone metabolism (Idris et al.

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2005, 2008; Ofek et al. 2006; Tam et al. 2006, 2008). The cannabinoid system consists of G-protein coupled cannabinoid receptors, CB1 and CB2, and their endogenous ligands. This system is well known to tune important steps of cell communication in the brain and presumably also in bone (Idris et al. 2005, 2008; Ofek et al. 2006; Tam et al. 2006, 2008). The CB1 receptor is mainly expressed in the nervous system whereas CB2 is strongly expressed in immune cells. The main endogenous lipid ligands are arachidonic acid derivatives like arachidonoylethanolamine (anandamide, AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Sugiura et al. 1995). These endocannabinoids are synthesized on demand and released to the extracellular space. After binding to their receptors, downstream signalling events are responsible for inhibition of further release of e.g., neurotransmitters (Wilson and Nicoll 2001) and cytokines/chemokines (Karsak et al. 2007; Klein et al. 1998).

In the last years, a complete and functionally relevant bone endocannabinoid system was identified and it has been shown that bone tissue produces very high levels of endocannabinoids (Tam et al. 2008). Osteoclasts and osteoblasts express CB2 receptors (Ofek et al. 2006), and the 2-AG synthesizing enzymes diacylglycerol lipase α and β (Tam et al. 2008), whereas CB1 receptors were so far just identified in osteoclasts (Idris et al. 2005). For CB2 receptors it was shown that they regulate the activity of bone cells. Osteoblasts stimulated with a CB2 specific agonist showed a reduced expression of RANKL (Ofek et al. 2006). The same agonist stimulated the proliferation of wild-type primary calvarial osteoblasts through a Gi/o protein-dependent mechanism, but inhibited the proliferation and differentiation in monocytic cells undergoing osteoclastic differentiation. In contrast, it was shown that CB2 receptor antagonists/inverse agonists inhibit the osteoclast formation and activity (Idris et al. 2008). Interestingly, CB2 deficient mice ($Cnr2^{-/-}$) have a marked age-related trabecular bone loss and cortical expansion, which is reminiscent of human osteoporosis (Ofek et al. 2006).

Indeed, human genetic association studies have demonstrated a role of the CB2 gene (*CNR2*) in osteoporosis. In a French case-control sample, a significant association of osteoporosis with SNPs and haplotypes encompassing the *CNR2* locus has been shown (Karsak et al. 2005). Two of the associated SNPs were in adjacent nucleotides ("double SNP" rs2502992–rs2501432) within the coding region of CB2 and result in a non-conservative missense variant (Gln63Arg). This variant is probably functionally relevant as demonstrated by a differentially endocannabinoid-induced inhibition of T lymphocyte proliferation (Sipe et al. 2005). An independent genetic study could replicate the association of one *CNR2* SNP with BMD in a large Japanese sample of pre- and postmenopausal women (Yamada et al. 2007).

In the present study, we intended to extend and independently confirm these findings by testing whether the *CNR2* influence is also detectable (1) in a healthy and ethnically homogeneous family sample allowing for a family-based association approach and (2) on bone sites and bone phenotypes not analyzed before. To this aim, we have examined 16 SNPs encompassing the extended *CNR2* locus in a family sample of the Chuvashian population. As indicators of bone strength and fragility we used hand radiographic BMD and BBRI, representing a component of the cross-sectional moment of inertia.

Materials and methods

Population sample

We studied 574 adults, 290 men and 277 women, ranging between 18 and 90 years of age and belonging to 126 two- to four-generation pedigrees. The subjects were uniformly distributed with respect to age between 20 and 70 years. The pedigrees comprised 3–14 persons. The studied individuals were all Chuvashians (Caucasians, descendents of Bulgaric tribes) from small villages in the Chuvasha and Bashkirostan autonomies of the Russian Federation. Their population is demographically stable and they have lived there for centuries. The environmental conditions, particularly dietary influences, have been relatively constant and genetic flow from the surrounding populations has been small (El'chinova et al. 2002). Further details on this relatively isolated population are given elsewhere (Livshits et al. 2002). Our genetic fieldwork in Chuvasha consisted of physical examination, health questionnaire conducted by a native speaker, anthropometric measurements, hand radiographs, and blood samples. All diagnostic measures were in compliance with the Helsinki Declaration. Written informed consent was obtained after due approval by the Tel-Aviv University ethics committee.

