
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Nutrition 89 (2021) 111340

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Applied nutritional investigation

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

Lei Fan Ph.D. [a], Xiangzhu Zhu M.D. [a], Yinan Zheng Ph.D. [b], Wei Zhang Ph.D. [b], Douglas L. Seidner M.D. [c], Reid Ness M.D. [d], Harvey J. Murff M.D. [e], Chang Yu Ph.D. [f], Xiang Huang Ph.D. [a], Martha J. Shrubsole Ph.D. [a], Lifang Hou Ph.D. [b], Qi Dai Ph.D. [a,*]

[a] Department of Medicine, Division of Epidemiology, Vanderbilt Epidemiology Center, Vanderbilt University School of Medicine, Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, Tennessee, USA
[b] Department of Preventive Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA
[c] Center for Human Nutrition, Department of Gastroenterology, Hepatology and Nutrition, Digestive Disease and Surgical Institute, Cleveland Clinic, Cleveland, Ohio, USA
[d] Department of Medicine, Division of Gastroenterology, Vanderbilt School of Medicine, Nashville, Tennessee, USA
[e] Division of Geriatric Medicine, General Internal Medicine and Public Health, Vanderbilt University School of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA
[f] Department of Biostatistics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

ARTICLE INFO

*Article History:*
Received 4 December 2020
Received in revised form 22 April 2021
Accepted 26 April 2021

*Keywords:*
Magnesium
TMPRSS2
Methylation changes
COVID-19
SARS-CoV-2

ABSTRACT

*Objectives:* 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.


## Introduction

As of December 4, 2020, in the United States alone, coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), had led to 14.2 million confirmed cases and 276 000 deaths. Up to 20% of symptomatic individuals will progress to severe or critical illness, ranging from hospitalization to death, whereas some with mild symptoms may also experience a variably prolonged period of recovery with long-term complications [1]. Current antiviral pharmaceutical therapeutics, such as remdesivir, targeting hospitalized patients with

This study was supported by R01 CA149633 (to Qi Dai and Chang Yu) and R01 CA202936 (to Qi Dai and Lifang Hou) from the National Cancer Institute, Department of Health and Human Services, as well as the Ingram Cancer Center Endowment Fund. Data collection, sample storage, and processing were partially conducted by the Survey and Biospecimen Shared Resource, which is supported in part by P30CA68485. Clinical visits to the Vanderbilt 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). All authors have no conflicts of interest. The Personalized Prevention of Colorectal Cancer Trial (PPCCT) was registered at clinicaltrials.gov as NCT01105169.
*Corresponding author: Tel.: +1 (615) 936-0707.
*E-mail address:* qi.dai@vanderbilt.edu (Q. Dai).

https://doi.org/10.1016/j.nut.2021.111340
0899-9007/

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 $D_3$ within the recommended dietary allowance plus vitamin $B_{12}$ 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 $D_3$ 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

### *Participants, 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 $2 \times 2$ factorial randomized controlled trial conducted at Vanderbilt University Medical Center, Nashville, Tennessee. The detailed study design has been reported previously [9,41]. In brief, participants ages 40 to 85 y were recruited, including 236 with adenomas or hyperplastic polyps diagnosed from 1998 to 2014 and 14 polyp-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 $\geq$ 700 mg/d and $<$ 2000 mg/d and in whom the Ca:Mg intake ratio was $\geq$ 2.6 based on the average of the first two baseline 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 [9,42–44]. 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 Infinium 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 following 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 Infinium 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

present study, we kept all 32 CpG sites related to the *TMPRSS2* gene selected by the EPIC array for the 224 participants whose methylation data passed the quality-control steps.

*Statistical analyses*

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 $\chi^2$ 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 *TMPRSS2* 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 $\alpha$ level of 0.05. The data analyses used R software (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 (mean (SD) based on pill counts were 96.1% (8.3%) and 95.9% (10.2%); $P = 0.37$ for the difference between the arms). The mean (SD) Ca:Mg ratios for the treatment and placebo arms after Mg and placebo supplementation were, respectively, 2.27 (0.13) and 3.87 (1.46; *P* for difference $< 0.001$), based on the two 24-h dietary recalls performed at baseline, and remained stable at 2.13 (0.68) and 3.51 (1.32), respectively (*P* for difference $< 0.001$) based on the four 24-h dietary recalls conducted over the 12-wk period of the trial.

