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Magnitude nutritional investigation

Methylation treatment on methylation changes of transmembrane serine protease 2 (TMPRSS2)

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Objective: The viral entry of SARS-CoV-2 requires host-expressed TMPRSS2 to facilitate the viral spike protein priming. This study aims to test the hypothesis that magnesium (Mg) treatment leads to DNA methylation changes in TMPRSS2.

Methods: This study is nested within the Personalized Prevention of Colorectal Cancer Trial, a double-blind 2×2 factorial randomized controlled trial, which enrolled 250 participants from Vanderbilt University Medical Center.

Results: We found that 12 wk of personalized Mg treatment significantly increased 5-methylcytosine methylation at cg16371860 (TSS1500, promoter) by 7.2% compared to the placebo arm (decreased by 0.1%) in those ages < 65 y. The difference remained statistically significant after adjusting for age, sex, and baseline methylation as well as correction for false discovery rate (adjusted P = 0.014). Additionally, Mg treatment significantly reduced 5-hydroxymethylcytosine levels at cg26337277 (close proximity to TSS200 and the 5′ untranslated region, promoter) by 2.3% compared to an increase of 7.1% in the placebo arm after adjusting for covariates in those ages < 65 y (P = 0.003). The effect remained significant at a false discovery rate of 0.10 (adjusted P = 0.088).

Conclusions: Among individuals ages < 65 y with calcium-to-magnesium intake ratios equal to or over 2.6, reducing the ratio to around 2.3 increased 5-methylcytosine modifications (i.e., cg16371860) and reduced 5-hydroxymethylcytosine modifications (i.e., cg26337277) in the TMPRSS2 gene. These findings, if confirmed, provide another mechanism for the role of Mg intervention in the prevention of COVID-19 and treatment of early and mild disease by modifying the phenotype of the TMPRSS2 genotype.

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ABSTRACT

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COVID-19 have not achieved statistically significant benefits on mortality in randomized trials [2–5]. While awaiting global vaccination for SARS-CoV-2 to end the COVID-19 pandemic and confirmation that vaccination provides complete protection in adults and against multiple SARS-CoV-2 variants [6–8], the National Institutes of Health highlights an urgent need for interventions which can be administered early during the course of infection to prevent disease progression to severe COVID-19, speed recovery, and prevent long-term complications [1].

In a precision-based randomized trial [9], we reported that improved magnesium (Mg) status led to an optimal level of vitamin D. A recent trial further confirmed this finding that Mg treatment improves vitamin D status [10]. We also previously found that high circulating levels of vitamin D were prospectively related to reduced risk of cardiovascular mortality only when Mg intake was adequate [11]. Based on our findings, a recent study found that a combination treatment of Mg and vitamin D2 within the recommended dietary allowance plus vitamin B2 reduced the risk of severe illness from COVID-19 by 80% [12]. Although cross-sectional studies investigating the correlation between vitamin D levels and COVID-19 severity and mortality [13] have been inconclusive, and a large-scale prospective cohort study in the UK Biobank found that serum 25-hydroxyvitamin D (25(OH)D) was not related to risk of severe COVID-19 after adjustment for confounding factors [14]. A recent open-label trial using calcifediol (i.e., 25(OH)D) treatment starting at 64 times the recommended dietary allowance [15] substantially reduced the need for intensive care unit care among hospitalized COVID-19 patients [16]. However, in a recent randomized trial conducted among hospitalized patients with COVID-19, a single high dose of vitamin D3 did not significantly reduce hospital length of stay compared with placebo [17].

In addition to optimizing 25(OH)D levels, Mg activates the conversion from 25(OH)D to 1,25-dihydroxyvitamin D, the active form of vitamin D [18]. People with “Mg-dependent vitamin-D-resistant rickets” [19] caused by Mg deficiency were resistant to vitamin D treatment alone, with no change in blood measures of 1,25-dihydroxyvitamin D at doses up to 600 000 IU [18]. However, they dramatically responded to Mg treatment, particularly treatment of Mg plus vitamin D [18,20]. Collectively, these varied findings support the hypothesis that the synergistic interaction between Mg and vitamin D may substantially reduce the doses required for vitamin D to reduce the severity of COVID-19. Since 79% of US adults do not meet the recommended dietary allowance of Mg [21], using the combination prophylaxis strategy of vitamin D plus Mg could be a crucial strategy for treating COVID-19.

