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Abstract. Background: Primary findings from a recent study reported that magnesium supplementation significantly reduced stress in severely stressed subjects with low magnesemia, and additional vitamin B6 enhanced this effect. The mechanism by which combining magnesium and vitamin B6 leads to reduced stress in these subjects remains to be elucidated. This secondary analysis investigated the impact of magnesium and vitamin B6 supplementation and perceived stress on erythrocyte magnesium levels, as a marker of body magnesium status. **Methods**: This was a secondary analysis from an 8-week randomized controlled trial comparing oral magnesium (300 mg) and magnesium-vitamin B6 (300 mg + 30 mg) supplementation. Stress level and erythrocyte magnesium level at baseline, and change in erythrocyte magnesium and serum vitamin B6 levels at weeks 4 and 8, were analyzed. **Results**: Overall, 264 subjects were randomized to treatment and had evaluable Depression Anxiety Stress Scale scores (132 in each treatment arm). At baseline, stress scores, and mean serum magnesium, erythrocyte magnesium, and serum vitamin B6 concentrations were similar between arms. Although not significant between groups, a significant increase over time in erythrocyte magnesium levels was observed in the subgroup of subjects with low baseline erythrocyte magnesium levels (<1.6 mmol/L) following treatment with magnesium and magnesium-vitamin B6 (week 4:0.21 mmol/L [95% confidence interval (CI), 0.10 to 0.31], p = 0.0003; and 0.13 mmol/L [95% CI, 0.02 to 0.23], p = 0.0233, respectively). Change from baseline in circulating vitamin B6 levels at weeks 4 and 8 in the magnesium-vitamin B6 supplemented group (314.96 nmol/L [95%CI, 294.61 to 335.31]) was significantly different (p < 0.0001) compared with the magnesium supplemented group (-0.39 nmol/L [95% CI, -20.73 to 19.94]). **Conclusion**: Magnesium alone and magnesium-vitamin B6 provided statistically significant increases in erythrocyte magnesium in subjects with low magnesium status

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(<1.6mmol/L). Vitamin B6 supplementation did not further increase magnesium levels.

Key words: biomarkers of magnesium status, randomized controlled trial, magnesium supplementation, vitamin B6 supplementation, perceived stress

Physiological stress, both mental and physical, has been cautiously linked to low magnesium levels. Hormones, such as catecholamines and corticosteroids, released during times of stress, increase the movement of magnesium out of cells. This leads to increased excretion of magnesium from the body, and in turn, a decrease in serum magnesium levels [1, 2]. Low magnesium status can reduce the body's resistance to stress by increasing secretion of stress-associated hormones, such as cortisol, which can in turn cause magnesium levels to decrease more, further decreasing the resistance to stress, creating a vicious circle [3].

As the second most abundant intracellular cation in the body [4, 5], magnesium is required for a variety of fundamental biochemical reactions and cellular functions [6], and plays an essential role in many biological processes, including being involved in over 300 enzymatic reactions [4]. In addition, magnesium regulates a variety of essential physiological functions, including neuromuscular, cardiovascular, immune and hormonal functions, and plays a role in maintaining cellular membrane stability [7, 8]. In healthy subjects, a balance exists between intestinal magnesium absorption and urinary excretion, which increases when there is an excess of circulating magnesium [9, 10]. Thus, the movement of magnesium out of cells during episodes of stress can lead to magnesium loss [1, 2], which can result in magnesium deficiency [1].

Vitamin B6 has a variety of important roles in the body, including serving as a cofactor for over 100 enzymes involved in amino acid metabolism, one-carbon reactions, glycogenolysis and gluconeogenesis, heme synthesis, niacin formation, lipid metabolism, neurotransmitter synthesis, and hormone action [11]. No trials to date have evaluated the direct effect of vitamin B6 on stress levels. However, findings from a number of preclinical studies have indicated that vitamin B6 may possess stress-reducing properties, including modulating neurotransmitters associated with depression and anxiety, reducing blood pressure, and reducing the physiological impact of corticosteroid secretion [12].

