

Genetic risk factors for decreased bone mineral accretion in children with asthma receiving multiple oral corticosteroid bursts

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Background: Long-term intermittent oral corticosteroid (OCS) use in children with asthma leads to significant decreases in bone mineral accretion (BMA).

Objective: We aimed to identify genetic factors influencing OCS dose effects on BMA in children with asthma.

Methods: We first performed a gene-by-OCS interaction genome-wide association study (GWAS) of BMA in 489 white participants in the Childhood Asthma Management Program trial who took short-term oral prednisone bursts when they experienced acute asthma exacerbations. We selected the top-ranked 2000 single nucleotide polymorphisms (SNPs) in the GWAS and determined whether these SNPs also had *cis*-regulatory effects on dexamethasone-induced gene expression in osteoblasts.

Results: We identified 2 SNPs (rs9896933 and rs2074439) associated with decreased BMA and related to the tubulin γ pathway. The rs9896933 variant met the criteria for genome-wide significance ($P = 3.15 \times 10^{-8}$ in the GWAS) and is located on the intron of tubulin folding cofactor D (*TBCD*) gene. The rs2074439 variant ($P = 2.74 \times 10^{-4}$ in the GWAS) showed strong *cis*-regulatory effects on dexamethasone-induced tubulin γ gene expression in osteoblasts ($P = 8.64 \times 10^{-4}$).

Interestingly, we found that BMA worsened with increasing prednisone dose as the number of mutant alleles of the 2 SNPs increased.

Conclusions: We have identified 2 novel tubulin γ pathway SNPs, rs9896933 and rs2074439, showing independent interactive effects with cumulative corticosteroid dose on BMA in children with asthma receiving multiple OCS bursts. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Asthma, bone mineral density, child, corticosteroids

Bone mineral accretion (BMA) is defined as the accrual in bone mineral density (BMD) over time. Because adults normally lose BMD as they age, the achievement of BMD that occurs during childhood and adolescence is regarded as an important factor contributing to osteoporosis.¹ Therefore factors affecting BMA during this critical period might also increase the risk of osteoporosis later in life. The adverse effects of oral corticosteroids (OCSs) on bone health have been well known for many years, and even intermittent OCS use can affect BMA in childhood. Our group previously reported that frequent OCS use in children with asthma led to significant decreases in BMA.^{2,3}

Advances in genomic technology enable us to integrate gene expression data with genotype data. Through this integration, we significantly increase the possibility of identifying functional genetic variants underlying disease pathogenesis. For example, we observed dexamethasone-specific *cis*-regulation of tenascin C (*TNC*) expression in the primary osteoblast.⁴ We then performed an association study among 6 expression single nucleotide polymorphisms (SNPs) within the *TNC* locus and examined increases in lung function after 2 months of inhaled corticosteroid (ICS) treatment in children with asthma. Finally, we demonstrated that this heritable *cis*-regulation of *TNC* expression explained the difference in response to ICSs by showing that 4 of 6 SNPs were significantly associated with lung function increases.⁴ Using a cellular expression quantitative trait locus (eQTL) approach, we have also recently identified several SNPs associated with ICS response at a genome-wide level.⁵

The purpose of the present study was to identify genetic factors with the potential to substantially modify the effects of OCSs on BMA in children with asthma receiving multiple intermittent OCS courses over time. We initially performed a genome-wide association study (GWAS) of OCS-induced BMA changes observed in children with asthma participating in the Childhood Asthma Management Program (CAMP) trial.⁵ They were followed for a mean of 4.3 years and took short-term oral prednisone bursts when they experienced acute exacerbations. To refine the potential for these SNPs to have a functional effect on bone, we determined whether the top-ranked SNPs had *cis*-regulatory

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Abbreviations used

APPL1: Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1
 BMA: Bone mineral accretion
 BMD: Bone mineral density
 CAMP: Childhood Asthma Management Program
 eQTL: Expression quantitative trait locus
 F-SNP: Functional Single Nucleotide Polymorphism
 GWAS: Genome-wide association study
 ICS: Inhaled corticosteroid
 LD: Linkage disequilibrium
 OCS: Oral corticosteroid
 PAK2: p21 protein-activated kinase 2
 SNP: Single nucleotide polymorphism
 TBCD: Tubulin folding cofactor D
 TNC: Tenascin C
 TUBG1: Tubulin γ 1

effects on corticosteroid-induced gene expression in osteoblast cells. Finally, noting that 2 of the associated variants mapped to the same pathway, we tested the combined effects of the 2 SNPs on BMA.

METHODS

The CAMP trial was approved by the institutional review boards of the participating institutions, and informed consent was obtained from the participating children and their parents.

CAMP BMA measurements

A total of 1041 children with mild-to-moderate asthma aged 5 to 12 years were randomized to budesonide, nedocromil, or placebo and followed for a mean of 4.3 years in the CAMP trial.⁶ For asthma exacerbations, oral prednisone bursts were prescribed per protocol as follows: 2 mg/kg per day (up to 60 mg) for 2 days followed by 1 mg/kg per day (up to 30 mg) for 2 days, with an option to continue dosing if improvement was insufficient. BMD measurements (in grams per square centimeter) of the lumbar spine (L1-L4) were performed yearly during the study period by means of dual-energy x-ray absorptiometry with the Hologic (Waltham, Mass) QDR-1500 (at 6 centers) or the Lunar (Madison, Wis) DPX (at 2 centers). Hologic dual-energy x-ray absorptiometry machines were further divided by the use of pencil-beam or fan-beam measurements. Detailed methods to convert Lunar measures to Hologic values and to adjust deviations between pencil- and fan-beam measurements were described in our previous report.³ BMA (in grams per square centimeter per year) represents the average gain of BMD over time and was calculated as follows:

(BMD at 4 years' follow-up – BMD at baseline)/4 years.

A 4-year follow-up was selected because this approximated the end of the trial. BMD z scores were calculated by using CAMP internal references.²

GWAS of BMA

Genotyping was done with the HumanHap550v3 BeadChip or the Infinium HD 140 Human610-Quad BeadChip (Illumina, San Diego, Calif). SNPs showing significant deviation from Hardy-Weinberg equilibrium ($P < .001$), SNPs with low minor allele frequency (<0.05), and SNPs with high rates of missing data (>0.01) were excluded. Using a linear regression gene-environment interaction model as implemented in PLINK (<http://pngu.mgh.harvard.edu/purcell/plink/>),⁷ we performed a GWAS to test the following model:

BMA ~ Genotype (additive genetic model) + Cumulative dose of OCS + Genotype*cumulative dose of OCS + Covariates.