The viral entry of SARS-CoV-2 depends on binding of the viral spike proteins to cellular receptors (i.e., angiotensin-converting enzyme), which requires host-expressed transmembrane serine protease 2 (TMPRSS2) to facilitate spike protein priming [22]. In vitro studies have shown that a TMPRSS2 inhibitor partially blocked SARS-CoV-2 from entering into lung epithelial cells [22]. Animal studies have found that TMPRSS2 knockout mice infected with H1N1 influenza had minimal infection and largely attenuated disease severity [23]. Supporting the findings in mice, a recent cohort study found that TMPRSS2 expression levels and genetic variants played an essential role in modulating COVID-19 severity [24]. The effect is also likely through regulation of the coagulation cascade and arterial thrombosis [25–27], one key factor for COVID-19 severity and mortality [28,29]. TMPRSS2 expression is influenced by both genetic variants and epigenetic changes such as DNA methylation. 5-methylcytosine (5-mC), the methylation of the fifth carbon of cytosines, has been shown to be associated with transcription repression [30,31], whereas 5-hydroxymethylcytosine (5-hmC) is specifically enriched in tissue-specific enhancers [32] and critical in maintaining active gene expression [33,34]. DNA methylation changes are inducible by environmental exposure, including nutrients [35,36]. In addition to optimizing vitamin D synthesis and metabolism, Mg affects the metabolism of α-ketoglutarate [37,38], one key factor for the ten-eleven translocation (TET) enzymes [39], which catalyze the oxidation of 5-mC to 5-hmC in an active demethylation pathway [40]. We therefore aimed to test the hypothesis that Mg treatment leads to DNA methylation changes (i.e., 5-mC and 5-hmC) in TMPRSS2 in the Personalized Prevention of Colorectal Cancer Trial, a precision-based magnesium supplementation trial.

**Materials and methods**

**Patients, randomization, and blinding**

This is an ancillary study nested in a parent study, the Personalized Prevention of Colorectal Cancer Trial (PPCCT; NCT01105169 at ClinicalTrials.gov). The PPCCT is a double-blind 2 × 2 factorial randomized controlled trial conducted at Vanderbilt University Medical Center, Nashville, Tennessee. The detailed study design has been reported previously [41]. In brief, participants ages 40 to 85 y were recruited, including 236 with adenomas or hyperplastic polyps diagnosed from 1998 to 2014 and 14 polyposis-free individuals with a high risk of colorectal cancer. Dietary and total intakes of calcium (Ca) and Mg, and the Ca:Mg ratios, were derived from six 24-h dietary recalls over the course of the trial, including intakes of Ca and Mg supplements. Eligible participants were those who had a Ca intake ≥ 700 mg/d and < 2000 mg/d and in whom the Ca: Mg ratio was ≥ 2.6 based on the average of the six 24-h dietary recalls. Eligible participants were randomized to Mg glycinate or placebo (microcrystalline cellulose) capsules. The Mg treatment used a personalized dose of Mg supplementation that would reduce the Ca:Mg intake ratio to around 2.3, suggested by previous studies [5,42–44]. Participants, study investigators, and staff were blinded to the assigned interventions. Blood samples were collected and processed at each clinic visit. Anthropometric measurements (weight, height, waist and hip circumference) were measured at least twice at each clinic visit.

We randomized 265 participants and allocated them to the Mg treatment arm or the placebo arm. Fifteen participants withdrew consent before taking Mg treatment or placebo; 250 randomized participants started treatment, and 239 completed the trial, with 11 finishing only part of the study before withdrawing [9]. Six of the withdrawals were due to self-reported adverse events (four withdrawals in the treatment arm and two in the placebo arm). One of them had donated blood at baseline and at the end of the trial. Therefore, in the present study, 240 participants were included who had blood collected at baseline and at the end of the trial.

**Measurement of 5-mC and 5-hmC at single resolution for the TMPRSS2 gene**

All 240 participants who were enrolled in the PPCCT and had blood DNA samples available at baseline and the end of the trial were included in the present study to examine the effect of personalized Mg treatment on methylation modifications in the TMPRSS2 gene. In order to minimize between-batches variation, samples were randomly organized in treatment–placebo (i.e. one treatment arm with one placebo arm) sets (four samples in each set: two from before and two from after treatment). Lab staff were blinded to sample status (i.e., treatment versus placebo, pretreatment versus posttreatment).