**Table 1**
Descriptive characteristics of treatment vs. placebo at baseline

| Characteristic | Placebo ($n = 120$) | Treatment ($n = 120$) | *P* |
|---|---|---|---|
| Age, y | 61.3 ± 8.2 | 60.3 ± 7.7 | 0.48* |
| Sex (male/female) | 51.7/48.3 | 54.2/45.8 | 0.70† |
| BMI, kg/m$^2$ | 30.6 ± 6.6 | 29.9 ± 6.1 | 0.48* |
| Systolic blood pressure, mm Hg | 128.3 ± 14.2 | 126.3 ± 15.4 | 0.31 |
| Diastolic blood pressure, mm Hg | 74.7 ± 9.2 | 75.3 ± 8.2 | 0.65 |
| eGFR, mL/min/1.73 m$^2$ | 78.9 ± 14.9 | 81.7 ± 13.8 | 0.13* |
| Smoking status | | | 0.27† |
| Never | 49.6 | 60.0 | |
| Ever | 42.0 | 33.3 | |
| Current | 8.4 | 6.7 | |
| Drinking status | | | 0.24† |
| Never | 32.5 | 42.5 | |
| Ever | 20.8 | 20.0 | |
| Current | 46.7 | 37.5 | |
| Physically active ≥ 2 d/wk | 77.5 | 85.0 | 0.14† |
| Education under college | 9.99 | 10.0 | 0.41† |
| Race (white/non-white) | 99.2/0.8 | 98.3/1.7 | 0.56† |
| Daily nutrient intake | | | |
| Total energy, kcal | 2108.2 ± 604.5 | 2084.3 ± 547.0 | 0.62* |
| Total Ca, mg | 1251.0 ± 358.6 | 1299.8 ± 332.0 | 0.20* |
| Total Mg, mg | 337.7 ± 98.7 | 363.8 ± 96.7 | 0.03* |
| Ca:Mg intake ratio | 3.9 ± 1.5 | 3.7 ± 0.9 | 0.32* |

BMI, body mass index; Ca, calcium; eGFR, estimated glomerular filtration rate; Mg, magnesium
All values are expressed as mean ± SD (continuous) or percentage (categorical)
*Wilcoxon test.
†Pearson's $\chi^2$ test.

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 *TMPRSS2* 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 *TMPRSS2* compared to placebo among participants ages < 65 y. To the best of our knowledge, no study has evaluated how to modify *TMPRSS2* cytosine modification, nor examined the effect of Mg treatment on cytosine modification in *TMPRSS2*.

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]. Hypermethylation 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 *TMPRSS2* expression.

Given that host cell protein TMPRSS2 plays an essential role in facilitating SARS-CoV-2 entry, higher expression of *TMPRSS2* may relate to

**Table 2**
Changes in cytosine modifications (CpG sites) in *TMPRSS2* by magnesium treatment versus placebo stratified by age