BMD measurements

The method has been described previously (Livshits et al. 2002). Briefly, standard plain radiographs of both hands were taken in the postero-anterior position with the X-ray source positioned 60 cm above, at an exposition time of 5–10 s, 100–150 mA, and 50 kv. Both hands and a bone reference control wedge were placed on the same film-containing plate to avoid variation of film development. BMD was measured on 18 bones (metacarpal, proximal, and middle phalanges × 3 fingers × 2 hands). The index of mineralization was measured in units of optical density (1 mg/mm^2), and the range of density measurement varied between 0 and

25 mg/mm^2. Each radiograph was converted into a digital image using the computer-attached scanner. All measurements on digitalized radiographs were carried out using UTHSCSA ImageTool Version 3.0 for Windows software package (http://ddsdx.uthscsa.edu/dig/itdesc.html) with the scripts written by one of us (I.M). The original data for each bone were standardized. The average for 18 bones was adjusted for age and sex variation.

BBRI measurements

It is widely accepted that although BMD is considered as the best predictor of osteoporotic fracture risk, it takes into account only in part the bone cross-sectional area that is an important determinant of both bone compression strength and of bending breaking resistance (e.g., Gatti et al. 2001; Kaptoge et al. 2008). BBRI is one of the geometrical indices that can be used to assess the cross-sectional moment of inertia in long bones (e.g., Kaptoge et al. 2008; Livshits et al. 2003). This procedure was reported in detail elsewhere (Kalichman et al. 2008). From the obtained digital gray-scale images (1,200 × 1,800 pixels with a resolution of 150 DPI) several measurements of bone size were taken. BBRI was measured on 12 bones (metacarpal and proximal phalanges × 3 fingers × 2 hands). Our program enabled us to mark the bone edge of the measured bone by a computerized contour line and to subsequently process the inside of the contour to get a number of quantitative bone shape and geometry traits of interest. The trait list included measurements of the bone's midshaft diameter (D) and medullary canal width (MCW), which were used to estimate BBRI = [(D4 − MCW4)/D]. The original data for each bone were standardized. The average for 12 bones was adjusted for age and sex variation.

Genotyping, quality control, and haplotype reconstruction

DNA was prepared from peripheral blood lymphocytes by standard techniques, the choice of SNPs has been described previously (Karsak et al. 2005). All but the "double SNP" (rs2502992 and rs2501432) were genotyped by PCR amplification using the ABI Assays-On-Demand/Design™ SNP Genotyping Products (Karsak et al. 2005). Genotyping of the "double SNP" was performed by Pyrosequencing™ on the PSQ™ HS 96A System (Biotage AB, Uppsala, Sweden). Primer sequences for the assays are published (Karsak et al. 2005) or are available upon request. Amplification conditions were standard as specified by the supplier. Controls were included to exclude mix-ups and other errors during genotyping. Thus, each plate contained a well with DNA-free reaction mix to detect contamination with DNA. Another well contained a dedicated DNA, which was expected to yield identical genotypes for all plates genotyped for a given genetic variant.

Statistical analysis

Preliminary statistical analyses were conducted using the STATISTICA 7 package for Windows (Statsoft Inc., USA). All original bone-specific phenotypes measured on digitalized radiographs were first standardized (*Z*-scores) using the trait specific mean and standard deviation in the whole sample. Next, we calculated the mean BMD values for all 18 hand bones, and mean BBRI for 12 bones, for each individual in the sample. Thus, two complex phenotypes were produced: (1) mean BMD and (2) mean BBRI. Both phenotypes subsequently were adjusted for age and according to sex as described in detail previously (Kalichman et al. 2008; Livshits et al. 2002).

The preliminary analysis also included BMD regression on the number of the selected alleles and comparison of the phenotype distributions of different homozygotes.

Statistical genetic analysis: we conducted the joint quantitative trait—SNP association analysis by means of transmission disequilibrium tests (TDTs). Specifically, we used two tests: the family-based association test, FBAT proposed by (Horvath et al. 2001) and implemented in the FBAT program and the orthogonal test (OT) proposed by (Abecasis et al. 2000) and included in the QTDT program. Both these TDT-like tests, in different ways, take advantage of families having more than one offspring. It has been shown recently that implementation of different TDTs give more reliable results than using one test only (Ermakov et al. 2008). The linkage disequilibrium (LD) pattern for SNPs in the *CNR2* region was calculated using the MAN-6 package for Windows (Malkin and Ginsburg 2006). The same software was used for haplotype reconstruction.

