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## Magnesium status and supplementation influence vitamin D status and metabolism: results from a randomized trial

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### ABSTRACT

**Background:** Previous in vitro and in vivo studies indicate that enzymes that synthesize and metabolize vitamin D are magnesium dependent. Recent observational studies found that magnesium intake significantly interacted with vitamin D in relation to vitamin D status and risk of mortality. According to NHANES, 79% of US adults do not meet their Recommended Dietary Allowance of magnesium.

**Objectives:** The aim of this study was to test the hypothesis that magnesium supplementation differentially affects vitamin D metabolism dependent on baseline 25-hydroxyvitamin D [25(OH)D] concentration.

**Methods:** The study included 180 participants aged 40–85 y and is a National Cancer Institute independently funded ancillary study, nested within the Personalized Prevention of Colorectal Cancer Trial (PPCCT), which enrolled 250 participants. The PPCCT is a double-blind 2 × 2 factorial randomized controlled trial conducted in the Vanderbilt University Medical Center. Doses for both magnesium and placebo were customized based on baseline dietary intakes. Subjects were randomly assigned to treatments using a permuted-block randomization algorithm. Changes in plasma 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>], 25-hydroxyvitamin D<sub>2</sub> [25(OH)D<sub>2</sub>], 1,25-dihydroxyvitamin D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>2</sub>, and 24,25-dihydroxyvitamin D<sub>3</sub> [24,25(OH)<sub>2</sub>D<sub>3</sub>] were measured by liquid chromatography–mass spectrometry.

**Results:** The relations between magnesium treatment and plasma concentrations of 25(OH)D<sub>3</sub>, 25(OH)D<sub>2</sub>, and 24,25(OH)<sub>2</sub>D<sub>3</sub> were significantly different dependent on the baseline concentrations of 25(OH)D, and significant interactions persisted after Bonferroni corrections. Magnesium supplementation increased the 25(OH)D<sub>3</sub> concentration when baseline 25(OH)D concentrations were close

to 30 ng/mL, but decreased it when baseline 25(OH)D was higher (from ~30 to 50 ng/mL). Magnesium treatment significantly affected 24,25(OH)<sub>2</sub>D<sub>3</sub> concentration when baseline 25(OH)D concentration was 50 ng/mL but not 30 ng/mL. On the other hand, magnesium treatment increased 25(OH)D<sub>2</sub> as baseline 25(OH)D increased.

**Conclusion:** Our findings suggest that optimal magnesium status may be important for optimizing 25(OH)D status. This trial was registered at [clinicaltrials.gov](https://clinicaltrials.gov) as NCT03265483. *Am J Clin Nutr* 2018;108:1249–1258.

**Keywords:** magnesium, vitamin D metabolism, interaction, calcium-to-magnesium ratio, randomized clinical trial

### INTRODUCTION

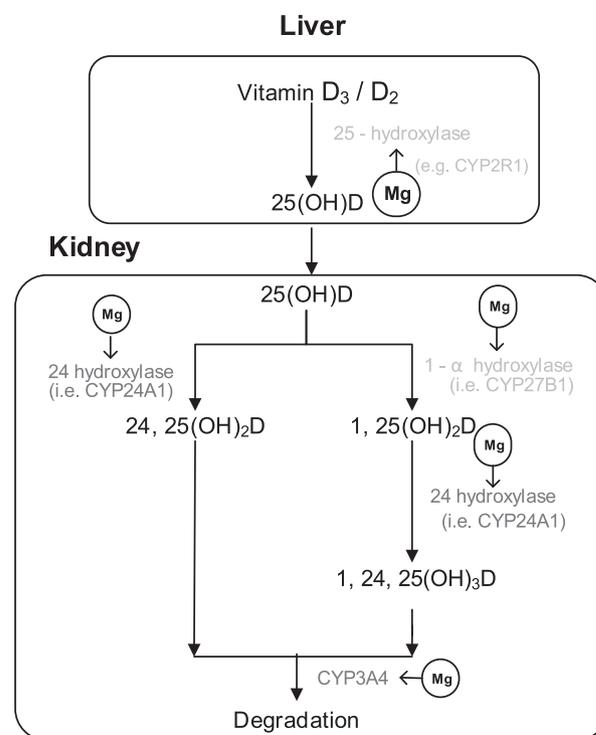
Epidemiologic studies and randomized trials have generated inconsistent findings on the role of vitamin D in bone fractures (1) and extraskeletal chronic diseases (2), such as colorectal adenoma recurrence (3), colorectal cancer incidence (4), total cancer incidence (5, 6), and cardiovascular disease (CVD) (7). Large-scale randomized trials testing vitamin D supplementation with cancer and CVD as primary outcomes are ongoing (2, 8). One striking observation is that a large portion of the interperson heterogeneity in circulating 25-hydroxyvitamin D [25(OH)D] concentrations is unexplained (9).

The 2015 Dietary Guidelines Advisory Committee determined that magnesium is underconsumed relative to the Estimated Average Requirement and is one of the shortfall nutrients in the US population (10). According to the NHANES, 79%

of US adults do not meet their Recommended Dietary Allowance of magnesium (11). For patients with “Mg-dependent vitamin-D-resistant rickets” (12), characterized by reduced 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] and impaired parathyroid response (13), intramuscular infusion with ≤600,000 IU vitamin D alone did not lead to any improvements in biochemical measures of vitamin D deficiency. However, magnesium supplementation did substantially reverse the resistance to vitamin D treatment (12–14). Furthermore, we reported from observational studies in the general US population that magnesium intake significantly interacted with vitamin D intake in affecting vitamin D status, and also interacted with circulating 25(OH)D in the risk of CVD mortality and possibly colorectal cancer mortality (15). The potential interaction between magnesium and vitamin D was supported by 2 subsequent studies, including a Finnish cohort study (16) and a study using a mouse model (17).

Previous studies indicate that magnesium status affects concentrations of cytochrome P450 (CYP) enzymes (18). Cytochrome P450 enzymes include not only the vitamin D-activating enzymes [i.e., 25-hydroxylase (e.g., CYP2R1) and 1 $\alpha$ -hydroxylase (i.e., CYP27B1)] but also vitamin D-deactivating enzymes [i.e., 24-hydroxylase (i.e., CYP24A1 and CYP3A4)]. 25-Hydroxylase synthesizes 25(OH)D from vitamin D<sub>3</sub> or vitamin D<sub>2</sub> in the liver, and then 1 $\alpha$ -hydroxylase synthesizes active 1,25(OH)<sub>2</sub>D from 25(OH)D in the kidney. 24-Hydroxylase metabolizes both 25(OH)D and 1,25(OH)<sub>2</sub>D to inactive forms: 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D, respectively. Finally, CYP3A4 (19) degrades 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D (20) (Figure 1). Both in vitro and in vivo studies have shown that 1 $\alpha$ -hydroxylase and 24-hydroxylase are magnesium dependent (21, 22).