In addition to the possible effects that vitamin B6 could be having on stress, it has been proposed that vitamin B6 facilitates cellular uptake of magnesium [13, 14], limiting its excretion and optimizing its effectiveness, given that magnesium is primarily an intracellular cation. In the primary findings from a recently published study by Pouteau *et al.* [15], magnesium supplementation was reported to reduce psychological stress, measured using the Depression Anxiety Stress Scale (DASS-42) which assesses negative emotional states of depression, anxiety, and tension/stress, in populations with low serum magnesium levels. Furthermore, the addition of vitamin B6 to magnesium was shown to enhance this positive treatment effect in severely stressed subjects [15].

With this in mind, this manuscript outlines the findings of secondary and exploratory analyses of the trial by Pouteau *et al.* [15]. The objective of the analyses was to compare the effect of the oral magnesium-vitamin B6 combination with that of magnesium alone on magnesium levels in erythrocytes, as a biomarker for body magnesium status, and serum vitamin B6 levels, and to determine if baseline stress levels, as measured by the DASS-42, have any impact on these findings.

Methods

Trial design

This 8-week, Phase IV, randomized, controlled, investigator-blinded, parallel-group trial was originally designed to compare an oral magnesium-vitamin B6 combination with magnesium alone in stressed healthy adults with suboptimal serum magnesium levels. The full details of this trial (EudraCT Number: 2015-003749-24), with a focus on the primary and key secondary endpoints, have been recently published [15]. Thus, only an overview of the trial methodology

is provided here, as far as is relevant for the objectives of this secondary post-hoc analysis.

In this trial, subjects were randomized 1:1 to treatment with either the oral magnesium-vitamin B6 combination (300 mg as magnesium lactate dihydrate and 30 mg daily, respectively) or magnesium alone (300 mg daily as magnesium lactate dihydrate). Randomization was stratified by sex and performed via an interactive web response system. Investigators remained blinded with regard to the assigned study treatment until the database lock.

Standard protocol approvals, registration, and subject consent

This trial was carried out in compliance with the recommendations of the Declaration of Helsinki and the International Conference of Harmonisation (ICH) guidelines for Good Clinical Practice (GCP), all applicable laws, rules, and regulations. The protocol also complied with the laws and regulations, as well as any applicable guidelines, from France, where the trial took place. Additionally, the Ethical Committee of Clermont-Ferrand University Hospital, France (*Comité de Protection des Personnes Sud Est 6, Clermont-Ferrand, France*) granted ethical approval and all subjects provided written informed consent.

Patient and public involvement statement

Subjects were not involved in developing the research outcomes or design of this study; however, the DASS-42 stress scale is a self-assessment tool, allowing the subjects to provide their assessment of their stress, depression, and anxiety scales over the course of the study and the associated outcomes.

Subjects

Eligible subjects were adults aged 18–50 years with moderate to extremely severe stress at screening (*table 1*), defined as having a DASS-42 stress subscale score of >18. Subjects included in the analysis also presented with suboptimal serum magnesium levels within the range of 0.45 mmol/L and 0.85 mmol/L. The serum magnesium cutoff of 0.85 mmol/L has been previously determined as the lower limit adjusted to a value for health in a trial of over 15,000 subjects [16].

Additional inclusion criteria were a body mass index (BMI) of >18.5 and $\leq$29.9 kg/m2, and the use of an effective method of contraception during the trial period for female subjects. Key exclusion criteria included: exposure to therapies prohibited by the protocol (including levodopa, quinidine, and proton-pump inhibitors) within 3 months prior to screening; concomitant conditions or diseases that could make subjects nonevaluable for the primary endpoint; severe hypomagnesemia (defined as serum magnesium of $\leq$0.45 mmol/L); participant-reported moderate or severe kidney failure; confirmed diagnosis of type 1 or 2 diabetes; any known addiction to drugs and alcohol; alcohol intake of $\geq$3 drinks per day.