Results

Basic descriptive characteristics, including age and body size/mass traits, of the studied sample for each sex are presented in Table 1. Additionally, we provide as an example main statistics for BMD and BBRI of selected bones of the third finger, averaged between right and left hands. The table also shows the sex specific averages for the standardized and combined BMD and BBRI on all measured bones. As expected, males had consistently higher BMD and BBRI measurements. Both combined variables were correlated significantly with age ($p < 0.001$, both traits) regardless of sex, and showed only moderate correlation (0.31–0.34, $p < 0.001$) with each other.

Statistical-genetic analyses in the Chuvashian population have shown a highly significant ($p < 0.001$) familial aggregation of each of the two studied phenotypes

Table 1 Basic descriptive statistics of the studied sample

Phenotype	Men ($N = 290$)				Women ($N = 277$)			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Age (years)	45.471	17.012	18	89	45.131	16.660	18	90
Height (m)	1.661	0.068	1.483	1.894	1.543	0.059	1.389	1.775
Weight (kg)	64.663	11.220	41.1	100.1	60.540	12.691	35.5	101.3
BMI (kg/m^2)	23.402	3.584	16.28	36.22	25.422	5.253	15.778	43.25
BMD_mc3 (mg/mm^2)	4.772	0.736	2.94	7.20	4.210	0.781	2.39	7.10
BMD_pr3 (mg/mm^2)	5.737	0.677	3.89	7.93	4.989	0.738	3.31	6.71
BMD_md3 (mg/mm^2)	4.365	0.585	2.66	6.4	3.751	0.584	2.415	5.155
BMD18 (standard)	0.438	0.824	−1.463	3.106	−0.461	0.961	−2.869	2.843
BBRI_mc3	726.907	168.011	368.28	1,310.65	490.020	121.209	240.97	852.79
BBRI_pr3	1,104.007	244.813	368.02	1,729.25	685.574	162.709	255.51	1,139.66
BBRI12 (standard)	0.740	0.776	−1.040	3.052	−0.759	0.543	−2.099	0.800

BMD—average of hand BMD measurement on both hands for metacarpal bone (mc3), proximal (pr3), and middle (md3) phalanges at finger 3; BMD18 (standard)—mean for BMD measurements at 18 bones, standardized for bone specific mean and variance prior combining together. We used similar definitions for BBRI

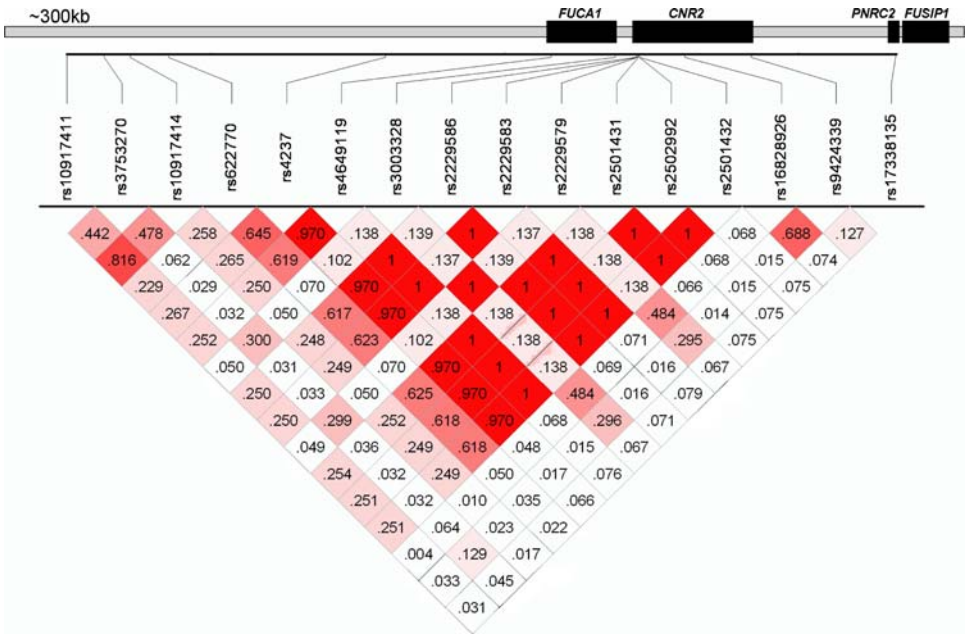

Fig. 1 Representation of linkage disequilibrium (LD) structure in the *CNR2* region in the Chuvashian population. Location of known genes in a region of approx. 300 kb on human chromosome 1p36.11 including the position of 16 typed single nucleotide polymorphisms (SNPs). Pairwise r^2 values between markers are shown as calculated by the program Man-6. Note the presence of a region of strong LD between SNPs rs4237 and rs2501432 including the *CNR2* coding region. *FUCA1*, alpha-L-fucosidase; *CNR2*, cannabinoid receptor type 2; *PNRC2*, proline-rich nuclear receptor coactivator 2; *FUSIP1*, FUS interacting protein (serine–arginine rich) 1