| CpG site | | | Change from baseline | | | | | | *P1* | *P2* | *P3* | FDR1 | FDR2 | FDR3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Placebo | | | Treatment | | | | | | | | |
| | | | Mean ± SD | Median (25%, 75%) | % change in mean | Mean ± SD | Median (25%, 75%) | % change in mean | | | | | | |
| cg16371860 | 5-mC | Overall | −0.001 ± 0.025 | −0.003 (−0.014, 0.014) | 0.2 | 0.007 ± 0.024 | 0.006 (−0.006, 0.02) | 4.5 | 0.011 | 0.010 | 0.019 | 0.121 | 0.124 | 0.271 |
| | | Ages < 65 y | −0.002 ± 0.022 | −0.002 (−0.012, 0.013) | −0.1 | 0.012 ± 0.022 | 0.011 (−0.001, 0.022) | 7.2 | 0.000 | 0.000 | 0.000 | 0.007 | 0.007 | 0.014 |
| | | Ages ≥ 65 y | −0.001 ± 0.031 | −0.003 (−0.020, 0.015) | 0.7 | −0.004 ± 0.025 | −0.004 (−0.017, 0.008) | −1.6 | 0.650 | 0.735 | 0.633 | 0.951 | 0.930 | 0.981 |
| | 5-hmC | Overall | 0.000 ± 0.002 | 0.000 (−0.001, 0.002) | 10.8 | −0.001 ± 0.002 | −0.001 (−0.001, 0.001) | −1.4 | 0.001 | 0.003 | 0.071 | 0.037 | 0.049 | 0.849 |
| | | Ages < 65 y | 0.000 ± 0.002 | 0.000 (−0.001, 0.002) | 10.9 | −0.001 ± 0.002 | −0.001 (−0.001, 0.001) | −3.7 | 0.002 | 0.002 | 0.014 | 0.044 | 0.047 | 0.218 |
| | | Ages ≥ 65 y | 0.000 ± 0.002 | 0.001 (−0.001, 0.002) | 10.5 | −0.000 ± 0.002 | −0.000 (−0.001, 0.001) | 3.8 | 0.325 | 0.488 | 0.423 | 0.743 | 0.958 | 0.883 |
| cg26337277 | 5-mC | Overall | 0.000 ± 0.002 | 0.000 (−0.001, 0.000) | N/A | 0.000 ± 0.001 | 0.000 (0.000, 0.000) | N/A | 0.759 | 0.827 | 0.325 | 0.899 | 0.913 | 0.743 |
| | | Aged < 65 | −0.000 ± 0.002 | 0.000 (−0.001, 0.000) | N/A | −0.000 ± 0.002 | 0.000 (−0.000, 0.000) | N/A | 0.678 | 0.676 | 0.991 | 0.868 | 0.865 | 0.991 |
| | | Aged ≥ 65 | 0.000 ± 0.002 | 0.000 (−0.001, 0.001) | N/A | 0.000 ± 0.001 | 0.000 (0.000, 0.000) | N/A | 0.275 | 0.310 | 0.143 | 0.874 | 0.919 | 0.943 |
| | 5-hmC | Overall | 0.000 ± 0.001 | 0.000 (−0.001, 0.001) | 6.1 | −0.000 ± 0.001 | 0.000 (−0.001, 0.000) | −1.8 | 0.002 | 0.003 | 0.012 | 0.037 | 0.049 | 0.386 |
| | | Aged < 65 | 0.000 ± 0.001 | 0.000 (−0.001, 0.001) | 7.1 | −0.000 ± 0.001 | 0.000 (−0.001, 0.000) | −2.3 | 0.003 | 0.003 | 0.003 | 0.044 | 0.047 | 0.088 |
| | | Aged ≥ 65 | 0.000 ± 0.001 | 0.000 (−0.000, 0.001) | 3.9 | −0.000 ± 0.001 | 0.000 (−0.001, 0.000) | −0.6 | 0.302 | 0.358 | 0.979 | 0.743 | 0.969 | 0.844 |

5-hmC, 5-hydroxymethylcytosine; 5-mC, 5-methylcytosine; FDR, false discovery rate (0.10); Mg, magnesium; NA, % change in mean was not available due to extremely low levels of pretreatment methylation; *P1*, *P* value for crude general linear model; *P2*, *P* value for general linear model adjusted for age and sex; *P3*, *P* value for general linear model adjusted for age, sex, and baseline methylation

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 $D_3$, 25-hydroxyvitamin $D_2$, 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 PPCCT, 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.

## References


[1] Kim PS, Read SW, Fauci AS. Therapy for early COVID-19: a critical need. JAMA 2020;324:2149–50.

[2] Wang Y, Zhang D, Du G, Du R, Zhao J, Jin Y, et al. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. Lancet 2020;395:1569–78.