Participants could follow their regular diet during the study period; however, participants were requested not to take drugs known to impact Mg status (magnesium-containing salts, levodopa or tetracyclines, phosphate or calcium salts, nonsteroidal anti-inflammatory drugs, quinidine-based treatments, or aminoglycosides, cisplatin, cyclosporine, and diuretics), or not to consume any food supplement containing vitamins and/or minerals, Mg-rich water or juices ($\leq$ 2 glasses per day), dark chocolate (<50 g per day).

Assessments and endpoints

The primary trial endpoints are described in detail in the primary publication of Pouteau *et al.* [15]. The objective of the post-hoc analysis described here was to compare the effect of the magnesium-vitamin B6 combination with that of magnesium alone on erythrocyte magnesium levels and serum vitamin B6 levels by analyzing: serum (at baseline) and erythrocyte magnesium levels, and any correlation between them, change in levels of erythrocyte magnesium following treatment at week 4 and week 8, and change in serum vitamin B6 levels following treatment at week 4 and week 8.

A total of 35 mL of venous blood was extracted from each subject and collected into dry tubes (for serum magnesium and vitamin B6) or a heparin tube (for erythrocyte magnesium). The blood samples were centrifuged within 2 hours of collection to separate serum/plasma and erythrocytes. The erythrocytes were washed with 0.9% sodium chloride three times, then aliquoted into Eppendorf tubes and conserved at $-20\,^{\circ}$C until biochemical analysis. Serum samples were transferred into dry tubes: one sample was immediate-

Table 1. Subject demographics and baseline characteristics.

Parameter	Magnesium-vitamin B6 (n = 132)	Magnesium (n = 132)	Total (n = 264)
Mean age, years (±SD)	31.2 (8.4)	32.1 (8.6)	31.6 (8.5)
Female, N (%)	98 (74.2)	97 (73.5)	195 (73.9)
Mean weight, kg (±SD)	65.7 (11.0)	65.3 (10.0)	65.5 (10.5)
DASS-42 stress, mean (±SD)[1]	27.7 (7.3)	27.6 (7.0)	27.7 (7.1)
Serum magnesium dosage at screening visit (mmol/L)[1,2]			
Mean (±SD)	0.80 (0.04)	0.80 (0.03)	0.80 (0.04)
Median	0.82	0.82	0.82
[Q1:Q3]	[0.78:0.82]	[0.78:0.82]	[0.78:0.82]
[Min:Max]	[0.66:0.84]	[0.68:0.84]	[0.66:0.84]
Missing n (%)	0 (0)	0 (0)	0 (0)
Erythrocyte magnesium dosage at baseline (mmol/L)[1]			
Mean (±SD)	1.83 (0.27)	1.84 (0.35)	1.83 (0.31)
Median	1.80	1.79	1.79
[Q1:Q3]	[1.67:1.96]	[1.65:1.98]	[1.66:1.97]
[Min:Max]	[1.17:3.11]	[0.73:3.26]	[0.73:3.26]
Missing n (%)	0 (0)	0 (0)	0 (0)
Serum vitamin B6 dosage at baseline (nmol/L)[1]			
Mean (±SD)	50.58 (68.76)	46.54 (27.63)	48.56 (52.27)
Median	41.00	38.50	41.00
[Q1:Q3]	[27.00:61.00]	[26.00:62.50]	[26.00:61.00]
[Min:Max]	[5.00:780.20]	[8.00:155.00]	[5.00:780.20]
Missing n (%)	1 (0.8)	0 (0)	1 (0.4)

The percentages are calculated compared with filled data.
[1]mITT population; [2]Quintile: QU1: 0.78; QU2: 0.80; QU3: 0.82; QU4: 0.83.
mITT: modified Intent-To-Treat; Q1, quartile 1; Q3, quartile 3; SD, standard deviation.

ly used for magnesium level assessment and a second serum sample was kept at –20 °C, protected from light, until vitamin B6 level assessment.