Based on these observations, we hypothesize that magnesium supplementation interacts with baseline circulating 25(OH)D concentrations in affecting biomarkers of vitamin D synthesis and metabolism. In other words, we hypothesize that magnesium



**FIGURE 1** Magnesium and vitamin D metabolism. Dark gray indicates deactivating enzymes, and light gray indicates activating enzymes. CYP, cytochrome P450; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)<sub>2</sub>D, 1,25-dihydroxycholecalciferol; 24,25(OH)<sub>2</sub>D, 24,25-dihydroxyvitamin D; 1,24,25(OH)<sub>3</sub>D, 1,24,25-trihydroxyvitamin D.

supplementation has different relations with vitamin D synthesis and metabolism dependent on the baseline circulating 25(OH)D concentration. To test this hypothesis, we conducted an ancillary study within the Personalized Prevention of Colorectal Cancer Trial (PPCCT), which is a double-blind, placebo-controlled, randomized controlled trial (23) testing the association of magnesium supplementation with colorectal carcinogenesis among 250 participants.

## METHODS

### Participants and randomization

This is a US National Cancer Institute (NCI)-funded ancillary study (registered at [clinicaltrials.gov](http://clinicaltrials.gov) as NCT03265483) nested in the parent study, the PPCCT (registered at [clinicaltrials.gov](http://clinicaltrials.gov) as NCT01105169). The PPCCT is a double-blind 2 × 2 factorial randomized controlled trial (23) conducted at the Vanderbilt University Medical Center, Nashville, TN. The Vanderbilt Survey Research Shared Resource enrolled the participants. A modified R program was used to generate the randomization schedule by Chang Yu, one of the principal investigators of the PPCCT. The randomization procedure used randomized blocks of 2 or 4 to allocate subjects in a 1:1 ratio to 2 treatment arms—magnesium treatment or placebo—within 3 strata defined by the transient receptor potential cation channel, subfamily M, member 7 (*TRPM7*) genotype: GG, GA, and AA. Eligible subjects were enrolled sequentially and were assigned sequentially to receive magnesium treatment or placebo according to the

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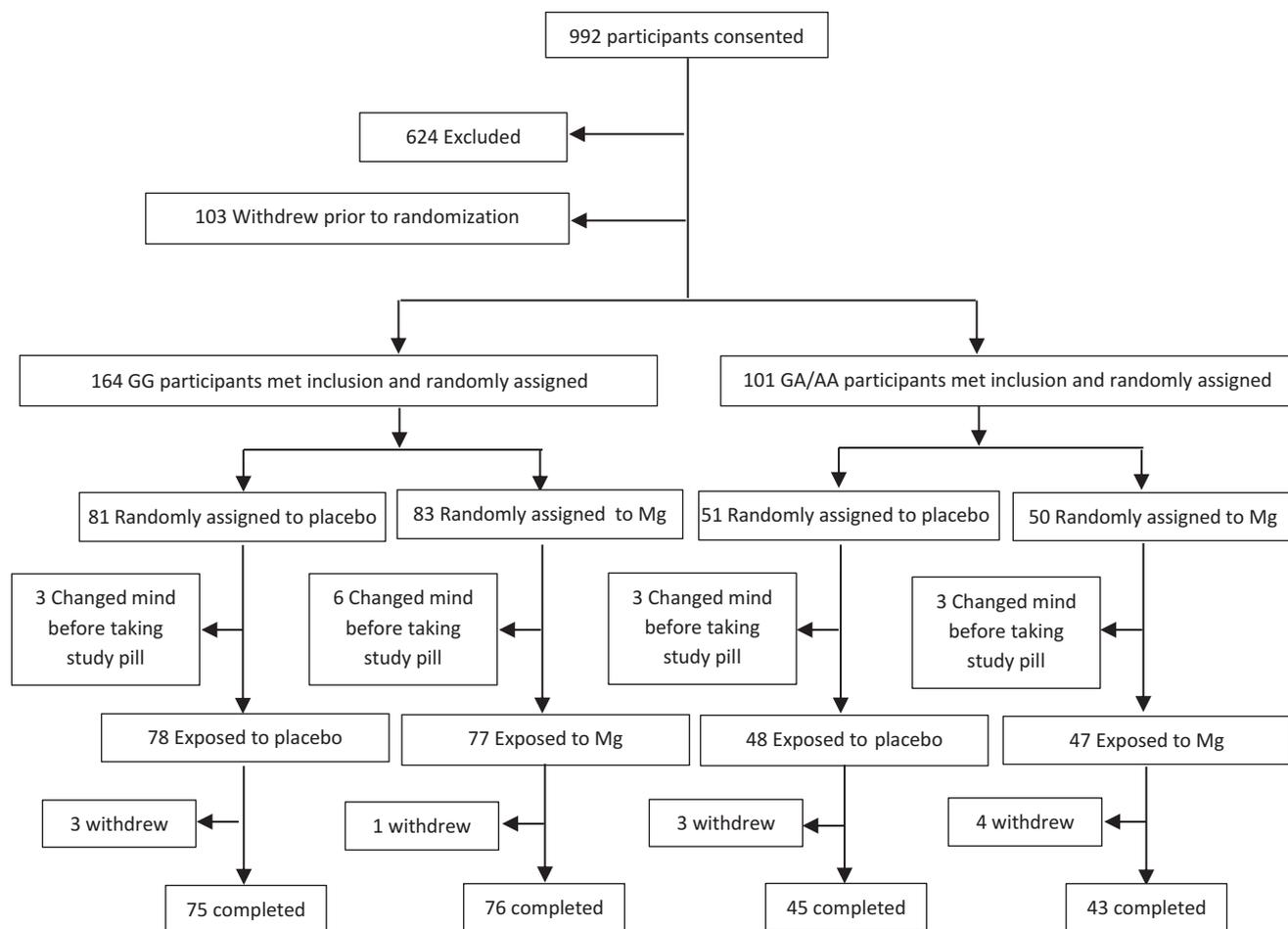
Clinical Research Center were supported in part by the Vanderbilt CTSA grant UL1 RR024975 from NCRR/NIH. The parent study data were stored in Research Electronic Data Capture (REDCap), and data analyses (VR12960) were supported in part by the Vanderbilt Institute for Clinical and Translational Research (UL1TR000445).

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Abbreviations used: CVD, cardiovascular disease; CYP, cytochrome P450; eGFR, estimated glomerular filtration rate; LCMS, liquid chromatography-mass spectrometry; NCI, National Cancer Institute; NIST, National Institute of Standards and Technology; PPCCT, Personalized Prevention of Colorectal Cancer Trial; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; *TRPM7*, transient receptor potential cation channel, subfamily M, member 7; 1,25(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; 1,25(OH)<sub>2</sub>D<sub>2</sub>, 1,25-dihydroxyvitamin D<sub>2</sub>; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; 24,25(OH)<sub>2</sub>D<sub>3</sub>, 24,25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D<sub>2</sub>, 25-hydroxyvitamin D<sub>2</sub>; 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>.