[3] Goldman JD, Lye DCB, Hui DS, Marks KM, Bruno R, Montejano R, et al. Remdesivir for 5 or 10 days in patients with severe Covid-19. N Engl J Med 2020;383:1827–37.

[4] Wilkinson E. RECOVERY trial: the UK covid-19 study resetting expectations for clinical trials. BMJ 2020;369:m1626.

[5] Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the treatment of Covid-19—final report. N Engl J Med 2020;383:1813–26.

[6] Rondy M, El Omeiri N, Thompson MG, Levêque A, Moren A, Sullivan SG. Effectiveness of influenza vaccines in preventing severe influenza illness among adults: a systematic review and meta-analysis of test-negative design case-control studies. J Infect 2017;75:381–94.

[7] Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, et al. Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell 2020;182. 812–27.e19.

[8] Li Q, Wu J, Nie J, Zhang L, Hao H, Liu S, et al. The impact of mutations in SARS-CoV-2 spike on viral infectivity and antigenicity. Cell 2020;182. 1284–94.e9.

[9] Dai Q, Zhu X, Manson JE, Song Y, Li X, Franke AA, et al. Magnesium status and supplementation influence vitamin D status and metabolism: results from a randomized trial. Am J Clin Nutr 2018;108:1249–58.

[10] Vázquez-Lorente H, Herrera-Quintana L, Molina-López J, Gamarra-Morales Y, López-González B, Miralles-Adell C, et al. Response of vitamin D after magnesium intervention in a postmenopausal population from the province of Granada, Spain. Nutrients 2020;12:2283.

[11] Deng X, Song Y, Manson JE, Signorello LB, Zhang SM, Shrubsole MJ, et al. Magnesium, vitamin D status and mortality: results from US National Health and Nutrition Examination Survey (NHANES) 2001 to 2006 and NHANES III. BMC Med 2013;11:187.

[12] Tan CW, Ho LP, Kalimuddin S, Cherng BPZ, Teh YE, Thien SY, et al. Cohort study to evaluate the effect of vitamin D, magnesium, and vitamin B12 in combination on progression to severe outcomes in older patients with coronavirus (COVID-19). Nutrition 2020;79–80:111017.

[13] Ali N. Role of vitamin D in preventing of COVID-19 infection, progression and severity. J Infect Public Health 2020;13:1373–80.

[14] Hastie CE, Mackay DF, Ho F, Celis-Morales CA, Katikireddi SV, Niedzwiedz CL, et al. Vitamin D concentrations and COVID-19 infection in UK Biobank. Diabetes Metab Syndr Clin Res Rev 2020;14:561–5.

[15] Rizzoli R. Vitamin D supplementation: upper limit for safety revisited? Aging Clin Exp Res 2021;33:19–24.

[16] Entrenas Castillo M, Entrenas Costa LM, Vaquero Barrios JM, Alcalá Díaz JF, López Miranda J, Bouillon R, et al. Effect of calcifediol treatment and best available therapy versus best available therapy on intensive care unit admission and mortality among patients hospitalized for COVID-19: a pilot randomized clinical study. J Steroid Biochem Mol Biol 2020;203:105751.

[17] Murai IH, Fernandes AL, Sales LP, Pinto AJ, Goessler KF, Duran CSC, et al. Effect of a single high dose of vitamin D3 on hospital length of stay in patients with moderate to severe COVID-19: a randomized clinical trial. JAMA 2021;325:1053–60.

[18] Reddy V, Sivakumar B. Magnesium-dependent vitamin-D-resistant rickets. Lancet 1974;1:963–5.

[19] Rude RK, Adams JS, Ryzen E, Endres DB, Niimi H, Horst RL, et al. Low serum concentrations of 1,25-dihydroxyvitamin D in human magnesium deficiency. J Clin Endocrinol Metab 1985;61:933–40.

[20] Fuss M, Bergmann P, Bergans A, Bagon J, Cogan E, Pepersack T, et al. Correction of low circulating levels of 1,25-dihydroxyvitamin D by 25-hydroxyvitamin D during reversal of hypomagnesaemia. Clin Endocrinol (Oxf) 1989;31:31–8.