(adjusted for age and sex) with heritability estimates (h^2) of 0.38 for BMD (Livshits et al. 2002) and 0.60 for BBRI (Livshits et al. 2003). This pattern is also well seen in the present sample (Suppl. Fig. 1) showing significant mid-parent/offspring correlations for daughters and sons separately.

To test our hypothesis, we genotyped 16 SNPs covering a region of approximately 300 kb and encompassing the *CNR2* gene (Fig. 1) with its single coding exon 2 and its 37 kb upstream located non-coding exon 1. The SNPs rs2229586 (at position 24,073,490, NCBI Build 36.1) to rs9424339 (at position 24,110,549) are located within the *CNR2* gene. The exact localization and genomic distribution of all used polymorphisms were described elsewhere (Karsak et al. 2005). None of the SNP's genotype distributions deviated significantly ($p \geq 0.05$) from the Hardy–Weinberg-Equilibrium (HWE). We calculated pairwise linkage disequilibrium (LD) coefficients (r^2) for the SNP pairs and found that several of the tested SNPs were in almost complete LD (Fig. 1), resulting in a similar haplotype block structure as in our former case-control study in a French population (Karsak et al. 2005). This haplotype block included SNPs from rs4237 (at position 23,986,716) to rs2501432 (at position 24,074,507). A comparison with the minor allele frequencies (MAF) of the French study is shown in Suppl. Table 1.

Table 2 Hand BMD (age-adjusted) statistics for rs2502992 genotypes

Genotype on -1 strand	A/A			A/G			G/G			*p* value	
	N	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	Allele number*	Homozygotes difference**
All	87	0.190	1.106	252	0.039	1.045	187	−0.096	0.928	0.047	0.019
Male	42	0.154	1.105	130	−0.076	1.025	101	−0.023	0.936	0.513	0.182
Female	45	0.224	1.118	122	0.001	1.070	86	−0.181	0.917	0.031	0.019

Mean and SD are genotype specific mean and standard deviation

N sample size

* *p* value for regression of a phenotypic value on the number of A alleles

** *p* value for difference in mean phenotypic value between the different homozygotes, *t* test

To test the association of selected markers with hand BMD and BBRI, adjusted for age and sex, we first calculated genotype-specific means for the amino acid exchanging "double SNP" (GG/AA). Linear regression of the BMD and BBRI on the number of G alleles and ANOVA of the alternative homozygotes revealed a statistically significant trend (Table 2). In agreement with this, five additional SNPs showed basically the same trend separately in men and women suggesting very little sex specific genetic effects (Suppl. Tables 2a, and 2b).

Next we used two different transmission disequilibrium tests, the FBAT and OT, for the calculation of an allelic association of all tested SNPs with BMD and BBRI, respectively. Both, BMD and BBRI showed nominally significant association with many of the markers including the "double SNP", which had a MAF_A of 40% (Table 3). For the BMD phenotype, the corresponding *p* values were 0.015 and 0.017. The BBRI association analysis resulted in even higher significance values: FBAT = 0.0056 and OT = 0.0015. Other polymorphisms, which were in almost complete LD to the "double SNP", reached similar significance levels (Table 3). Of interest is the marker rs4237, located about 80 kb downstream (i.e., telomeric) of the *CNR2* gene which is also in high LD to SNPs in the *CNR2* coding region (Fig. 1). The association tests with this marker were also consistently significant for both phenotypes (Table 3) reaching the best *p* values (FBAT = 0.007 and OT = 0.008) with the BMD phenotype.

To adjust our TDT results for multiple testing, we used the false discovery rate (FDR) approach proposed by Benjamini and Yekutieli (2001), for multiple testing under dependency (Table 3). The total number of different null-hypotheses for both traits and for all examined SNP was 64. With FDR = 0.05, 28 tests remained significant after correction for multiple testing and even with FDR as low as 0.015, seven tests remained significant (Table 3). These seven SNPs were all in nearly complete LD and included the "double SNP".