TABLE I. Baseline characteristics of subjects enrolled in CAMP

Total no.	489
Male sex, no. (%)	302 (61.7)
Age (y), mean (SD)	8.8 (2.1)
Height (cm), mean (SD)	132.0 (19.8)
BMI (kg/m ²), median (IQR)	17.1 (15.8-19.7)
BMD (g/cm ²), median (IQR)	0.782 (0.683-0.935)
Vitamin D (ng/mL), median (IQR)	36.6 (28.8-47.9)
Tanner stage, no. (%)	
I/II/III/IV/V	353 (72.2)/100 (20.4)/24 (4.9)/11 (2.2)/1 (0.3)
Cumulative dose of prednisolone (mg), median (IQR)	360 (120-900)
BMA (g/cm ² /y), median (IQR)	0.038 (0.024-0.063)

BMI, Body mass index; IQR, interquartile range.

Based on our previous report, age, sex, BMD, height, body mass index, serum vitamin D concentration, and Tanner stage measured at baseline were included as covariates.³ Because we focused on the interactive effect between genotype and cumulative OCS dose on BMA, we reported P values from the “genotype*cumulative dose of OCS” term. Only the data from white children were used in the present study because of potential issues with population stratification.

Cis-regulatory effect on corticosteroid-induced gene expression in osteoblasts

We used data from our previous study.⁴ In brief, expression profiling of human primary bone cells derived from 113 donors (51 female and 62 male donors undergoing total hip or knee replacement at the Uppsala University Hospital, Uppsala, Sweden) and stimulated with dexamethasone (100 nmol/L) for 24 hours was measured by using Illumina HumRef-8v2 BeadChips. Genotyping was done with the Illumina HapMap550K Duo chip, as previously described.⁴ We performed an association test between corticosteroid-induced gene expression and SNPs by using a linear regression model implemented in SNPTEST software (<http://www.stats.ox.ac.uk/~marchini/software/gwas/gwas.html>).⁸ An additive effect of SNP was assumed, and 2 covariates (year of birth and sex) were included in a regression model. Cis-regulatory SNPs were defined when SNPs were located in a 1-Mb window flanking the target gene or within the target gene itself, and their P values were less than .05.

Statistical analysis

Pearson correlation and simple linear regression analysis were done to evaluate relations between the cumulative dose of OCS and BMA, according to the genotypes. Multiple linear regression models were constructed to test effects of cumulative OCS dose on BMA adjusted by baseline covariates. All analyses were performed with R (version 3.0.2) software (www.r-project.org).

RESULTS

A total of 489 white subjects were included in the GWAS of OCS-induced BMA. Table I summarizes baseline characteristics. The median number of OCS bursts was 3 (range, 0-27). The cumulative dose of OCS showed a significantly negative association with BMA after adjustment for covariates (β coefficient = -3.036×10^{-6} and $P = 7.96 \times 10^{-5}$, see Table E1 in this article's Online Repository at www.jacionline.org). A total of 44,319 SNPs with P values of less than .05 in GWASs were identified. Table II shows 9 SNPs that met the threshold for genome-wide significance ($P < 1 \times 10^{-7}$) for the interaction of genotype with corticosteroid dose on BMA. Quantile-quantile and Manhattan plots for the interaction results

TABLE II. Nine SNPs with genome-wide significance identified in the GWAS of OCS-induced BMA

Rank	SNP	Chromosome	Gene	β Coefficient	P value
1	rs7003550	8	Intergenic	-8.15×10^{-6}	1.46×10^{-8}
2	rs9896933	17	<i>TBCD</i>	-8.56×10^{-6}	3.15×10^{-8}
3	rs7599706	2	Intergenic	-1.49×10^{-5}	3.98×10^{-8}
4	rs12447718	16	<i>ZFPM1</i>	-1.31×10^{-5}	4.26×10^{-8}
5	rs4368243	18	Intergenic	-5.99×10^{-6}	6.35×10^{-8}
6	rs2316527	7	<i>DPP6</i>	-1.13×10^{-5}	7.63×10^{-8}
7	rs4484658	8	Intergenic	-8.90×10^{-6}	8.09×10^{-8}
8	rs7506840	18	<i>LOC100130480</i>	-1.11×10^{-5}	8.95×10^{-8}
9	rs10485681	20	Intergenic	-8.63×10^{-6}	9.91×10^{-8}

DPP6, Dipeptidyl-peptidase 6; *ZFPM1*, zinc finger protein, *FOG* family member 1.

TABLE III. Three SNPs showing *cis*-regulatory effects on corticosteroid-induced gene expression in osteoblasts among the top-ranked 2000 SNPs in the GWAS

SNP	GWAS (P value)	<i>Cis</i> -regulatory effect in osteoblasts	
		Gene induced by dexamethasone	P value
rs949637	.0008123	<i>FLJ16171</i>	.0008897
rs2074439	.0002743	<i>TUBG1</i>	.0008635
rs2030737	.0002953	<i>KY</i>	.0001267

FLJ16171, FLJ16171 protein (on chromosome 5q35); *KY*, kyphoscoliosis peptidase (on chromosome 3q22.2).

are presented in Fig E1 in this article's Online Repository at www.jacionline.org. Power for the interaction analysis was calculated by using Quanto software 1.2.4 (<http://biostats.usc.edu/Quanto.html>). Quanto is a program for computing either power or required sample size for association studies of genes, environmental factors, and gene-environment interaction.^{9,10} An additive genetic model and α value (2-sided) of .05 were assumed. Minor allele frequency, mean and variance of BMA in CAMP subjects, genetic effect (β coefficient of the "genotype" term in our regression model), and environmental effect (β coefficient of the "cumulative dose of OCS" term in our regression model) were obtained from our results. For rs9896933, an estimated power was 0.68 for main (gene) effect and 0.58 (2 *df* test) for interaction (gene-environment interaction; ie, interaction between genotype and cumulative dose of OCS). In osteoblasts a total of 14,982 SNPs showed *cis*-regulatory effects on corticosteroid-induced gene expression. We selected the top-ranked 2000 SNPs in the GWAS and found that 3 SNPs among them showed *cis*-regulatory effects on corticosteroid-induced gene expression in osteoblasts (Table III).