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**FIGURE 2** Flow diagram of trial recruitment and retention. AA, GA, GG, *TRPM7* genotypes. *TRPM7*, transient receptor potential cation channel, subfamily M, member 7.

randomization schedule. Participants, study investigators, and staff were all blinded to the assigned interventions. The blinding was implemented through the Vanderbilt Investigational Drug Service. A research pharmacist at the Drug Service maintained the randomization schedule and was the only person who was aware of the actual interventions. A total of 265 participants were randomly assigned and allocated to either the magnesium treatment or placebo arm. Of these, 15 withdrew their consent before taking the magnesium treatment or placebo. Thus, 250 participants were randomly assigned and started the treatments. Among them, 239 completed the trial, with the other 11 participants finishing only part of the study before withdrawing (see **Figure 2**). We recruited the first participant on 21 March 2011 and completed recruitment on 27 January 2016, because we had fulfilled the primary recruitment aim for the PPCCT.

The primary aim of the PPCCT was to examine the effects of magnesium supplementation and magnesium-*TRPM7* genotype interaction on the expression of biomarkers (i.e., *TRPM7*, mixed lineage kinase domain-like pseudokinase (MLKL), Ki67:Bax, Ki67:terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), cyclooxygenase-2) in colorectal mucosa. The secondary outcomes in the PPCCT included serum magnesium, body magnesium status, C-reactive protein, 25(OH)D, and urinary excretion of prostaglandin E2 metabolite. In the PPCCT,

we proposed to measure total 25(OH)D using an ELISA-based approach. However, following our novel finding of magnesium–vitamin D interaction from an observational study published in 2013 (15), we submitted a separate grant application to the NCI for an ancillary study. We proposed to measure 5 vitamin D metabolites in the ancillary study. The analysis of this ancillary study is reported herein.

Participants, aged 40–85 y, were recruited from Vanderbilt patient sources as follows: 1) 236 individuals with adenomas or hyperplastic polyps diagnosed from 1998 to 2014 or 2) 14-polyp free individuals with high risk of colorectal cancer. All participants had a calcium intake of  $\geq 700$  and  $< 2000$  mg/d, and their calcium-to-magnesium intake ratio was  $> 2.6$ , measured using two 24-h dietary recalls. A list of exclusion criteria was applied: a history of colectomy, inflammatory bowel disease, any organ transplantation, cancer other than nonmelanoma skin cancer, gastric bypass, chronic renal diseases (glomerular filtration rate  $< 50 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ), hepatic cirrhosis, chronic ischemic heart disease, diarrhea, type 1 diabetes, and pituitary dwarfism; current use of lithium carbonate therapy, blood anticoagulant drugs, digoxin, and licorice; and without contact information and informed consent.

For the current study, funded by an independent NCI project, 180 participants who had completed the PPCCT study by

October 2015 were selected. This included 90 women and 90 men (87 participants in the treatment arm and 93 in the placebo arm). The parent PPCCT was still blinded for primary outcomes; thus, an unblinded independent statistician outside of the study team conducted all the statistical analyses for the current report.

### Interventions and precision-based dosing strategy

Two 24-h dietary recalls were performed for all participants at the baseline of the PPCCT. Based on their baseline intakes of calcium and magnesium as well as their calcium-to-magnesium intake ratio, each participant was assigned to a customized dose of magnesium supplementation that would reduce the calcium-to-magnesium intake ratio to  $\sim 2.3$ , as suggested by several previous studies (23–27). The mean recorded intake from 24-h recalls was used to estimate the baseline intakes of calcium and magnesium, and the calcium-to-magnesium ratio. Placebos of microcrystalline cellulose were made to appear identical to magnesium capsules. The capsules, which were made of gelatin, were filled by the Vanderbilt Investigational Pharmacy personnel following USP 797 conditions according to the compounding instructions. The intervention period was designed to be 12 wk. The Vanderbilt Clinical Pharmacist in the Investigational Drug Service dispensed the capsules.

There were 4 additional 24-h dietary recalls conducted for all participants during the intervention period, with 2 taking place during weeks 1–6 and the other 2 taking place during weeks 6–12. Participants were scheduled for 3 clinic visits (weeks 1, 6, and 12). Information on the participant's use of medications and nutritional supplements, and other information on health and diet, was collected at each clinic visit. Blood samples were collected and processed at each clinic visit. Anthropometric measurements (weight, height, and waist and hip circumferences) were measured at least twice at each clinic visit.

### Vitamin D metabolite assay and kidney function

Blood was collected from a forearm vein at each clinic visit after participants had fasted for  $\geq 8$  h. Both serum and plasma were rapidly cooled and frozen at  $-80^{\circ}\text{C}$  before biochemistry analysis. In order to minimize potential errors caused by batch effects, samples were randomly organized into sets that included  $\geq 1$  pair of pre- and postsamples from a participant in the treatment arm and  $\geq 1$  pair of pre- and postsamples from the placebo arm. The samples were shipped on dry ice overnight to AAF's laboratory. This assay of vitamin D metabolites is validated by participation in quality-assurance programs organized by DEQAS (the Vitamin D External Quality Assessment Scheme) and the National Institute of Standards and Technology (NIST). In the current study, plasma samples were used as described previously (28). To control for batch-to-batch variability, samples for each set were analyzed in the same laboratory run. A pool of quality-control samples was added to each batch of samples to be assayed. Laboratory staff were blinded to the samples' status (in treatment or placebo arms or quality control) to eliminate bias.

1,25-Dihydroxyvitamin D<sub>2</sub> [1,25(OH)<sub>2</sub>D<sub>2</sub>] and 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] were extracted from plasma using an ALPCO (Laboratory Equipment Supplier in Salem, New Hampshire) immunoextraction kit following the manufacturer's protocols. In brief, 550  $\mu\text{L}$  plasma was centrifuged in a 1.5-mL microcentrifuge tube at  $13,500 \times g$  for 10 min. Next, 500  $\mu\text{L}$  of the clear plasma supernatant was transferred to an ImmunoTube with 10  $\mu\text{L}$  internal standard solution [1,25(OH)<sub>2</sub>D<sub>3</sub>-d<sub>3</sub> at 10 ng/mL in methanol]. The ImmunoTube was then capped, placed on a rotator, and mix-rotated (rotated end over end) at room temperature for 1 h. After the rotation mixing, the tube was placed in a 13-  $\times$  75-mm test tube and centrifuged at  $600 \times g$  for 1 min, followed by removal of the cover and the outlet of the tubes, before being further centrifuged at  $600 \times g$  for 2 min to remove the liquid. The ImmunoTube was then washed 3 times with 500  $\mu\text{L}$  WASHSOL, followed by elution of the analytes with 250  $\mu\text{L}$  ELUREAG. The eluant was collected and dried under a nitrogen flow. The derivatization reaction was carried out by adding 50  $\mu\text{L}$  4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) solution (0.5 mg/mL in anhydrous acetonitrile) to the dried sample and mixing it manually on a vortex at 2000 rpm for 10 s. The vial was then capped and incubated at room temperature for 1 h. The reaction was quenched by adding 50  $\mu\text{L}$  deionized water, and the resulting mixture was transferred to HPLC inserts and subjected to liquid chromatography–mass spectrometry (LCMS) analysis.