In our former work we identified a three-marker haplotype spanning exon 2 and a small part of intron 1 of *CNR2* showing a highly significant association with osteoporosis (Karsak et al. 2005). We therefore tested the association of this haplotype (rs2229583–rs2501431–rs16828926) in the present study. Nominally significant results were obtained by both tests and for both phenotypes ($FBAT_{BBRI}$ and $OT_{BBRI} = 0.003$ and $FBAT_{BMD} = 0.026$ and $OT_{BMD} = 0.068$), and the significance of associations was comparable with *p* values obtained by SNP-based analysis. The risk haplotype T–A–G had a frequency of 57%. The strongest *p* value (FBAT = 0.007) for BMD was reached by the haplotype C–G–G with a population frequency of 30%. Thus, the haplotype analysis confirmed positive association results with the single markers for both hand bone phenotypes.

Discussion

In this study, we analyzed an association of SNPs in and around the *CNR2* gene with two quantitative hand bone strength phenotypes, i.e., mineral density (BMD) and geometrical properties (BBRI), in an ethnically homogeneous family-based sample of healthy Chuvashian individuals (people of Bulgaric ancestry) living along the Volga river in Russia.

Our results showed that several SNPs in *CNR2* were significantly associated with both phenotypes, BMD and BBRI. This work presents the first evidence of association of this genomic locus with bone characteristics in a healthy family sample, indicating that *CNR2* polymorphisms are also relevant for bone strength in healthy individuals with a highly significant familial aggregation. This result thus confirmed and extended the positive association in case-control samples for postmenopausal osteoporosis and low BMD in French women (Karsak et al. 2005) and in pre-and postmenopausal Japanese women (Yamada et al. 2007). The significant association of the *CNR2* locus with bone strength phenotypes observed across three quite different genetic/ethnic backgrounds supports the evidence for a functional involvement of *CNR2* in bone metabolism in a more general sense. The present results and their interpretation

Table 3 Summary of the association analyses in the Chuvashian population at the *CNR2* locus

SNP	Chromosomal position (NCBI Build 36.1)	MAF	Traits			
			BMD		BBRI	
			FBAT	OT	FBAT	OT
rs10917411	23878052	0.42	0.1033	0.1001	0.2597	0.2890
rs3753270	23890811	0.24	0.5842	0.3469	0.9309	0.8328
rs10917414	23899805	0.40	0.0528	0.0573	0.2665	0.2772
rs622770	23912853	0.45	0.3736	0.3236	0.0458	0.0306
rs4237	23986716	0.40	0.0069**	0.0082*	0.0026**	0.0010***
rs4649119	24044130	0.40	0.0217	0.0260	0.0041**	0.0011***
rs3003328	24066413	0.09	0.6907	0.5531	0.0563	0.0113*
rs2229586	24073490	0.40	0.0150*	0.0171*	0.0037**	0.0010***
rs2229583	24073570	0.41	0.0193*	0.0216*	0.0034**	0.0009***
rs2229579	24073749	0.09	0.7234	0.5863	0.0897	0.0237
rs2501431	24074230	0.40	0.0156*	0.0174*	0.0042**	0.0012***
rs2502992	24074506	0.40	0.0154*	0.0168*	0.0056**	0.0015***
rs2501432	24074507	0.40	0.0154*	0.0168*	0.0056**	0.0015***
rs16828926	24087717	0.13	0.9533	0.8869	0.0530	0.0401
rs9424339	24110549	0.16	0.7570	0.7947	0.4046	0.4206
rs17338135	24159484	0.42	0.5144	0.6769	0.0476	0.0464

For the cannabinoid receptor type 2 (*CNR2*) locus, 16 genotyped single nucleotide polymorphisms (SNP) are shown. Polymorphisms located in the *CNR2* coding exon are shown in bold; rs numbers, the chromosomal position (NCBI Build 36.1), and the minor allele frequencies (MAF) are given. Provided are the *p* values for two different tests, the family-based association test (FBAT) and the orthogonal test (OT). Two quantitative traits were used for this study, the bone mineral density (BMD) and the breaking bending resistance index (BBRI), which were adjusted for age and sex. We used the False Discovery Rate (FDR) approach proposed by Benjamini and Yekutieli (2001), for multiple testing under dependency

* *p* values significant at a FDR level of 0.05

** *p* values significant at a FDR = 0.03

*** *p* values significant at a FDR = 0.015

is strengthened by the fact that we for the first time observed a significant association in a family-based association study, which is supposed to be well protected against possible stratification and intermixture effects in genetic association studies.