We focused on 2 SNPs, rs9896933 (minor allele frequency, 0.173 [ranked second in the GWAS]; $P = 3.15 \times 10^{-8}$) and rs2074439 (minor allele frequency, 0.34 [ranked 1256th in the GWAS]; $P = 2.74 \times 10^{-4}$), given that (1) rs9896933 is located on the intron of the tubulin folding cofactor D (*TBCD*) gene and is in tight linkage disequilibrium (LD; ie, highly correlated) with rs3785522, a nonsynonymous coding SNP on *TBCD* ($r^2 = 0.945$; Fig 1, A and B); (2) rs2074439 is located on chromosome 17q21 (Fig 1, B), which was previously identified as a QTL associated with BMD in European pedigrees¹¹; (3) the tubulin γ 1 (*TUBG1*) gene regulated by rs2074439 showed significantly reduced expressions in osteoblasts after incubation with

dexamethasone for 24 hours⁴; and (4) a biologic interaction between *TUBG1* and *TBCD* has been reported.¹² Negative correlations between cumulative dose of OCS and BMA stratified by rs9896933 and rs2074439 genotype were shown (see Figs E2 and E3 in this article's Online Repository at www.jacionline.org). We did not demonstrate significant epistasis between rs9896933 and rs2074439. However, we found that an interaction between rs9896933 and the cumulative dose of OCS and an interaction between rs2074439 and the cumulative dose of OCS were still significant when both terms were put into a regression model together as follows:

BMA $\sim$ Cumulative dose of OCS + rs9896933 genotype (additive model) + rs2074439 genotype (additive model) + Cumulative dose of OCS*rs9896933 genotype + Cumulative dose of OCS*rs2074439 genotype + Covariates (see Table E2 in this article's Online Repository at www.jacionline.org).

This meant that both interactions were independently significant when adjusting for each other. Fig 2 shows correlations between the cumulative dose of OCS and BMA stratified by the number of mutant alleles of rs9896933 and rs2074439. A dosage effect of mutant alleles on a slope obtained from simple linear regression analysis was observed (see Fig E4 in this article's Online Repository at www.jacionline.org). Table E3 in this article's Online Repository at www.jacionline.org shows β coefficient and *P* values of association between cumulative dose of OCS and BMA in each group obtained from multiple linear regression analysis adjusted by baseline covariates.

We also evaluated whether rs9896933 showed the same interactive effect with ICSs on BMA in asthmatic children without OCS bursts. For this, we compared BMA stratified by rs9896933 of subjects who were randomized to ICSs with that of subjects who were randomized to placebo. We found that rs9896933 genotype was not significantly associated with BMA in both groups (see Fig E5 in this article's Online Repository at www.jacionline.org).

DISCUSSION

For their unrivaled anti-inflammatory and immunomodulatory effects, OCSs are recommended in the current treatment guidelines for pediatric asthma.¹³⁻¹⁶ However, OCSs frequently cause many adverse outcomes, including osteoporosis. Multiple OCS bursts over time have been associated with lower BMD in children with asthma.^{2,3} Various factors, including sex, age, height, and pubertal stage, are known to affect BMA associated with OCS use in children.¹⁷ However, there has been no study evaluating a genetic influence on BMA induced by OCS bursts in