Genomic DNA was extracted from buffy coat fractions collected using a QIAamp DNA Mini Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer’s protocol [45]. DNA quality was examined using standard molecular biology protocols. We used the TET-assisted bisulfite (TAB)-Array, which combines TET-assisted bisulfite conversion with the Infinum methylation EPIC array (EPIC array), which interrogates approximately 850 000 CpG or non-CpG methylation sites to differentiate 5-hmC and 5-mC signals at base resolution [46]. Our detailed approach has been reported previously [46–48]. Using this technique, we were able to differentiate 5-mC from 5-hmC.

The β values for 5-mC and 5-hmC were estimated using the maximum-likelihood estimate from the paired bisulfite conversion and TAB-treated samples [39]. The follow-up quality control steps were taken: We excluded low-quality probes, where the number of beads was below three or the detection P value was above 0.05 [49]. We also excluded CpGs with a detection rate < 95% and samples with a percentage of low-quality methylation measurements ≤ 5% or an extremely low signal of BS probes [49]. We then excluded extreme outliers, as defined by Tukey’s method [50], based on average total signal value across CpG probes. The remaining samples were preprocessed using the R package ENmix to improve accuracy and reproducibility [49]. Dye bias was corrected using regression on a logarithm of internal control probes [51], and quantile normalization was applied to the signal for Infinum I or II probes. Finally, the extreme outliers of β value (i.e., the proportion of methylated signal in total signals, from 0 to 1) across samples, defined by Tukey’s method, were set as missing. In total, the TAB-Array data for 224 participants out of 240 passed the seven quality-control steps. In the
Continuous demographic variables (mean ± SD) and categorical demographic variables (percentage) were compared between the treatment and placebo arms. The Wilcoxon rank-sum test was conducted to evaluate continuous variables, and Pearson χ² tests were conducted to compare categorical variables. Linear regression models were fitted to examine the effect of Mg treatment on overall changes of cytosine modifications (5-mC and 5-hmC) in the TMRPSS2 gene in three models—model 1: crude value; model 2: sex and age; model 3: sex, age, and baseline methylation. We also conducted stratified analyses by age, because older adults are at increased risk for severe illness and mortality from COVID-19 [52]. All P values are two sided, and statistical significance was determined using an α level of 0.05. The data analyses used R (version 3.5.1).

Results

The baseline demographic characteristics of the 240 participants are presented in Table 1. The treatment arm was not significantly different from the placebo arm for baseline demographic variables of age, sex, smoking status, alcohol drinking status, physical-activity status, educational achievement, race, daily intake of total energy, total Ca, intake ratio of Ca to Mg, and factors related to cardiovascular events, including body mass index body mass index, blood pressure, and estimated glomerular filtration rate.

The mean daily dose of personalized Mg supplementation was 205.59 mg, with a range from 77.25 to 389.55 mg. Adherence to the pill regimen was very high for both the placebo and treatment arms with a range from 77.25 to 389.55 mg. Adherence to the pill regimen was very high for both the placebo and treatment arms (mean (SD) based on pill counts were 96.1% (8.3%) and 95.9% (10.2%)).

Shown in Supplemental Table A.1 and A.2 are the effects of personalized Mg treatment on cytosine modifications 5-mC and 5-hmC, respectively, in TMRPSS2 for 32 CpG sites. We found that compared to the placebo arm, Mg treatment significantly increased 5-mC at the cg16371860 CpG site (P = 0.011), even after adjusting for age and sex (P = 0.010) and further adjusting for baseline 5-mC levels (P = 0.019; Table 2). However, the significance disappeared after false discovery rate (FDR) correction at 0.10 based on the Benjamini–Hochberg approach. In stratified analyses by age (Table 2), we found that personalized Mg treatment significantly increased 5-mC methylation at cg16371860 (TSS1500, promoter) by 7.2% compared to the placebo arm (decreased by 0.1%) in those ages < 65 y. The difference remained statistically significant after adjustment for age, sex, and baseline methylation as well as FDR correction (FDR-adjusted P = 0.014). The effect of Mg treatment was not significant in those ages ≥ 65 y. On the other hand, we found that Mg treatment affected 5-hmC levels at cg16371860 compared to the placebo arm after adjusting for age and sex, but the effect was not significant after further adjustment for baseline levels. In the stratified analysis by age, in those ages < 65 y the effect of Mg treatment remained after adjusting for sex, age, and baseline levels, but disappeared at an FDR of 0.10.