In previous studies, the BMD was measured by dual-energy X-ray absorptiometry (DEXA) on lumbar spine (L2–L4) and femoral neck (Karsak et al. 2005; Yamada et al. 2007) or on total body and trochanter by DEXA and the distal radius BMD by peripheral quantitative computed tomography (Yamada et al. 2007). In our work, BMD was assessed using hand radiographs. This is a well-established method, which has been used for the assessment of hand skeletal status for over 40 years. A modification of this method implementing digital X-ray radiogrammetry (DXR), which was also applied here, is known for more than 10 years (e.g., Bouxsein et al. 1997). It calculates peripheral BMD from hand radiographs and has as high precision as DEXA of clinically important skeletal sites (hip, spine). Thus the in vitro precision for direct DXR recently tested on four different X-ray equipments ranged from 0.14 to 0.30%, expressed as coefficient of variations (CV%) and from 0.0012 to 0.0028 g/cm^2, expressed as smallest detectable difference (Hoff et al. 2009). Comparison of DEXA and DXR in 28 women with osteoporosis defined by DEXA at the femoral neck and in 28 women with normal BMD showed that DXR has excellent precision of 0.004 g/cm^2 compared with 0.021 and 0.015 g/cm^2 obtained by DEXA at the hip and spine, respectively (Elliot et al. 2005). Our study was not designed to evaluate risk of fracture at spine or at hip but we chose the present phenotypes to test for a more general effect of the selected polymorphisms looking at different skeletal sites. In addition to high precision and the analysis of additional bones the implementation of the present method has some more advantages: (1) it is highly relevant clinically for risk of forearm fracture (e.g., Reed et al. 2004), (2) it measures simultaneously numerous bones, 18 used here, and (3) it allows to measure other important bone strength related phenotypes, e.g., BBRI as used in this paper. Note that only 10% ($R^2 = 0.102$) of BBRI variation was attributable to BMD in this sample.

Our findings (Table 3) suggest that the *CNR2* association with BMD is independent of the method of quantification

and skeletal localization of bones investigated, and confirmed by BBRI that showed even higher significance values of association with the *CNR2* polymorphisms. This implies in turn that different bone-strength features are modulated by *CNR2* polymorphisms.

Calculation of the mean BMD for homo- and heterozygotes of the non-synonymous "double SNP" in the coding region of the CB2 receptor revealed a significant difference in bone density between individuals with the two alternative homozygous genotypes (AA vs. GG). Consistent with previous studies (Karsak et al. 2005), individuals with the GG genotype have a significantly reduced hand BMD. In line with this result, we could show that heterozygotes for this "double SNP" showed an intermediate mean BMD value indicating that this polymorphism influences the bone phenotype in a quantitative manner. This trend was observed in both sexes and in the total sample, however, it reaches statistical significance only in females. Indeed, a possible limitation of our study is that the sample size did not provide sufficient power to investigate the effects of sex on the observed associations, which is an important issue in osteoporosis etiology and epidemiology. The size of our sample is well suited for the aims of the present family-based association study, providing a power of 0.8 to detect a genetic effect explaining <5% of the total trait's variability, yet, the size of our sample is not sufficient to carry out the corresponding analyses for each sex separately. However, we previously conducted additional statistical-genetic analysis showing that after adjustment of the variation of these phenotypes for sex and age the modifying effect of sex and especially menopausal status on the genetic transmission pattern became negligible (Livshits et al. 2002, 2003, 2004).

Functional effects of the two naturally occurring alleles were demonstrated in human T lymphocytes resulting in different inhibition of proliferation after cannabinoid treatment (Sipe et al. 2005) and future experiments will clarify whether bone cells also show different functional responses dependent on the genotype. Concerning putative functional mechanisms underlying the genetic association, in addition to a direct effect of the missense alteration as described above the positive finding with rs4237 may also be of interest. Indeed, this SNP, which is located more than 80 kb downstream of *CNR2*, showed the highest significance value for association with BMD (FBAT = 0.0069). Interestingly, a systematic study of the influence of genetic variation on gene expression identified a genomic region 20–140 kb downstream of the *CNR2* gene strongly influencing CB2 receptor expression (Dixon et al. 2007). The marker rs4237 is included in this regulatory cluster of SNPs and the T allele of this SNP showed a significantly lower expression of the *CNR2* transcript by −0.456 with a p value of 10^{-10}. Here and in our previous work, the same allele of this SNP was also associated with low BMD and with osteoporosis (Karsak et al. 2005). These results suggest that SNPs lying in cis to the *CNR2* gene like rs4237 may act as regulators of *CNR2* expression and that these regulators are significantly associated with bone density variation and other components of bone strength.