25-Hydroxyvitamin D<sub>2</sub> [25(OH)D<sub>2</sub>], 25-hydroxyvitamin D<sub>3</sub>, and 24,25-dihydroxyvitamin D<sub>3</sub> [24,25(OH)<sub>2</sub>D<sub>3</sub>] were extracted from plasma by liquid-liquid extraction. In brief, 100  $\mu\text{L}$  plasma was mixed gently with 100  $\mu\text{L}$  internal standard solution 25-hydroxyvitamin D<sub>3</sub>-d<sub>6</sub> at 260 ng/mL in methanol], followed by incubation at room temperature for 15 min. The mixture was then extracted with 1 mL hexanes by mixing on a vortex at 1750 rpm for 5 min, followed by centrifugation at  $1037 \times g$  for 5 min to separate the 2 layers. The upper layer was dried completely under a nitrogen flow and treated with PTAD as described above, followed by LCMS analysis. To the dried sample we added 50  $\mu\text{L}$  PTAD solution, which was manually mixed on a vortex at 2000 rpm for 10 s. The vial was then capped and incubated at room temperature for 1 h. The reaction was quenched by adding 50  $\mu\text{L}$  deionized water, and the resulting mixture was transferred to HPLC inserts and subjected to LCMS analysis.

LCMS was performed using a model Accela ultra HPLC system coupled with a Q Exactive Orbitrap Mass Spectrometer and a CTC PAL autosampler (all from Thermo Fisher). Aliquots of 25  $\mu\text{L}$  of the above mixture for 25(OH)D and 24,25(OH)<sub>2</sub>D<sub>3</sub> and 40  $\mu\text{L}$  for 1,25(OH)<sub>2</sub>D were injected into an Agilent SB C18 column (50  $\times$  2.1 mm, 1.8  $\mu\text{m}$ ; Agilent) with a precolumn filter (0.2  $\mu\text{m}$ ; Thermo Fisher). Gradient elution was performed at a flow rate of 300  $\mu\text{L}/\text{min}$  with the use of 0.1% formic acid in H<sub>2</sub>O (A) and 0.1% formic acid in acetonitrile (B) as follows: 0- to 10.0-min linear gradient from 60% A to 20% A; hold at the same ratio for 1 min; then go back to the first line condition and equilibrate for 5 min. The total HPLC time including equilibration was 15 min. Mass analysis was performed in positive electrospray target SIM mode, under the following conditions: (+) electrospray ionization (ESI) spray voltage 4.5 kV, capillary transfer temperature  $350^{\circ}\text{C}$ , heated electrospray ionization (HESI) heater temperature  $350^{\circ}\text{C}$ , sheath gas flow rate

30 units, auxiliary gas 5 units, in-source collision induced dissociation (CID) 5 eV. Quantitation of all analytes was performed with Xcalibur software within 5 ppm of the calculated exact masses  $\{[M + H]^+$ :  $25(\text{OH})\text{D}_2 = 570.36920$ ;  $25(\text{OH})\text{D}_3 = 558.36902$ ;  $25(\text{OH})\text{D}_3\text{-d}_6 = 564.40668$ ;  $24,25(\text{OH})_2\text{D}_3 = 574.36393\}$ , and the detection limit for these analytes was 2–22 pg/mL  $\{[M + H]^+$ :  $1,25(\text{OH})_2\text{D}_3 = 574.36393$ ;  $1,25(\text{OH})_2\text{D}_3\text{-d}_3 = 577.38276$ ;  $1,25(\text{OH})_2\text{D}_2 = 586.39393\}$ ; the detection limit for these analytes was 1 pg/mL. Consistent with previous studies (29), we found that 96.7% of participants had undetectable concentrations of  $1,25(\text{OH})_2\text{D}_2$ . The CVs for intrabatch variation were 5.58, 7.24, 7.74, and 9.38 for  $1,25(\text{OH})_2\text{D}_3$ ,  $25(\text{OH})\text{D}_2$ ,  $25(\text{OH})\text{D}_3$ , and  $24,25(\text{OH})_2\text{D}_3$ , respectively. The corresponding CVs for interbatch variations were 5.57, 7.34, 5.31, and 2.72, respectively. The concentrations of  $25(\text{OH})\text{D}_3$  were 26.8 and 28.7 ng/mL, respectively, when the NIST-assigned values were 24.1 and 28.3 ng/mL.

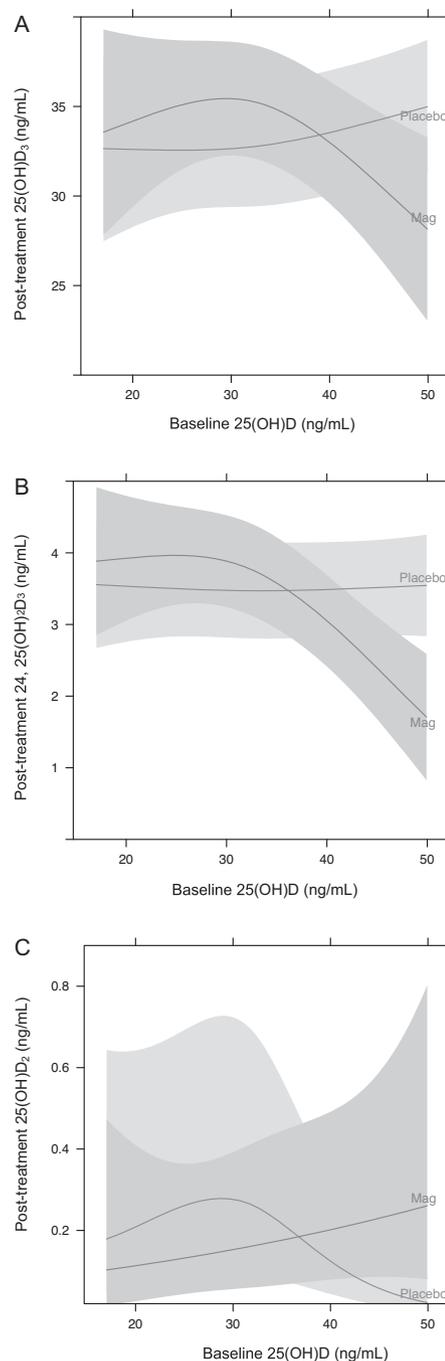
Serum creatinine was measured by a kinetic alkaline picrate method with the use of a Cobas Mira Plus clinical autoanalyzer and a kit from Randox Laboratories; CVs were <6%. An estimated glomerular filtration rate (eGFR) based on serum creatinine was obtained by using the modified 4-variable Modification of Diet in Renal Disease study equation (30).