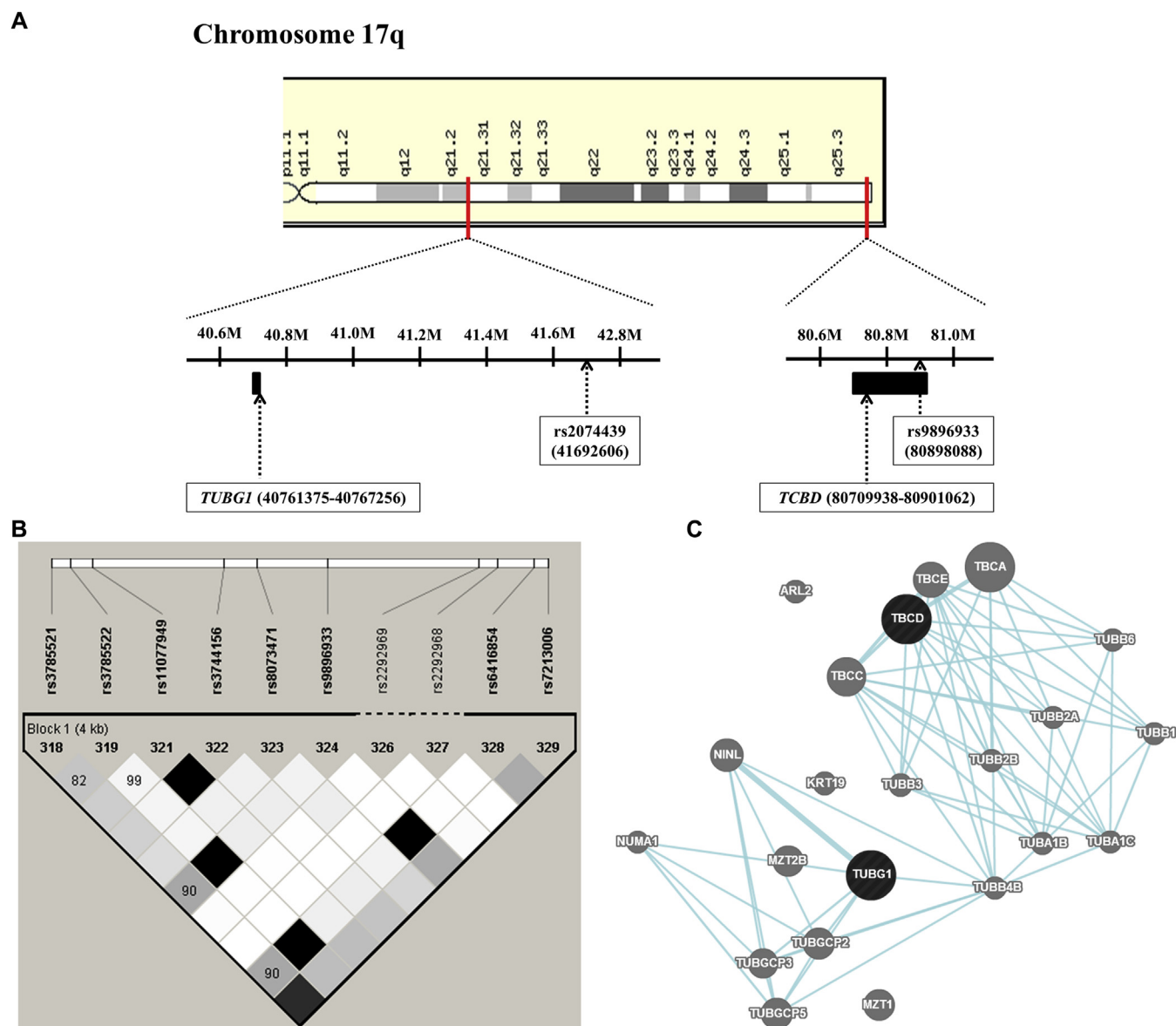


FIG 1. Position of identified SNPs and genes on chromosome 17q. **A**, Rs9896933, rs2074439, *TCBD*, and *TUBG1*. **B**, LD plot between rs9896933 and rs3785522 (HapMap CEPH population). **C**, Analysis of potential pathway interactions between *TBCD* and *TUBG1* through GeneMania (www.genemania.org).

children with asthma. In the present study we identified 2 SNPs of potential functional significance (rs9896933 and rs2074439) that were involved in the γ tubulin pathway and had significant interactive effects with a cumulative dose of OCS on BMA in children with asthma. For asthmatic children without OCS bursts, effects of those 2 SNPs on BMA were not different between those assigned to ICS and those assigned to placebo. However, for the proper interpretation, we should make it clear that the medium ICS dose (total daily dose of budesonide was 400 μ g) was administered in the CAMP study.⁶ Notably, the association between cumulative dose of OCS and lower BMA increased as the number of mutant alleles of 2 the SNPs increased.

The rs9896933 variant is located on the intron of *TBCD* and is in tight LD with rs3785522, a nonsynonymous coding SNP in *TBCD*. We evaluated the functional implication of rs3785522 using the Functional Single Nucleotide Polymorphism (F-SNP), a

Web-based tool (<http://compbio.cs.queensu.ca/F-SNP/>) integrating information obtained from 16 bioinformatics tools and databases about the functional effects of SNPs.¹⁸ F-SNP indicated 0.912 for the FS score of rs3785522. The FS score is a functional SNP scoring system provided by F-SNP to distinguish features of disease-related SNPs versus neutral SNPs.¹⁹ The median FS score for neutral SNPs is 0.1764, whereas for disease-related SNPs, the median score increased to 0.5.¹⁹ Thus rs3785522 might be functionally important. Similarly, through its association with corticosteroid-induced changes in expression of *TUBG1*, rs2074439 (or a variant in LD with it) represents a regulatory variant in the γ tubulin pathway.

Osteoblasts that synthesize new bone matrix on bone-forming surfaces play an important role in bone homeostasis.²⁰ Decreased bone formation is an important mechanism for corticosteroid-induced BMD loss.²¹ Therefore corticosteroid effects on

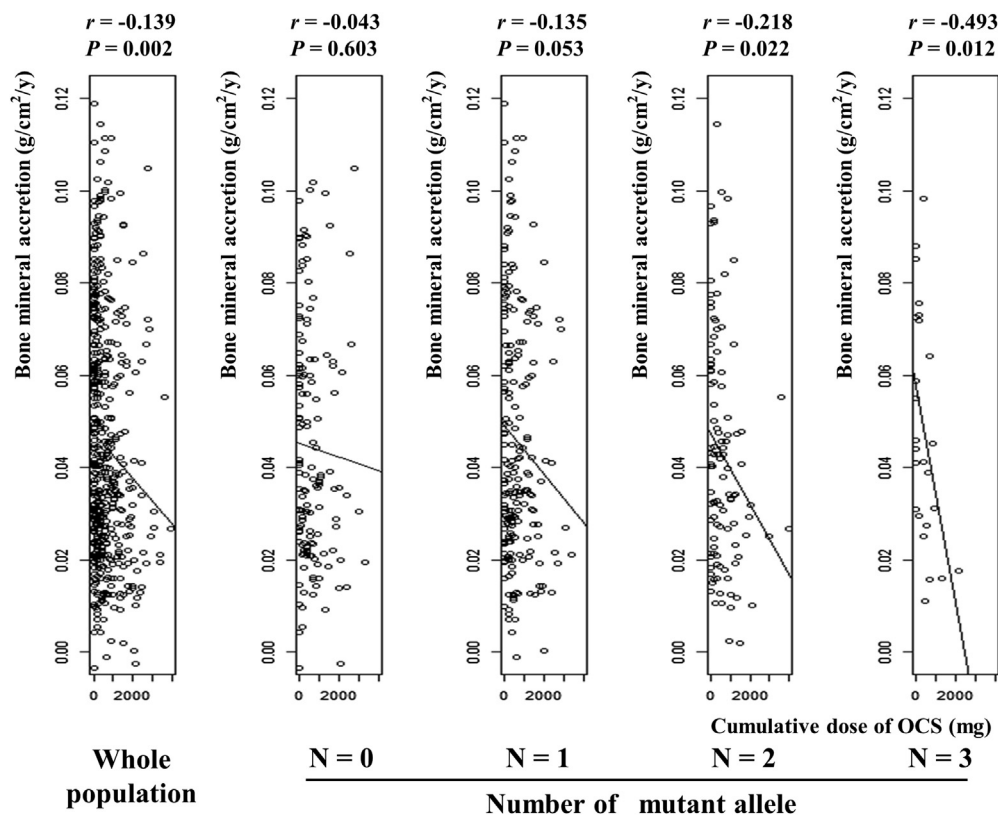


FIG 2. Correlations between cumulative dose of OCS and BMA stratified by the number of mutant alleles of rs9896933 and rs2074439. Regression lines of the whole population, subjects without any mutant alleles, subjects with 1 mutant allele, subjects with 2 mutant alleles, and subjects with 3 mutant alleles are shown. The slope obtained from simple linear regression analysis becomes steeper as the number of mutant alleles of the 2 SNPs increases. P values were obtained from Pearson correlation analysis.

osteoblasts have been extensively evaluated. Corticosteroids prevent terminal osteoblast differentiation,²² induce osteoblast apoptosis,²³ and inhibit osteoblast production of bone matrix.²⁴ Although these effects are known, the role of microtubules on corticosteroid-induced BMD loss in osteoblasts has not been previously elucidated.