It has to be mentioned that recent genome-wide association studies (GWAS) did not report the *CNR2* locus as one of the major susceptibility loci for BMD or osteoporosis (Richards et al. 2008; Styrkarsdottir et al. 2008). Whether this locus would show some weaker evidence for association, which just does not reach genome-wide significance in the cohorts studied up to now, is not evident from the published data. There is, however, an even more serious problem concerning a comparison of these studies: indeed, both platforms used in the current GWAS studies (i.e., the Illumnia 300/370/550 k and the Affymetrix 5.0 arrays) show a gap in SNP coverage of about 200 kb completely including *CNR2* and the neighbouring genes. This gap is probably caused by a large sequence gap in one of the previous NCBI Genome Builds, which was the basis for SNP selection for the above mentioned genotyping arrays. This means that the *CNR2* locus is not adequately covered in the published GWAS and that future studies applying novel arrays (e.g., Illumina 1 M-duo and Affymetrix 6.0), which cover this locus, are needed to finally judge a possible contribution of common variants of *CNR2* in BMD/osteoporosis susceptibility in the analysed patient cohorts. In addition, to reduce the probability of a false positive association also by statistical means, we adjusted our TDT results for multiple testing by implementing the FDR approach for multiple testing under dependency (Table 3). After correction for multiple testing with FDR = 0.05, 28 tests remained significant. Moreover, for seven SNPs, which were in almost complete LD and included the "double SNP", the results of the tests remained significant with FDR as low as 0.015 (Table 3).

In conclusion, our results confirm and substantially extend previous studies about the association of *CNR2* and bone phenotypes by investigating different skeletal sites, different bone phenotypes, and by applying a family-based association approach in a healthy family sample of Chuvashian descent.

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References

Abecasis GR, Cardon LR, Cookson WO (2000) A general test of association for quantitative traits in nuclear families. Am J Hum Genet 66:279–292

Benjamini Y, Yekutieli D (2001) The control of the false discovery rate in multiple testing under dependency. Ann Stat 29:1165–1188

Bouxsein ML, Michaeli DA, Plass DB, Schick DA, Melton ME (1997) Precision and accuracy of computed digital absorptiometry for assessment of bone density of the hand. Osteoporos Int 7:444–449

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258:1946–1949

Dixon AL, Liang L, Moffatt MF, Chen W, Heath S, Wong KC, Taylor J, Burnett E, Gut I, Farrall M, Lathrop GM, Abecasis GR, Cookson WO (2007) A genome-wide association study of global gene expression. Nat Genet 39:1202–1207

El'chinova GI, Roshchina Iu V, Zinchenko RA, Zinchenko SP, Ginter EK (2002) Population genetic study of the Alatyr region of the Republic of Chuvashiia. Genetika 38:251–258

Elliot JR, Fenton AJ, Young T, Mansfield A, Burton C, Wilkinson TJ (2005) The precision of digital X-ray radiogrammetry compared with DXA in subjects with normal bone density or osteoporosis. J Clin Densitom 8:187–190

Ermakov S, Malkin I, Keter M, Kobyliansky E, Livshits G (2008) Family-based association study of polymorphisms in the RUNX2 locus with hand bone length and hand BMD. Ann Hum Genet 72:510–518

Gatti D, Sartori E, Braga V, Corallo F, Rossini M, Adami S (2001) Radial bending breaking resistance derived by densitometric evaluation predicts femoral neck fracture. Osteoporos Int 12:864–869

Hoff M, Dhainaut A, Kvien TK, Forslind K, Kalvesten J, Haugeberg G (2009) Short-time in vitro and in vivo precision of direct digital X-ray radiogrammetry. J Clin Densitom 12:17–21

Horvath S, Xu X, Laird NM (2001) The family based association test method: strategies for studying general genotype–phenotype associations. Eur J Hum Genet 9:301–306

Idris AI, van 't Hof RJ, Greig IR, Ridge SA, Baker D, Ross RA, Ralston SH (2005) Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors. Nat Med 11:774–779

Idris AI, Sophocleous A, Landao-Bassonga E, van't Hof RJ, Ralston SH (2008) Regulation of bone mass, osteoclast function, and ovariectomy-induced bone loss by the type 2 cannabinoid receptor. Endocrinology 149:5619–5626