### Statistical analyses

Based on 3 earlier clinical studies (12–14), we estimated that we needed only 12 individuals/arm to have 80% power to detect the magnesium–vitamin D interaction. However, the earlier clinical studies were conducted in those with severe magnesium deficiency, whereas our randomized trial was conducted in those at risk of magnesium deficiency. To be conservative, we also conducted a power estimation based on the NHANES data used in our previous report. Because NHANES is conducted in the general US population, very low cutoffs were selected to define low intakes of vitamin D and magnesium to estimate the power. We understand that our power estimation could not take into account the effect of sun exposure on  $25(\text{OH})\text{D}$ .

Previous data from NHANES (15) have shown that in subjects with low daily vitamin D intake ( $\leq 40$  IU), serum  $25(\text{OH})\text{D}$  was  $20.0 \pm 9.2$  ng/mL (mean  $\pm$  SD) with a high daily magnesium intake ( $> 420$  mg) compared with  $17.9 \pm 8.8$  ng/mL with a low daily magnesium intake ( $\leq 225$  mg), whereas in subjects with high vitamin D intake ( $> 1000$  IU), serum  $25(\text{OH})\text{D}$  was  $27.3 \pm 7.8$  ng/mL with high magnesium intake compared with  $18.7 \pm 15.7$  ng/mL with low magnesium intake. Assuming an SD of 8 ng/mL for serum  $25(\text{OH})\text{D}$ , 90 subjects in the magnesium treatment and 90 in the placebo group will give us 83.2% power to detect a difference of 7 ng/mL in the effect of magnesium supplement intake between subjects with a low baseline  $25(\text{OH})\text{D}$  concentration and those with a high baseline  $25(\text{OH})\text{D}$  concentration, with a 2-sided type I error rate of 0.05 based on a *t* test.

Summary statistics for continuous variables (mean  $\pm$  SD, median, and IQR) and categorical variables (count and percentage) were reported for the 2 randomly assigned arms. The Wilcoxon rank sum test was conducted to evaluate whether pretreatment values were different between the 2 arms



**FIGURE 3** Post-treatment plasma vitamin D metabolite concentrations calculated by a linear model. Concentrations were adjusted for age, sex, baseline BMI, eGFR, total  $25(\text{OH})\text{D}$ , and blood collection season among 87 participants randomly assigned to the magnesium treatment arm and 93 participants randomly assigned to the placebo arm. The solid curves represent point estimates and the gray regions represent 95% CIs. (A) Adjusted to  $25(\text{OH})\text{D}_3 = 31.37$  ng/mL, age = 60 y, sex = female, BMI ( $\text{kg}/\text{m}^2$ ) = 29.1, baseline GFR =  $78 \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ , blood sample collection season = summer. (B) Adjusted to  $25(\text{OH})\text{D}_3 = 3.095$  ng/mL, age = 60 y, sex = female, BMI = 29.1, baseline GFR =  $78 \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ , blood sample collection season = summer. (C) Adjusted to  $25(\text{OH})\text{D}_2 = 0.485$  ng/mL, age = 60 y, sex = female, BMI = 29.1, baseline GFR =  $78 \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ , blood sample collection season = summer. eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; Mag, magnesium;  $25(\text{OH})\text{D}$ , 25-hydroxyvitamin D;  $25(\text{OH})\text{D}_2$ , 25-hydroxyvitamin  $\text{D}_2$ ;  $25(\text{OH})\text{D}_3$ , 25-hydroxyvitamin  $\text{D}_3$ ;  $24,25(\text{OH})_2\text{D}_3$ ,  $24,25$ -dihydroxyvitamin  $\text{D}_3$ .

(magnesium treatment or placebo) for continuous variables. Pearson chi-square tests were conducted to compare categorical variables between treatment arms. Ordinary linear regression models were fitted to examine the association of magnesium treatment with vitamin D metabolites, adjusting for age, sex, baseline eGFR, BMI, 25(OH)D, and other vitamin D metabolites and baseline sample collection season. We assumed a linear relation for baseline vitamin D metabolite and a smooth relation for other continuous variables using restricted cubic regression splines with 3 knots (knot locations were chosen at 5%, 50%, and 95% of sample quantiles). The interaction between treatment and baseline 25(OH)D was also included in the models. Log-transformation was conducted to appropriately fit the model in the analysis of 25(OH)D<sub>2</sub>. The fold-change of 25(OH)D<sub>2</sub> is presented in **Figure 3** and **Table 1**. We conducted an analysis with additional adjustment for smoking and drinking status but found that the results did not alter appreciably, and so present models without these adjustments. The data analysis used R 3.3.0 software (<https://www.r-project.org/>).

## RESULTS

In the parent study (PPCCT), 250 participants were allocated to either the magnesium treatment or placebo arm and began the treatment. After the treatments had begun, 11 of the 250 participants withdrew from the trial. Self-reported adverse events were responsible for 6 of the withdrawals, 4 of which were in the treatment arm and 2 were in the placebo arm. To note, the other 5 withdrew due to lack of time or interest.

The participants in the magnesium treatment arm did not significantly differ from those in the placebo arm with regard to averages or distributions for age, sex, BMI, eGFR, *TRPM7* genotype, smoking status, alcohol drinking status, physical activity status, education achievement, race, season when the baseline blood sample was collected, or vitamin D metabolites concentrations, including 25(OH)D, 25(OH)D<sub>3</sub>, 25(OH)D<sub>2</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and 24,25(OH)<sub>2</sub>D<sub>3</sub>. The baseline calcium-to-magnesium intake ratios were comparable between the placebo and magnesium treatment arms, although the baseline magnesium intake was higher in the treatment arm (**Table 2**). However, age, sex, BMI, and eGFR were still adjusted for in the subsequent analyses due to their important impact on vitamin D status. The mean daily dose of personalized magnesium supplementation was 205.52 mg, with a range from 77.25 to 389.55 mg. Compliance with the treatment regimen was very high for both the placebo and treatment arms (mean  $\pm$  SD values based on capsule counts were 97.3%  $\pm$  4.4% and 97.5%  $\pm$  3.9%, respectively;  $P = 0.83$  for difference between the arms). The mean  $\pm$  SD calcium-to-magnesium ratios for the treatment and placebo arms after administration of magnesium and placebo supplementation were 2.27  $\pm$  0.13 and 3.84  $\pm$  1.43, respectively ( $P < 0.001$  for difference between the arms), based on the two 24-h dietary recalls performed at baseline, and remained stable at 2.13  $\pm$  0.68 and 3.50  $\pm$  1.31, respectively ( $P < 0.001$  for difference between the arms), based on the four 24-h dietary recalls conducted over the 12-wk period of the trial.