Additionally, we found that Mg treatment reduced 5-hmC at cg26337277 (close proximity to TSS200 and 5’ untranslated region, promoter) compared to the placebo arm after adjusting for age, sex, and baseline levels (P = 0.012). However, the significant effect disappeared at an FDR of 0.10 (FDR-adjusted P = 0.386). In the stratified analysis by age, we found that Mg treatment significantly reduced 5-hmC levels by 2.3% compared to an increase of 7.1% in the placebo arm after adjusting for covariates in those ages < 65 y (P = 0.003). The effect remained significant at an FDR of 0.10 (adjusted P = 0.088). No effect was found in those ages ≥ 65 y.

Discussion

In this personalized precision-based randomized trial, we found that 12 wk of Mg treatment significantly increased 5-mC DNA methylation at the cg16371860 CpG site and decreased 5-hmC cytosine modification at the cg26337277 CpG site in TMRPSS2 compared to placebo among participants ages < 65 y. To the best of our knowledge, no study has evaluated how to modify Mg treatment on cytosine modification in TMRPSS2.

Cg16371860 is a CpG site located at a CpG-rich island 200 to 1500 base pairs upstream of the transcriptional start site (TSS1500), and cg26337277 CpG site resides in close proximity to (0–200 base pairs upstream of) TSS200 and the 5’ untranslated region. These loci are within the promoter region of the gene, therefore potentially playing a role in transcription initiation and regulation of gene expression. Although 70% to 80% of CpG sites of the human genome are methylated to maintain a stable molecular phenotype, regions of CpG island promoters of actively transcribed genes are frequently lowly methylated [53]. Hypomethylation of the promoter regions could impede transcription activity and repress related gene expression [54]. Similarly, reduced 5-hmC levels are also associated with decreased gene expression, because conversion from 5-mC to 5-hmC is an active demethylation process [55]. Our observations that Mg treatment induced increases in 5-mC methylation at cg16371860 and decreases in 5-hmC methylation at cg26337277 CpG in the promoter indicate a hindered process of transcription initiation and, subsequently, lower levels of TMRPSS2 expression.

Given that host cell protein TMRPSS2 plays an essential role in facilitating SARS-CoV-2 entry, higher expression of TMRPSS2 may relate to
higher viral loads in the host and, subsequently, worse clinical outcomes. This has been supported by a recent finding that black people, who are disproportionately affected by severe COVID-19 [56], have significantly higher average nasal epithelial gene expression of TMPRSS2 compared with other races and ethnicities [57]. High viral loads can further induce a cytokine storm, which triggers a violent inflammatory immune response that contributes to acute respiratory distress syndrome, multiple organ failure, and finally death in severe cases of SARS-CoV-2 infection [58,59]. The association between high viral loads and fatal clinical outcomes has been observed in both human influenza A (H5N1) [60] and SARS-CoV-2 [61].

In addition to viral entry, TMPRSS2 expression levels may affect disease severity by regulating the coagulation cascade and arterial thrombosis through the protease activated receptor-signaling pathway [25–27]. This is further supported by a recent study reporting that polymorphisms near TMPRSS2 are associated with thrombocyte count [26]. Moreover, nafamostat [62], a TMPRSS2 inhibitor, has already been used in clinical practice as an effective anticoagulant, and camostat mesylate [22], a closely related compound, are undergoing clinical trials to test their utility for COVID-19 treatment, further indicating the important role of TMPRSS2 in regulating thrombosis and, in turn, disease severity. Given that nearly half of people with COVID-19 pneumonia develop thrombotic complications [29], and deceased patients are distinctively characterized with widespread vascular thrombosis [28], TMPRSS2 could be a key target for interventional strategies in reducing COVID-19 severity and mortality. Since TMPRSS2 plays a similar role during influenza infection [23], the findings from the present study suggest that Mg status may also affect influenza infection and severity.