Microtubules are major components of the cytoskeleton and provide scaffolding, sequestering, and delivery functions. It is known that microtubules are involved in various signaling pathways, such as sonic hedgehog/Gli, Wnt/ β -catenin, G protein, and mitogen-activated protein kinase pathways which are related to skeletal development and homeostasis.^{25,26} Interestingly, recent reports noted that inhibition of microtubule assembly increases osteoblast differentiation *in vitro* and murine bone mass *in vivo*.^{27,28} Formation of new microtubule polymers from heterodimers of α and β tubulin is termed nucleation and requires another member of the tubulin family, γ tubulin.²⁹ γ Tubulin forms γ tubulin ring complexes with additional proteins, and these γ tubulin complexes might also be involved in regulating microtubule dynamics.³⁰ TBCD, which is coded by the *TBCD* gene, is one of the 5 tubulin-specific cochaperones A through E that mediate tubulin nucleation.³¹ TBCD plays a critical role in recruiting γ tubulin ring complexes and initiating microtubule growth, and excess of TBCD inhibits microtubule assembly.¹² Taken together, it is possible that a disruption in the γ tubulin pathway in osteoblasts might result in BMD changes and that these changes can be modified through genetic factors.

Interestingly, androgen was reported to promote γ tubulin recruitment and microtubule assembly in human fibroblasts,³² and pregnenolone was reported to bind to microtubule-associated protein 2 and to stimulate microtubule assembly.³³ As mentioned before, inhibition of microtubule assembly in osteoblasts is possibly linked to BMD decrease. Therefore it is also possible that corticosteroid-induced BMD loss is associated with the γ tubulin pathway.

An eQTL is a locus that explains a fraction of the genetic variance of a gene expression phenotype, and standard eQTL analysis is particularly useful for identifying pharmacogenomic loci.³⁴ However, eQTL might differ according to the cell type,³⁵ and thus functional validation with eQTLs is optimally conducted in cell lines that are relevant to the disease. We demonstrated an epistasis between the *cis*-eQTL SNP of *TUBG1* and the second most significant SNP in the GWAS, which is in tight LD with a possibly functional nonsynonymous coding SNP in *TBCD*. A biologic interaction between γ tubulin and TBCD was reported,¹¹ and we found that *TBCD* and *TUBG1* shared a common pathway by using the program GeneMania (<http://genemania.org/>; Fig 1, C). Combined, these findings suggest that 2 SNPs identified in the present study might have a causative role in OCS-induced BMA in children with asthma.

We believed that children with asthma were a relevant population to reveal genetic factors associated with BMA caused by corticosteroids because they carry little risk for BMD loss compared with the elderly or patients with other diseases.

However, we could not obtain BMA data from a similar group of asthmatic children for the replication of our results, which is an important limitation in generalizing our results. Confirmation in an independent cohort is warranted.

In addition, considering that only 3 of 2000 SNPs identified in the GWAS showed *cis*-regulatory effects, different pharmacologic profiles between dexamethasone and prednisone need to be mentioned. Dexamethasone is more potent and has a longer half-life compared with prednisone³⁶ and thus the involved genes and pathways might differ somewhat, although clinical responses to both drugs in reducing acute asthma exacerbations were not different.^{37,38}

In addition to the rs9896933 variant's relationship with the γ tubulin pathway, 8 other SNPs with genome-wide significance identified in GWASs of BMA were noted. Thus far, there have been no reports showing direct associations between these SNPs and BMD. However, rs7003550, the top-ranked SNP, is of particular interest. According to the SCAN database, rs7003550 significantly affects expression of the p21 protein-activated kinase 2 (*PAK2*; $P = 5 \times 10^{-5}$) and adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (*APPL1*; $P = .0001$) genes in white subjects.³⁹ The SCAN database provides summary information from eQTL mapping of HapMap SNPs to gene expression (evaluated by the Affymetrix exon array) in the full set of HapMap white subjects.⁴⁰ The PAKs are critical effectors that link Rho GTPases to cytoskeletal reorganization and have been reported to take a role in methylglyoxal-induced⁴¹ and citrinin-induced⁴² osteoblast apoptosis. The protein encoded by the *APPL1* gene has been shown to be involved in regulation of cell proliferation and in cross-talk between the adiponectin- and insulin-signaling pathways.⁴³ Interestingly, the effect of adiponectin on bone metabolism is mediated by *APPL1*.⁴⁴ Taken together, rs7003550 might be involved in OCS-induced decrease in BMA by modulating *PAK2* and *APPL1* gene expression.

In conclusion, we have identified novel variants that influence the magnitude of the effect of OCSs on BMA based on an integrative genomic approach. Two of these variants of potential functional significance map to the γ tubulin pathway and can provide novel functional insights into the mechanisms by which systemic OCSs affect bone health. More studies testing other diseases, such as ulcerative colitis, juvenile idiopathic arthritis, and other autoimmune diseases, that feature corticosteroids are needed to obtain a complete picture of the mechanisms involved and risk estimates in susceptible children.

Clinical implications: Our data suggest that genetic factors can contribute to decreased BMA in asthmatic children receiving OCSs.