The relations of magnesium treatment with the plasma concentrations of 25(OH)D<sub>3</sub> ( $P = 0.001$  for interaction), 25(OH)D<sub>2</sub> ( $P = 0.009$  for interaction), and 24,25(OH)<sub>2</sub>D<sub>3</sub> ( $P < 0.0001$  for interaction) were significantly different based on the baseline

plasma concentrations of 25(OH)D. The interactions were statistically significant after Bonferroni corrections. However, magnesium treatment did not interact significantly with baseline 25(OH)D in changing 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration ( $P = 0.25$  for interaction; see **Supplemental Figure 1**).

When the baseline 25(OH)D was higher, at  $\sim$ 30–50 ng/mL, magnesium treatment reduced 25(OH)D<sub>3</sub> (**Figure 3A**). At a baseline 25(OH)D concentration of 50 ng/mL, magnesium treatment caused a significantly reduced 25(OH)D<sub>3</sub> concentration compared with the placebo arm, with an estimated mean (95% CI) difference of  $-6.87$  ( $-11.30, -2.45$ ) (**Table 1**). At a baseline 25(OH)D of 30 ng/mg, but not 20 ng/mg, magnesium treatment led to a significantly elevated concentration of 25(OH)D<sub>3</sub> compared with the placebo arm, with an estimated mean (95% CI) difference of 2.79 (0.25, 5.34) (**Table 1**). The association of magnesium treatment with 24,25(OH)<sub>2</sub>D<sub>3</sub> was similar to the pattern observed for 25(OH)D<sub>3</sub> only when baseline 25(OH)D was higher, from 30 to 50 ng/mL (**Figure 3B**). A significant decrease in 24,25(OH)<sub>2</sub>D<sub>3</sub> was observed with supplementation at 50 ng/mL, but there were no signs of a treatment effect at the other concentrations (**Table 1**). Thus, there was no consistent evidence that magnesium supplementation increased vitamin D metabolite concentrations [i.e., 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub>] at lower concentrations. On the other hand, magnesium treatment increased concentrations of 25(OH)D<sub>2</sub> as the baseline 25(OH)D continuously increased (**Figure 3C**). Magnesium treatment led to a significant 8.39-fold (95% CI: 2.38-, 29.63-fold) increase in 25(OH)D<sub>2</sub> compared with placebo at a baseline 25(OH)D concentration of 50 ng/mL (**Table 1**).

## DISCUSSION

We found that magnesium supplementation interacted with baseline plasma concentrations of 25(OH)D in affecting the concentrations of 25(OH)D<sub>3</sub>, 25(OH)D<sub>2</sub>, and 24,25(OH)<sub>2</sub>D<sub>3</sub>. We found that magnesium supplementation reduced 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> when 25(OH)D concentrations were  $>30$  ng/mL, particularly at 50 ng/mL. These findings are novel. However, there was no consistent evidence that magnesium supplementation increased vitamin D metabolite concentrations [i.e., 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub>] at lower concentrations. Magnesium supplementation increased 25(OH)D<sub>3</sub> when 25(OH)D concentrations were at 30 ng/mL, but not 20 ng/mL, whereas magnesium supplementation did not increase 24,25(OH)<sub>2</sub>D<sub>3</sub>. Also, we found that the pattern of the magnesium association was different with 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>.

Both in vitro and in vivo studies have indicated that magnesium deficiency affects 1 $\alpha$ -hydroxylase (i.e., CYP27B1) and 24-hydroxylase (i.e., CYP24A1), which synthesize and metabolize 25(OH)D and 1,25(OH)<sub>2</sub>D, respectively (21, 22). Magnesium deficiency, which leads to reduced 1,25(OH)<sub>2</sub>D and impaired parathyroid hormone response (13), has been implicated in “Mg-dependent vitamin-D-resistant rickets” (12). However, magnesium supplementation substantially reversed the resistance to vitamin D treatment (12–15). These earlier studies were case reports or small, nonrandomized, placebo-controlled clinical studies conducted in patients with severe clinical magnesium deficiency. The current study was conducted in individuals almost without clinical symptoms of magnesium deficiency (i.e.,

**TABLE 1**Relation between magnesium treatment and vitamin D metabolism, by baseline 25(OH)D concentrations<sup>1</sup>

Baseline 25(OH)D	Group, ng/mL		Difference (treatment – placebo) (95% CI)	P
	Magnesium	Placebo		
25(OH)D <sub>3</sub> , ng/mL				
20	30.95 ± 2.18	29.37 ± 2.08	1.58 (–2.28, 5.44)	0.42
30	32.20 ± 1.56	29.41 ± 1.61	2.79 (0.25, 5.34)	0.03
40	29.76 ± 1.84	30.31 ± 1.80	–0.54 (–3.26, 2.18)	0.69
50	24.89 ± 2.74	31.76 ± 2.00	–6.87 (–11.30, –2.45)	0.002
24,25(OH) <sub>2</sub> D <sub>3</sub> , ng/mL				
20	4.35 ± 0.41	3.96 ± 0.39	0.39 (–0.39, 1.17)	0.33
30	4.31 ± 0.32	3.90 ± 0.32	0.41 (–0.10, 0.91)	0.11
40	3.47 ± 0.32	3.91 ± 0.32	–0.44 (–0.99, 0.11)	0.12
50	2.12 ± 0.44	3.97 ± 0.36	–1.85 (–2.70, –0.99)	<0.0001
25(OH)D <sub>2</sub> , ng/mL				
20	–2.22 ± 0.57	–1.65 ± 0.55	0.56 (0.19, 1.69) <sup>2</sup>	0.30
30	–1.94 ± 0.45	–1.38 ± 0.46	0.57 (0.27, 1.18) <sup>2</sup>	0.13
40	–1.68 ± 0.44	–2.13 ± 0.45	1.57 (0.72, 3.41) <sup>2</sup>	0.25
50	–1.43 ± 0.55	–3.56 ± 0.57	8.39 (2.38, 29.63) <sup>2</sup>	0.001

<sup>1</sup>Values are means ± SEMs unless otherwise indicated. Values are based on our multiple linear regression model, adjusting for age = 60 y, sex = male, BMI (kg/m<sup>2</sup>) = 30, baseline eGFR = 7 · min<sup>–1</sup> · 1.73 m<sup>–2</sup>, blood sample collection season = summer, baseline 25(OH)D<sub>3</sub> = 30 ng/mL or 24,25(OH)<sub>2</sub>D<sub>3</sub> = 4 ng/mL or 25(OH)D<sub>2</sub> = 0.5 ng/mL among 87 participants randomly assigned to the magnesium treatment arm and 93 participants randomly assigned to the placebo arm. The P values for the interactions between magnesium intake with baseline 25(OH)D in changing 25(OH)D<sub>3</sub>, 24,25(OH)<sub>2</sub>D<sub>3</sub>, and 25(OH)D<sub>2</sub> were 0.001, <0.0001, and 0.009, respectively. Total 25(OH)D, 25(OH)D<sub>3</sub>: 1 ng/mL = 2.4959 nmol/L; 25(OH)D<sub>2</sub>: 1 ng/mL = 2.4233 nmol/L. 24,25(OH)<sub>2</sub>D<sub>3</sub>, 24,25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D<sub>2</sub>, 25-hydroxyvitamin D<sub>2</sub>; 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>.