[21] Ervin RB, Wang CY, Wright JD, Kennedy-Stephenson J. Dietary intake of selected minerals for the United States population: 1999-2000. Adv Data. 2004;(341):1-5.

[22] Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell 2020;181. 271–80.e8.

[23] Hatesuer B, Bertram S, Mehnert N, Bahgat MM, Nelson PS, Pöhlmann S, et al. TMPRSS2 is essential for influenza H1N1 virus pathogenesis in mice. PLoS Pathog 2013;9:e1003774.

[24] Asselta R, Paraboschi EM, Mantovani A, Duga S. ACE2 and TMPRSS2 variants and expression as candidates to sex and country differences in COVID-19 severity in Italy. Aging (Albany NY) 2020;12:10087–98.

[25] Isermann B. Homeostatic effects of coagulation protease-dependent signaling and protease activated receptors. J Thromb Haemost 2017;15:1273–84.

[26] Lopera Maya EA, van der Graaf A, Lanting P, van der Geest M, Fu J, Swertz M, et al. Lack of association between genetic variants at *ACE2* and *TMPRSS2* genes involved in SARS-CoV-2 infection and human quantitative phenotypes. Front Genet 2020;11:613.

[27] Sidhu TS, French SL, Hamilton JR. Differential signaling by protease-activated receptors: implications for therapeutic targeting. Int J Mol Sci 2014;15:6169–83.

[28] Ackermann M, Verleden SE, Kuehnel M, Haverich A, Welte T, Laenger F, et al. Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in Covid-19. N Engl J Med 2020;383:120–8.

[29] Klok FA, Kruip MJHA, van der Meer NJM, Arbous MS, Gommers D, Kant KM, et al. Confirmation of the high cumulative incidence of thrombotic complications in critically ill ICU patients with COVID-19: an updated analysis. Thromb Res 2020;191:148–50.