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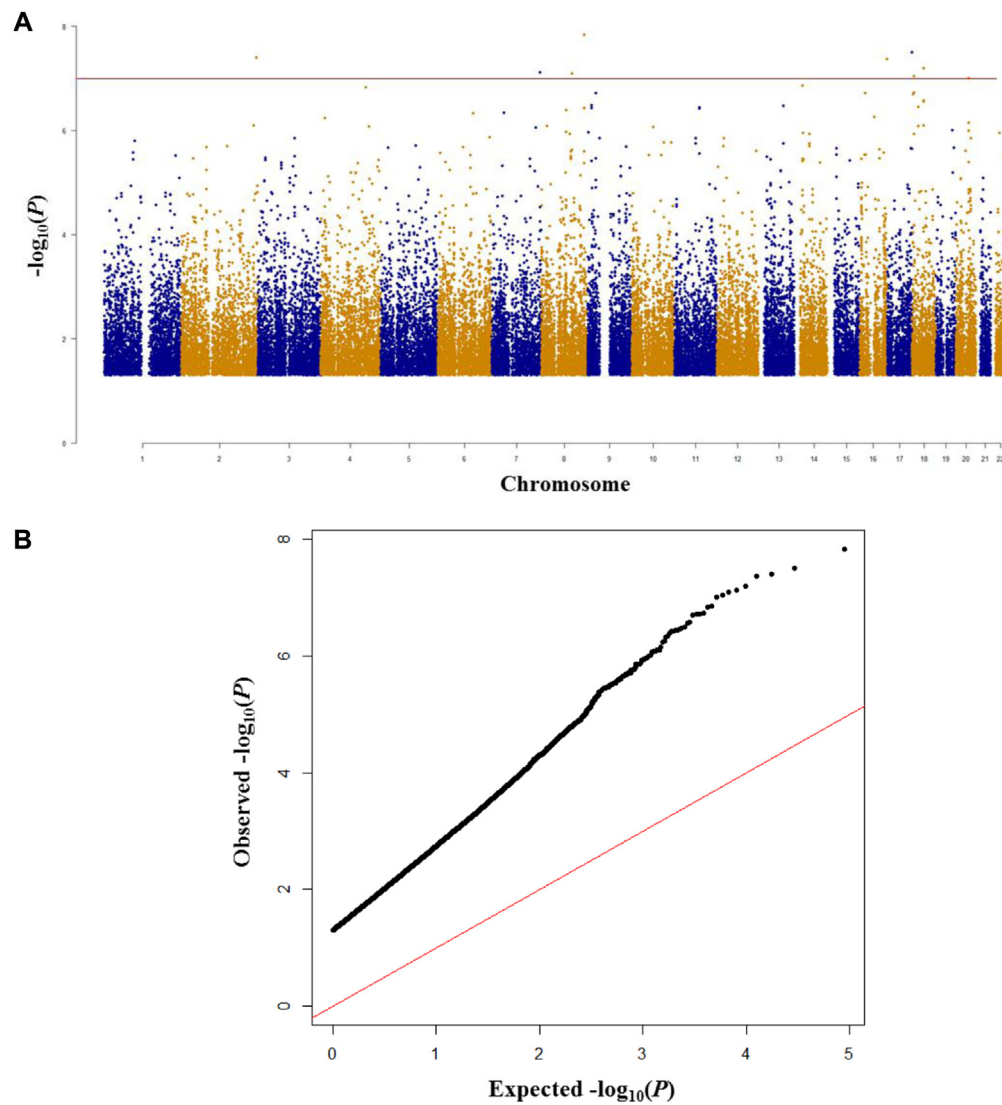


FIG E1. Quantile-quantile and Manhattan plots for interaction results. **A**, Quantile-quantile plot. *Red line*, Genome-wide significance. **B**, Manhattan plot.

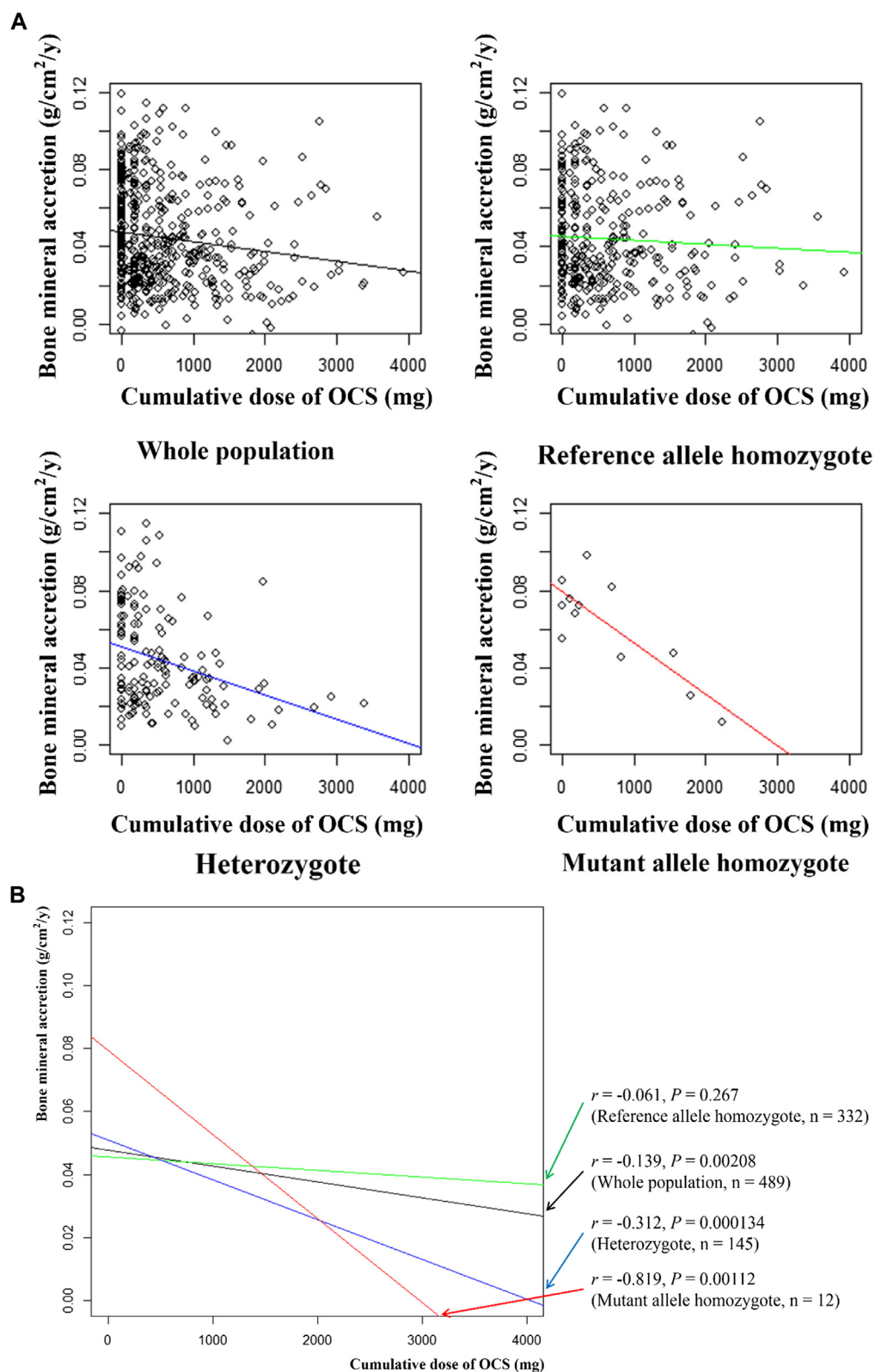


FIG E2. Correlations between cumulative dose of OCS and BMA stratified by genotype of rs9896933. **A**, Whole population, reference allele homozygotes, heterozygotes, and mutant allele homozygotes. **B**, Merge of individual regression lines. A slope obtained from simple linear regression analysis became steeper as the number of mutant alleles of the 2 SNPs increased. *P* values were obtained from Pearson correlation analysis.