<sup>2</sup>Log-transformation was conducted to appropriately fit the model in the analysis of 25(OH)D<sub>2</sub>. The fold-change of 25(OH)D<sub>2</sub> is presented.

only 1 participant had serum magnesium <1.7 mg/dL). In this population, we found that magnesium supplementation did not interact with baseline plasma 25(OH)D in affecting plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration. However, we found that magnesium supplementation significantly changed plasma 25(OH)D<sub>3</sub> concentration depending on the patient's baseline plasma 25(OH)D concentration. These findings are supported by a study conducted in the US general population (15). In that study, we reported that magnesium intake significantly interacted with the intake of vitamin D in relation to risk of both vitamin D deficiency and insufficiency in NHANES 2001–2006 (15). However, this previous observational study was a cross-sectional study, and the 25(OH)D<sub>2</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> metabolites were not measured.

Thus, the findings from the current study provide the first evidence in humans that magnesium supplementation reduces 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> when 25(OH)D is higher but may increase 25(OH)D<sub>3</sub> when 25(OH)D is lower. The precise molecular mechanism is not clear. One possible explanation is that magnesium supplementation affects both vitamin D-activating enzymes (i.e., CYP27B1 and CYP2R1) and vitamin D-deactivating enzymes [i.e., CYP24A1 and CYP3A4 (Figure 1)]. When baseline 25(OH)D is <30 ng/mL, the activity of CYP3A4 on vitamin D degradation is limited; thus, the relation of magnesium supplementation is primarily with vitamin D synthesis enzymes. When baseline 25(OH)D concentrations are >30 ng/mL, CYP3A4 activity starts to elevate and the activity is further enhanced by magnesium supplementation, which leads to a significant reduction in concentrations of 24,25(OH)<sub>2</sub>D<sub>3</sub>. In addition, the reduction in 24,25(OH)<sub>2</sub>D<sub>3</sub> seems stronger than the reduction in 25(OH)D<sub>3</sub>, indicating that the reduction in 25(OH)D<sub>3</sub> could be secondary to the 24,25(OH)<sub>2</sub>D<sub>3</sub> reduction.

Our observation about plasma 25(OH)D<sub>3</sub> is also consistent with previous reports. We found from the NHANES III cohort study that the longitudinal inverse associations between circulating 25(OH)D and total mortality, particularly due to CVD, were modified by magnesium intake (15). Following our findings, a borderline significant interaction between magnesium intake and circulating 25(OH)D in relation to total mortality was observed in a Finnish cohort study conducted in a population with low circulating 25(OH)D status and high magnesium intakes (16); and another study showed that magnesium treatment modified the association of vitamin D analogs with vascular calcification in mice with experimental chronic kidney disease (17). The most recent Institute of Medicine Report on Dietary Reference Intakes of Calcium and Vitamin D mentioned that several cohort studies found a U-shaped relation between plasma 25(OH)D concentration and risk of incident CVD (7). For example, in the Framingham Offspring Study, although a significant inverse association was found between plasma 25(OH)D and incident CVD, there was no additional reduction in risk once plasma 25(OH)D concentrations increased beyond 30 ng/mL (7, 31). This finding has been supported by 2 subsequent meta-analyses of cohort studies (8, 32, 33). Similarly, a reverse J-shaped association was found between plasma 25(OH)D and CVD mortality in a recent large cohort study in 247,574 participants (34); and concentrations of 25(OH)D at 28 ng/mL were associated with the lowest risk of CVD mortality. However, none of those studies examined the potential interaction between vitamin D status and magnesium status in relation to CVD risk. Because the 2015 Dietary Guidelines Advisory Committee determined that magnesium is underconsumed in the US population (10, 11), future studies are necessary to further

**TABLE 2**Descriptive characteristics of 180 participants at baseline<sup>1</sup>

	Magnesium treatment (n = 87)	Placebo (n = 93)	P
Age, y	60.4 ± 8.3	61.7 ± 8.3	0.30 <sup>2</sup>
Male sex, %	52	48	0.66 <sup>3</sup>
BMI, kg/m <sup>2</sup>	29.4 ± 6.0	30.3 ± 6.5	0.30 <sup>2</sup>
eGFR	81.0 ± 14.0	78.0 ± 15.0	0.16 <sup>2</sup>
TRPM7 genotype GG, %	65	68	0.64 <sup>3</sup>
Smoking status, %			0.21 <sup>3</sup>
Never	46	59	
Ever	44	33	
Current	10	8	
Drinking status, %			0.25 <sup>3</sup>
Never	44	32	
Ever	17	20	
Current	39	48	
Physically active ≥2 d/wk, %	84	78	0.33 <sup>3</sup>
Education less than college, %	10	9	0.91 <sup>3</sup>
White race, %	98	99	0.52 <sup>3</sup>
Family history of colorectal cancer, %	14	11	0.53 <sup>3</sup>
Daily nutrients intake, mg/d			
Total calcium	1327 ± 332	1236 ± 364	0.06 <sup>2</sup>
Total magnesium	366 ± 97	333 ± 96	0.01 <sup>2</sup>
Calcium-to-magnesium intake ratio	3.7 ± 0.9	3.9 ± 1.6	0.86 <sup>2</sup>
Season, %			0.82 <sup>3</sup>
Spring	16	20	
Summer	41	35	
Fall	26	27	
Winter	16	17	
Aspirin use, %	30	24	0.34 <sup>3</sup>
NSAID use, %	20	18	0.83 <sup>3</sup>
Plasma 25(OH)D, ng/mL	33.4 ± 10.2	32.0 ± 12.7	0.53 <sup>2</sup>
Plasma 25(OH)D <sub>3</sub> , ng/mL	32.3 ± 10.4	30.1 ± 11.3	0.20 <sup>2</sup>
Plasma 25(OH)D <sub>2</sub> , ng/mL	1.12 ± 2.31	2.84 ± 10.89	0.32 <sup>2</sup>
Plasma 1,25(OH) <sub>2</sub> D <sub>3</sub> , pg/mL	81.4 ± 50.9	84.2 ± 51.3	0.70 <sup>2</sup>
Plasma 24,25(OH) <sub>2</sub> D <sub>3</sub> , ng/mL	4.66 ± 3.71	3.82 ± 2.78	0.24 <sup>2</sup>

<sup>1</sup>Continuous variables are means ± SDs; categorical variables are percentages. eGFR, estimated glomerular filtration rate; NSAID, nonsteroidal anti-inflammatory drug; TRPM7, transient receptor potential cation channel, subfamily M, member 7; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; 24,25(OH)<sub>2</sub>D<sub>3</sub>, 24,25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D<sub>2</sub>, 25-hydroxyvitamin D<sub>2</sub>; 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>.