[30] Dunn BK. Hypomethylation: one side of a larger picture. Ann N Y Acad Sci 2003;983:28–42.
[31] Robertson KD, Wolffe AP. DNA methylation in health and disease. Nat Rev Genet 2000;1:11–9.
[32] Wei J, Beadell A, Zhang Z, Sharma G, Talwar R, Arensdorf P, et al. A human tissue map of 5-hydroxymethylcytosines exhibits tissue specificity through gene and enhancer modulation. Nat Commun 2020;11:6161.
[33] Jin S-G, Wu X, Li AX, Pfeifer GP. Genomic mapping of 5-hydroxymethylcytosine in the human brain. Nucleic Acids Res 2011;39:5015–24.
[34] Mariani CJ, Madzo J, Moen EL, Yesilkanal A, Godley LA. Alterations of 5-hydroxymethylcytosine in human cancers. Cancers (Basel) 2013;5:786–814.
[35] Bishop KS, Ferguson LR. The interaction between epigenetics, nutrition and the development of cancer. Nutrients 2015;7:922–47.
[36] Ryu M-S, Langkamp-Henken B, Chang S-M, Shankar MN, Cousins RJ. Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis. Proc Natl Acad Sci USA 2011;108:20970–5.
[37] Mithieux G, Vega FV, Riou JP. The liver glucose-6-phosphatase of intact microsomes is inhibited and displays sigmoid kinetics in the presence of alpha-ketoglutarate-magnesium and oxaloacetate-magnesium chelates. J Biol Chem 1990;265:20364–8.
[38] Panov A, Scarpa A. Independent modulation of the activity of alpha-ketoglutarate dehydrogenase complex by Ca2+ and Mg2+. Biochemistry 1996;35:427–32.
[39] Lu X, Zhao BS, He C. TET family proteins: oxidation activity, interacting molecules, and functions in diseases. Chem Rev 2015;115:2225–39.
[40] Klungland A, Robertson AB. Oxidized C5-methyl cytosine bases in DNA: 5-hydroxymethylcytosine; 5-formylcytosine; and 5-carboxycytosine. Free Radic Biol Med 2017;107:62–8.
[41] Liu S, Liu Q. Personalized magnesium intervention to improve vitamin D metabolism: applying a systems approach for precision nutrition in large randomized trials of diverse populations. Am J Clin Nutr 2018;108:1159–61.
[42] Rosanoff A, Dai Q, Shapses SA. Essential nutrient interactions: does low or suboptimal magnesium status interact with vitamin D and/or calcium status? Adv Nutr 2016;7:25–43.
[43] Dai Q, Shu X-O, Deng X, Xiang Y-B, Li H, Yang G, et al. Modifying effect of calcium/magnesium intake ratio and mortality: a population-based cohort study. BMJ Open 2013;3:e002111.
[44] Zhao J, Giri A, Zhu X, Shrubsole MJ, Jiang Y, Guo X, et al. Calcium:magnesium intake ratio and colorectal carcinogenesis, results from the prostate, lung, colorectal, and ovarian cancer screening trial. Br J Cancer 2019;121:796–804.
[45] Dai Q, Shrubsole MJ, Ness RM, Schlundt D, Cai Q, Smalley WE, et al. The relation of magnesium and calcium intakes and a genetic polymorphism in the magnesium transporter to colorectal neoplasia risk. Am J Clin Nutr 2007;86:743–51.
[46] Nazor KL, Boland MJ, Bibikova M, Klotzle B, Yu M, Glenn-Pratola VL, et al. Application of a low cost array-based technique—TAB-Array—for quantifying and mapping both 5mC and 5hmC at single base resolution in human pluripotent stem cells. Genomics 2014;104:358–67.
[47] Zeng C, Zhang Z, Wang J, Chiu BC-H, Hou L, Zhang W. Application of the high-throughput TAB-Array for the discovery of novel 5-hydroxymethylcytosine biomarkers in pancreatic ductal adenocarcinoma. Epigenomes 2019;3:16.
[48] Zhu X, Borenstein AR, Zheng Y, Zhang W, Seidner DL, Ness R, et al. Ca:Mg ratio, APOE cytosine modifications, and cognitive function: results from a randomized trial. J Alzheimers Dis 2020;75:85–98.
[49] Xu Z, Niu L, Li L, Taylor JA. ENmix: a novel background correction method for Illumina HumanMethylation450 BeadChip. Nucleic Acids Res 2016;44:e20.
[50] Tukey J W. Exploratory data analysis 1977;2:131–60.
[51] Xu Z, Langie SAS, De Boever P, Taylor JA, Niu L. RELIC: a novel dye-bias correction method for Illumina Methylation BeadChip. BMC Genomics 2017;18:4.
[52] Sanyaolu A, Okorie C, Marinkovic A, Patidar R, Younis K, Desai P, et al. Comorbidity and its impact on patients with COVID-19. SN Compr Clin Med 2020;Jun 25:1–8.
[53] Greenberg MVC, Bourc'his D. The diverse roles of DNA methylation in mammalian development and disease. Nat Rev Mol Cell Biol 2019;20:590–607.