Kalichman L, Malkin I, Bigman G, Matias R, Seibel MJ, Kobyliansky E, Livshits G (2008) Age-related changes in bone-strength-associated geometry indices in naive human population. Anat Rec (Hoboken) 291:835–844

Kaptoge S, Beck TJ, Reeve J, Stone KL, Hillier TA, Cauley JA, Cummings SR (2008) Prediction of incident hip fracture risk by femur geometry variables measured by hip structural analysis in the study of osteoporotic fractures. J Bone Miner Res 23:1892–1904

Karsak M, Cohen-Solal M, Freudenberg J, Ostertag A, Morieux C, Kornak U, Essig J, Erxlebe E, Bab I, Kubisch C, de Vernejoul MC, Zimmer A (2005) Cannabinoid receptor type 2 gene is associated with human osteoporosis. Hum Mol Genet 14:3389–3396

Karsak M, Gaffal E, Date R, Wang-Eckhardt L, Rehnelt J, Petrosino S, Starowicz K, Steuder R, Schlicker E, Cravatt B, Mechoulam R, Buettner R, Werner S, Di Marzo V, Tuting T, Zimmer A (2007) Attenuation of allergic contact dermatitis through the endocannabinoid system. Science 316:1494–1497

Klein TW, Friedman H, Specter S (1998) Marijuana, immunity and infection. J Neuroimmunol 83:102–115

Livshits G, Karasik D, Kobyliansky E (2002) Complex segregation analysis of the radiographic phalanges bone mineral density and their age-related changes. J Bone Miner Res 17:152–161

Livshits G, Yakovenko K, Kobyliansky E (2003) Quantitative genetic study of radiographic hand bone size and geometry. Bone 32:191–198

Livshits G, Deng HW, Nguyen TV, Yakovenko K, Recker RR, Eisman JA (2004) Genetics of bone mineral density: evidence for a major pleiotropic effect from an intercontinental study. J Bone Miner Res 19:914–923

Malkin I, Ginsburg E (2006) Program package for Mendelian analysis of pedigree data (MAN, Version 6). Department of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel Aviv University. Technical Report

Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K, Tam J, Attar-Namdar M, Kram V, Shohami E, Mechoulam R, Zimmer A, Bab I (2006) Peripheral cannabinoid receptor, CB2, regulates bone mass. Proc Natl Acad Sci USA 103:696–701

Reed MR, Murray JR, Abdy SE, Francis RM, McCaskie AW (2004) The use of digital X-ray radiogrammetry and peripheral dual energy X-ray absorptiometry in patients attending fracture clinic after distal forearm fracture. Bone 34:716–719

Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, Andrew T, Falchi M, Gwilliam R, Ahmadi KR, Valdes AM, Arp P, Whittaker P, Verlaan DJ, Jhamai M, Kumanduri V, Moorhouse M, van Meurs JB, Hofman A, Pols HA, Hart D, Zhai G, Kato BS, Mullin BH, Zhang F, Deloukas P, Uitterlinden AG, Spector TD (2008) Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet 371:1505–1512

Sipe JC, Arbour N, Gerber A, Beutler E (2005) Reduced endocannabinoid immune modulation by a common cannabinoid 2 (CB2) receptor gene polymorphism: possible risk for autoimmune disorders. J Leukoc Biol 78:231–238

Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, Jonsdottir T, Saemundsdottir J, Center JR, Nguyen TV, Bagger Y, Gulcher JR, Eisman JA, Christiansen C, Sigurdsson G, Kong A, Thorsteinsdottir U, Stefansson K (2008) Multiple genetic loci for bone mineral density and fractures. N Engl J Med 358:2355–2365

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89–97

Tam J, Ofek O, Fride E, Ledent C, Gabet Y, Muller R, Zimmer A, Mackie K, Mechoulam R, Shohami E, Bab I (2006) Involvement of neuronal cannabinoid receptor CB1 in regulation of bone mass and bone remodeling. Mol Pharmacol 70:786–792

Tam J, Trembovler V, Di Marzo V, Petrosino S, Leo G, Alexandrovich A, Regev E, Casap N, Shteyer A, Ledent C, Karsak M, Zimmer A, Mechoulam R, Yirmiya R, Shohami E, Bab I (2008) The cannabinoid CB1 receptor regulates bone formation by modulating adrenergic signaling. FASEB J 22:285–294

Wilson RI, Nicoll RA (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature 410:588–592

Yamada Y, Ando F, Shimokata H (2007) Association of candidate gene polymorphisms with bone mineral density in community-dwelling Japanese women and men. Int J Mol Med 19:791–801