<sup>2</sup>Wilcoxon test.

<sup>3</sup>Pearson chi-square test.

understand the clinical relevance of the finding from the current study.

In this study, we found that magnesium supplementation increased plasma concentrations of 25(OH)D<sub>2</sub>. Previous studies indicate that vitamin D-specific CYP enzymes (i.e., CYP2R1, CYP27B1, and CYP24A1) are unable to differentiate vitamin D<sub>2</sub> from vitamin D<sub>3</sub> (20). On the other hand, nonvitamin D-specific enzymes (i.e., CYP3A4) may degrade 24-vitamin D<sub>2</sub> more efficiently than 24-vitamin D<sub>3</sub> in the intestine, and this may provide an explanation for the lower toxicity of vitamin D<sub>2</sub> compared with vitamin D<sub>3</sub> compounds (20). Our findings indicate that magnesium supplementation may not only accelerate the metabolism and degradation of 25(OH)D<sub>3</sub> but also shift CYP3A4

to selectively degrade vitamin D<sub>3</sub> over vitamin D<sub>2</sub> when plasma 25(OH)D is high. Thus, our findings provide the first evidence that adequate magnesium status could potentially prevent vitamin D-related adverse events. Hypomagnesemia is often concurrent with hypocalcemia in humans (18). A number of previous clinical trials conducted in adults consistently indicated that high calcium supplementation increases urinary excretion of magnesium (35–38), whereas magnesium homeostasis is mainly regulated by kidney reabsorption. Thus, individuals with high calcium-to-magnesium intake ratios in their habitual diet are at high risk of magnesium deficiency. In several epidemiologic studies, calcium-to-magnesium intake ratios between 1.7 and 2.6 were reported to be critical for calcium and magnesium intakes to be protective against colorectal cancer, mortality due to CVD, and total mortality (23–27). In the US general adult population, >76% had calcium-to-magnesium intake ratios ≥2.6 based on the NHANES 2009–2010 data. In the current randomized trial, all participants at baseline had a calcium-to-magnesium intake ratio ≥2.6. A precision-based dosing strategy of magnesium supplementation was used to reduce the calcium-to-magnesium ratios in the diet to ~2.3. Thus, it is not clear if magnesium supplementation among those with calcium-to-magnesium ratios <2.6 would show similar changes in vitamin D metabolites, or if other magnesium dosing strategies would have the same vitamin D association.

The current study has several strengths, including the randomized, placebo-controlled design. Furthermore, a precision-based design was utilized. Thus, all the background intakes of magnesium and calcium from both diet and supplements were measured 2 times before and 4 times during the treatment, and a personalized dosing strategy of magnesium supplementation was provided to each individual. We found that the calcium-to-magnesium ratios remained stable. In addition, we had a high compliance with the study medication, and the dropout rate was very low. The study does, however, have some weaknesses. The primary concern is that this is an independent ancillary study. Thus, our study may not be powerful enough to detect the interactions. We did find that 3 of the interactions were statistically significant and remained significant after Bonferroni corrections; however, we cannot eliminate the possibility that we did not have sufficient statistical power to detect the interaction between magnesium supplementation and baseline 25(OH)D on 1,25(OH)<sub>2</sub>D<sub>3</sub>. The other concern is that there were only 2 participants with baseline 25(OH)D <12 ng/mL. Thus, we did not have the power to test how magnesium supplementation affects vitamin D synthesis and metabolism among those with overt vitamin D deficiency at baseline. However, the results did not change after removing these 2 individuals with overt vitamin D deficiency at baseline. Thus, our study only provides evidence of how magnesium supplementation affects the vitamin D status and metabolism among those without overt vitamin D deficiency. We did not measure magnesium concentrations in 24-h urine samples at baseline. This may have led to an underestimation in the measurement of magnesium intake amounts. Also, our findings might be explained by the risk of bias from “regression to the mean.” However, we found clearly different patterns in the changes of concentrations of 25(OH)D<sub>3</sub>, 24,25(OH)<sub>2</sub>D<sub>3</sub>, and 25(OH)D<sub>2</sub> after administration of the magnesium treatment compared with placebo. Also, we adjusted for baseline 25(OH)D<sub>3</sub>. In addition, we found that the

correlation between baseline blood 25(OH)D<sub>3</sub> and personalized dose of magnesium was minimal (−0.003 for all participants, −0.009 in the treatment group, and 0.004 in the placebo group). The standardization to NIST was reasonable for 25(OH)D<sub>3</sub>. However, we did not have data for 24,25(OH)<sub>2</sub>D<sub>3</sub>. Thus, interpretation of the 24,25(OH)<sub>2</sub>D<sub>3</sub> results should be done with caution. However, we found the effects for magnesium supplementation on 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> were similar when the baseline 25(OH)D was >30 ng/mL. In this ancillary study, to increase the sample size and efficiency, we included participants who completed the trial but not those who enrolled and withdrew. Thus, the analyses were not carried out on an intention-to-treat sample. We cannot eliminate the possibility that the significant effect of magnesium supplementation on 25(OH)D<sub>3</sub> at 30 ng/mL might be due to chance. Finally, the baseline intake amount of magnesium was significantly higher in the magnesium treatment arm than in the placebo arm, although the baseline calcium-to-magnesium ratio intake did not differ significantly by treatment arm. In the parent study, 236 of the enrolled participants had been previously diagnosed with colorectal adenomas or hyperplastic polyps. Although their polyps and adenomas were removed when they participated in the trial, cautious interpretation of our results is warranted, particularly regarding generalization of our findings.

In summary, among individuals with calcium-to-magnesium intake ratios  $\geq 2.6$ , who account for >76% of the US general adult population, magnesium supplementation increases 25(OH)D<sub>3</sub> but not 24,25(OH)<sub>2</sub>D<sub>3</sub> when baseline 25(OH)D concentrations are <30 ng/mL, but decreases the concentrations of both in a dose-response manner when baseline 25(OH)D concentrations are higher (from 30 to 50 ng/mL). On the other hand, magnesium treatment increases 25(OH)D<sub>2</sub> as baseline 25(OH)D concentrations increase. Our findings suggest that optimal magnesium status may be important for optimizing 25(OH)D status. Further dosing studies are warranted in appropriate animal models.

The authors' responsibilities were as follows—QD and MJS: contributed to the hypothesis development and to the manuscript preparation; QD, MJS, CY, and DLS: contributed to the study design; HN and XZ: were responsible for the data analysis; QD, XL, and AAF: contributed to the assay of the vitamin D metabolites; XZ, JEM, YS, XL, AAF, RBC, AR, HN, LF, HM, RMN, DLS, CY, and MJS: contributed to the data interpretation and manuscript revision; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

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