[54] Abdolmaleky HM, Smith CL, Faraone SV, Shafa R, Stone W, Glatt SJ, et al. Methylomics in psychiatry: modulation of gene-environment interactions may be through DNA methylation. Am J Med Genet B Neuropsychiatr Genet 2004;127B:51–9.
[55] Rodríguez-Aguilera JR, Ecsedi S, Goldsmith C, Cros M-P, Domínguez-López M, Guerrero-Celis N, et al. Genome-wide 5-hydroxymethylcytosine (5hmC) emerges at early stage of in vitro differentiation of a putative hepatocyte progenitor. Sci Rep 2020;10:7822.
[56] Carethers JM. Insights into disparities observed with COVID-19. J Intern Med 2020;289:463–73.
[57] Bunyavanich S, Grant C, Vicencio A. Racial/ethnic variation in nasal gene expression of transmembrane serine protease 2 (TMPRSS2). JAMA 2020;324:1567–8.
[58] Nile SH, Nile A, Qiu J, Li L, Jia X, Kai G. COVID-19: pathogenesis, cytokine storm and therapeutic potential of interferons. Cytokine Growth Factor Rev 2020;53:66–70.
[59] Li X, Geng M, Peng Y, Meng L, Lu S. Molecular immune pathogenesis and diagnosis of COVID-19. J Pharm Anal 2020;10:102–8.
[60] de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJD, Chau TNB, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 2006;12:1203–7.
[61] Magleby R, Westblade LF, Trzebucki A, Simon MS, Rajan M, Park J, et al. Impact of SARS-CoV-2 viral load on risk of intubation and mortality among hospitalized patients with coronavirus disease 2019. Clin Infect Dis 2020;Jun 30: ciaa851.
[62] Choi J-Y, Kang Y-J, Jang HM, Jung H-Y, Cho J-H, Park S-H, et al. Nafamostat mesilate as an anticoagulant during continuous renal replacement therapy in patients with high bleeding risk: a randomized clinical trial. Medicine (Baltimore) 2015;94:e2392.
[63] Stopsack KH, Mucci LA, Antonarakis ES, Nelson PS, Kantoff PW. TMPRSS2 and COVID-19: serendipity or opportunity for intervention? Cancer Discov 2020;10:779–82.
[64] Mahal BA, Berman RA, Taplin M-E, Huang FW. Prostate cancer-specific mortality across Gleason scores in black vs nonblack men. JAMA 2018;320:2479–81.
[65] Fowke JH, Koyama T, Dai Q, Zheng SL, Xu J, Howard LE, et al. Blood and dietary magnesium levels are not linked with lower prostate cancer risk in black or white men. Cancer Lett 2019;449:99–105.
[66] Dai Q, Motley SS, Smith JA, Concepcion R, Barocas D, Byerly S, et al. Blood magnesium, and the interaction with calcium, on the risk of high-grade prostate cancer. PLoS One 2011;6:e18237.
[67] Cooper ID, Crofts CAP, DiNicolantonio JJ, Malhotra A, Elliott B, Kyriakidou Y, et al. Relationships between hyperinsulinaemia, magnesium, vitamin D, thrombosis and COVID-19: rationale for clinical management. Open Heart 2020;7:e001356.
[68] Lima M de L, Pousada J, Barbosa C, Cruz T. [Magnesium deficiency and insulin resistance in patients with type 2 diabetes mellitus]. Arq Bras Endocrinol Metabol 2005;49:959–63. [in Portuguese].
[69] DiNicolantonio JJ, O'Keefe JH, Wilson W. Subclinical magnesium deficiency: a principal driver of cardiovascular disease and a public health crisis. Open Heart 2018;5:e000668.
[70] Maier JAM, Malpuech-Brugère C, Zimowska W, Rayssiguier Y, Mazur A. Low magnesium promotes endothelial cell dysfunction: implications for atherosclerosis, inflammation and thrombosis. Biochim Biophys Acta 2004;1689: 13–21.
[71] Minton K. Immunodeficiency: magnesium regulates antiviral immunity. Nat Rev Immunol 2013;13:548–9.
[72] Kuo C-L, Pilling LC, Atkins JL, Masoli JAH, Delgado J, Kuchel GA, et al. APOE e4 genotype predicts severe COVID-19 in the UK Biobank community cohort. J Gerontol A Biol Sci Med Sci 2020;75:2231–2.
[73] Michaelson DM. APOE ε4: the most prevalent yet understudied risk factor for Alzheimer's disease. Alzheimers Dement 2014;10:861–8.
[74] Sebastiani P, Gurinovich A, Nygaard M, Sasaki T, Sweigart B, Bae H, et al. APOE alleles and extreme human longevity. J Gerontol A Biol Sci Med Sci 2019;74:44–51.
[75] Rasmussen KL. Plasma levels of apolipoprotein E, APOE genotype and risk of dementia and ischemic heart disease: a review. Atherosclerosis 2016;255:145–55.