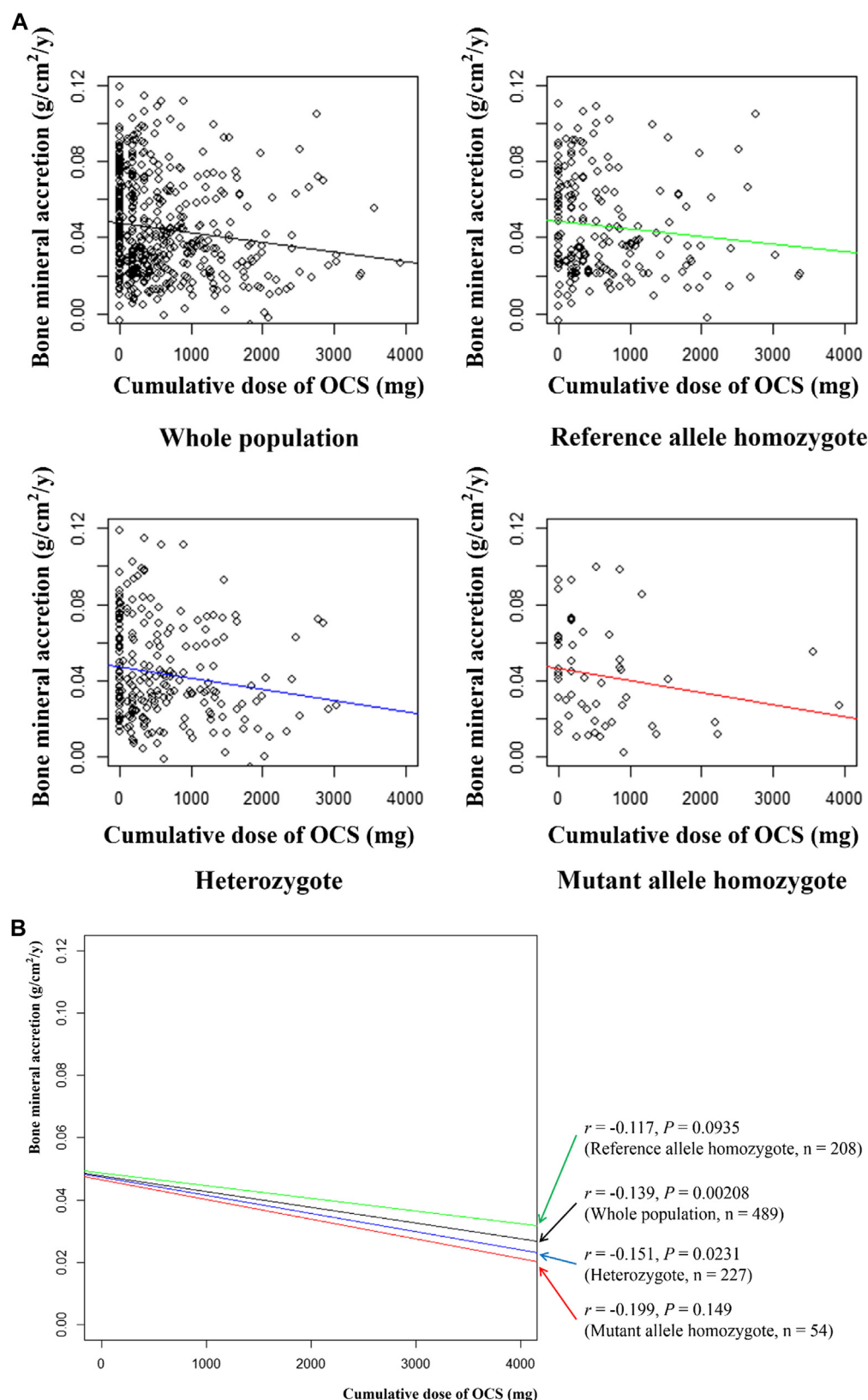


FIG E3. Correlations between cumulative dose of OCS and BMA stratified by genotype of rs2074439. **A**, Whole population, reference allele homozygotes, heterozygotes, and mutant allele homozygotes. **B**, Merge of individual regression lines. A slope obtained from simple linear regression analysis became steeper as the number of mutant alleles of the 2 SNPs increased. *P* values were obtained from Pearson correlation analysis.