Strikingly, TMPRSS2 was first identified as a key regulator in prostate cancer. Strongly up-regulated TMPRSS2 expression was observed in prostate cancer cell lines [63]. Consistent with racial disparities in nasal epithelial gene expression of TMPRSS2, black people also have higher incidence and mortality of prostate cancer than white people [64], further indicating that the high prostate cancer mortality among black people may be attributed to up-regulated TMPRSS2 expression. However, factors that may affect modified TMPRSS2 expression remain unknown. Previous evidence has found that black people have significantly lower levels of serum Mg or lower intake of Mg compared to white people [65], and low blood Mg levels and a high Ca:Mg ratio have been significantly associated with high-grade prostate cancer [66]. Our findings from the present study, in which Mg status was improved by modulating the Ca: Mg intake ratio to around 2.3, suggest a protective effect of Mg from severe COVID-19 in individuals ages < 65 y through regulating DNA methylation and demethylation modifications and subsequently suppressing TMPRSS2 expression.

Although no significant effect on TMPRSS2 methylation was observed in those ages ≥ 65 y, it is likely that Mg treatment may exert benefits through other mechanisms. First, vitamin D has been proposed to generate beneficial effects in acute respiratory distress syndrome through activation of the vitamin D receptor signaling pathway by reducing the cytokine storm, regulating the renin-angiotensin system, maintaining the integrity of the pulmonary epithelial barrier, and tapering down the increased coagulability [16]. Our and other studies have found that Mg optimized body vitamin D status and substantially reduced the dose requirement for vitamin D [9,19,67]. In a previous publication, we reported baseline and end-of-study levels of circulating vitamin D in a subgroup of 180 participants within the PPCCT suggesting that the effect of Mg treatment on plasma concentrations of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, and 24,25-hydroxyvitamin D_3 were significantly different depending on the baseline concentrations of 25-hydroxyvitamin D [9]. Second, Mg deficiency is a prevailing yet underrecognized driver for increased risks of
cardiometabolic diseases including diabetes [68], hypertension, coronary heart disease, heart failure, and thrombosis [69]. Previous studies have found that low Mg plays an essential role in promoting endothelial cell dysfunction and generating a proinflammatory, prothrombotic, and proatherogenic environment that could contribute to the pathogenesis of cardiovascular diseases and severe COVID-19 [70]. Third, Mg deficiency caused by a gene mutation has been shown to lead to reduced cytotoxic activity of T cells and increased viral load, but Mg treatment reduced B cells positive for Epstein–Barr virus, indicating that Mg is critical in antivirus immunity [71]. Finally, a study found that people with COVID-19 who carry the apolipoprotein E (ApoE) genotype were at a 2.3-fold increased risk of severe COVID-19 [72]. In addition to being a major risk factor for dementia [73], the ApoE genotype is strongly linked to lower human longevity [74], a genetic mark of early aging, and is linked to lower plasma ApoE [75]. We previously reported from a randomized trial that in those ages ≥ 65 y, Mg treatment improved cognitive function, particularly among older participants, via demethylation in the ApoE gene, which is expected to result in increased ApoE levels [48]. This study has several strengths, including the double-blinded randomized trial design. Furthermore, a precision-based design was used. Intakes of Mg and Ca from both diet and supplements were measured twice before and four times during the treatment, and a personalized dosing strategy of Mg supplementation was followed for each participant. The Ca:Mg ratios remained stable over the 12-wk study period. In addition, we had high adherence to the study medication, and the dropout rate was very low. The study has some weaknesses, though. The primary concern is that TMPRSS2 expression was not measured in the PP CCT, and thus the association between DNA methylation changes and level of TMPRSS2 expression and phenotype patterns is not confirmed. However, the observed DNA methylation changes were internally consistent, with increased 5-mC and reduced 5-hmc indicating a reduced level of TMPRSS2 expression.

Conclusions

In summary, in individuals ages < 65 y with Ca:Mg intake ratios ≥ 2.6, reducing Ca:Mg ratios to around 2.3 increased 5-mC modifications (i.e., cg16371860) and reduced 5-hmc modifications (i.e., cg26337277) in the TMPRSS2 gene. The National Institutes of Health has recently highlighted the crucial need for early intervention to reduce the likelihood of developing severe outcomes and reduce demand on the health care system [1]. These findings, if confirmed, provide another mechanism for the role of Mg intervention in the prevention of COVID-19 and treatment of early and mild disease by modifying the phenotype of the TMPRSS2 genotype in addition to affecting ApoE methylation in older people [48] and optimizing levels of vitamin D [9]. In addition, these findings indicate a possible mechanism of Mg in prostate cancer and influenza due to the critical role of the TMPRSS2 gene in those conditions.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2021.111340.

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