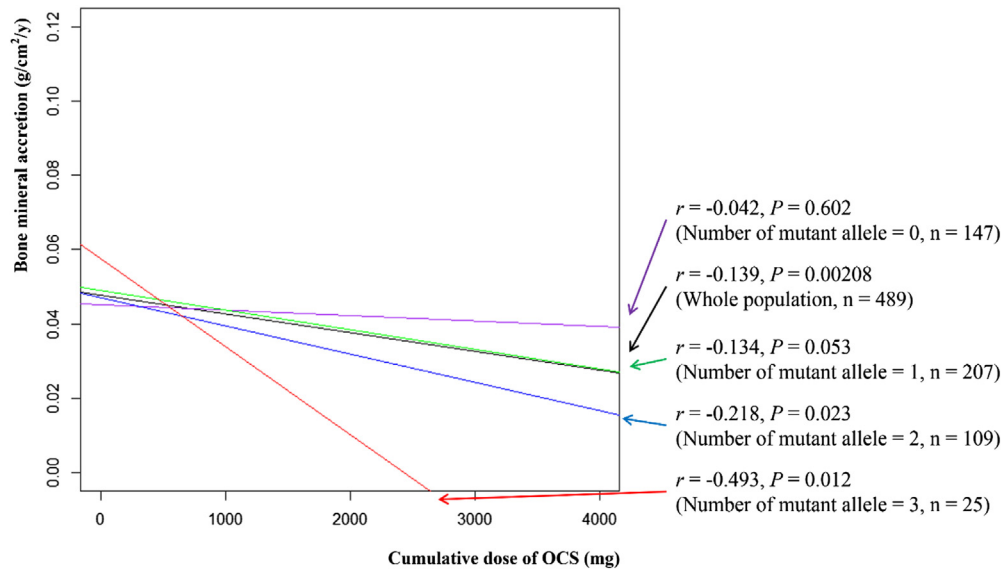


FIG E4. Correlations between cumulative dose of OCS and BMA stratified by number of mutant alleles of rs9896933 and rs2074439 (merge of individual regression lines). A slope obtained from simple linear regression analysis became steeper as the number of mutant alleles of the 2 SNPs increased. *P* values were obtained from Pearson correlation analysis.

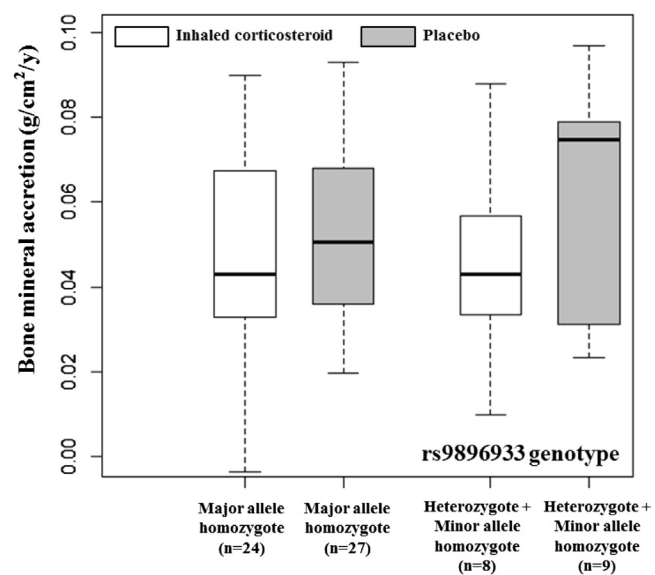


FIG E5. Comparison of BMA stratified by rs9896933 in asthmatic children without OCS bursts between those randomized to ICS and those randomized to placebo. Differences according to the treatment groups or genotypes of rs9896933 were not statistically significant.

TABLE E1. Relationship between variables and BMA in white CAMP subjects

Variables	β Coefficient	P value
Cumulative dose of prednisone	-3.04×10^{-6}	7.96×10^{-5}
Covariates measured at baseline		
Age	-7.19×10^{-4}	.149
Sex (female vs male)	0.00384	.002
BMD	0.148	$<2 \times 10^{-16}$
Height	-3.45×10^{-5}	.535
BMI	-1.94×10^{-4}	.357
Vitamin D	-1.55×10^{-5}	.669
Tanner stage 2 (vs 1)	0.00136	.379
Tanner stage 3 (vs 1)	-0.00137	.631
Tanner stage 4 (vs 1)	-0.0271	4.32×10^{-11}
Tanner stage 5 (vs 1)	-0.0470	1.52×10^{-4}

Multiple linear regression analysis.

BMI, Body mass index.

TABLE E2. β Coefficients and P values of associations between variables and BMA*

Variables	β Coefficient	P value
CumOCS	-1.074×10^{-6}	.3428
Age	9.954×10^{-4}	.0397
Female (vs male)	3.389×10^{-3}	.0004
BMD (z score)	0.1497	$<2 \times 10^{-16}$
Height	-3.635×10^{-5}	.5004
BMI	-1.224×10^{-4}	.5492
Serum concentration of vitamin D	2.392×10^{-5}	.4941
Tanner stage 2 (vs Tanner stage 1)	1.698×10^{-3}	.2585
Tanner stage 3 (vs Tanner stage 1)	-9.935×10^{-4}	.7180
Tanner stage 4 (vs Tanner stage 1)	-2.251×10^{-2}	3.570×10^{-10}
Tanner stage 5 (vs Tanner stage 1)	-4.541×10^{-2}	1.420×10^{-4}
rs2074439HE (vs rs2074439MA)	-5.411×10^{-4}	.7193
rs2074439MI (vs rs2074439MA)	-1.657×10^{-3}	.4655
rs9896933HE (vs rs9896933MA)	-6.734×10^{-3}	.0803
rs9896933MI (vs rs9896933MA)	-8.235×10^{-3}	1.880×10^{-5}
CumOCS:rs2074439HE (vs CumOCS:rs2074439MA)	-2.147×10^{-6}	.01754
CumOCS:rs2074439MI (vs CumOCS:rs2074439MA)	-8.666×10^{-6}	1.480×10^{-4}
CumOCS:rs9896933HE (vs CumOCS:rs9896933MA)	-1.260×10^{-5}	8.077×10^{-3}
CumOCS:rs9896933MI (vs CumOCS:rs9896933MA)	-7.525×10^{-6}	2.790×10^{-5}

CumOCS, Cumulative oral corticosteroid; BMI, body mass index; HE, heterozygote; MA, major allele homozygote; MI, minor allele homozygote.

*Regression model: BMA $\sim$ Cumulative dose of OCS + Age + Sex + BMD + Height + Body mass index + Serum concentration of vitamin D + Tanner stage + rs2074439 genotype (additive model) + rs9896933 genotype (additive model) + Cumulative dose of OCS*rs2074439 genotype + Cumulative dose of OCS*rs9896933.

TABLE E3. β Coefficients and P values of associations between cumulative dose of OCS and BMA stratified by the number of mutant alleles of rs9896933 and rs2074439

Group	β Coefficient	P value*
Whole population	-3.036×10^{-6}	7.96×10^{-5}
Subjects without mutant allele	-1.183×10^{-6}	.3504
Subjects with 1 mutant allele	-2.587×10^{-6}	.0298
Subjects with 2 mutant alleles	-7.178×10^{-6}	5.38×10^{-5}
Subjects with 3 mutant alleles	-2.871×10^{-5}	1.57×10^{-5}

*Adjusted by age, sex, BMD, height, body mass index, serum concentration of vitamin D, and Tanner stage measured at baseline.