Magnesium levels were analyzed using the Dimension Vista® System Flex® MG reagent cartridge (Siemens). This is a modified version of the methylthymol blue complexometric procedure, described by Connerty *et al.* [17]. Methylthymol blue forms a blue complex in the presence of magnesium and the amount of complex formed can be measured using a bichromatic endpoint method to determine the concentration of magnesium. Erythrocyte magnesium concentration was measured using a colorimetric method based on the formation of a colored complex of magnesium with xylidyl blue reagent, in alkaline solution (Eurofins Biomnis laboratory, Lyon, France). Serum vitamin B6, as the active form pyridoxal 5-phosphate, was analyzed using a HPLC reagent kit (Chromsystems® 31000), which produces a fluorescent vitamin B6 derivative that can be chromatographically determined using fluorescence detection with an isocratic HPLC system.

Statistical methods

For the purposes of this publication, only the statistical methodology undertaken in the secondary post-hoc analyses reported from this trial is detailed.

All analyses were performed in the modified Intent-To-Treat (mITT) population. Missing data for levels of erythrocyte magnesium and serum vitamin B6 were imputed at weeks 4 using baseline observation carried forward (BOCF) and at week 8 using a last observation carried forward (LOCF) approach, assuming the data were missing completely at random (MCAR).

For the post-hoc analyses, the change in serum vitamin B6 levels from baseline to week 4 and week 8 was measured by means of a repeated measures analysis of covariance (ANCOVA). The ANCOVA model included study visit, treatment and interaction between visit and treatment as fixed effects; sex and baseline value as covariates; and subject as a random effect. The Bonferroni technique was used to handle multiple comparisons of treatment effect at different timepoints. The raw values (baseline, week 4, week 8) of serum vitamin B6 levels were analyzed using a similar methodology as for the change from baseline (in this model, baseline value was not considered as a covariate). The same statistical model was applied for measuring erythrocyte magnesium levels.

Subgroups analyses for erythrocyte magnesium levels were also performed using the same methodology, in subjects with baseline erythrocyte magnesium levels of <1.6 and ≥1.6 mmol/L (i.e. an arbitrarily selected value representing a normal level of erythrocyte magnesium, accounting for levels observed in clinical practice).

Results

Demographics and baseline characteristics of subjects

Subject disposition has been fully reported previously [15]. Briefly, of 854 subjects screened, a total of 667 subjects with DASS-42 scores >18 had results for magnesemia, although a number of subjects were excluded in accordance with the exclusion criteria. Overall, 268 subjects were randomized into the trial, with 134 subjects in each treatment arm (magnesium-vitamin B6 and magnesium alone). Of the 268 randomized subjects, a total of 264 were administered at least one dose of study drug (ITT population). Overall, 132 subjects from each arm had evaluable DASS-42 stress scores at baseline and at least one other time point during treatment and were analyzed as part of the mITT. Of these, a total of 260 (98.5%) subjects completed the trial (130 per arm; three withdrew due to subject decision [two from the magnesium-vitamin B6 arm, one from the magnesium arm] and one withdrew due to an adverse event [magnesium only arm]).

Subject demographics and baseline characteristics were similar between arms, and can be found in *table 1*. There were no observed differences between study arms in mean serum magnesium levels at screening and mean erythrocyte magnesium levels at baseline. Mean (SD) vitamin B6 level was not significantly different in the magnesium-vitamin B6 combination group compared with the magnesium only group at baseline (50.6 [68.8] nmol/L and 46.5 [27.6] nmol/L, respectively). As indicated in *table 2*, no differences on DASS-42 stress scores were observed at baseline between quintiles of erythrocyte magnesium or quintiles of serum vitamin B6 levels.

Table 2. DASS-42 stress scores at baseline by erythrocyte magnesium and serum vitamin B6 levels at baseline (mITT population).

Parameter	Level (quintile) of erythrocyte magnesium dosage at baseline					
No. subjects, N	53	52	54	56	49	Total (N = 264)
Quintile, mmol/L	[0.73–1.62]	[1.62–1.74]	[1.74–1.86]	[1.86–2.04]	[2.04–3.26]	-
DASS-42 stress, Mean (±SD)	29.5 (6.3)	26.1 (7.6)	27.7 (7.4)	26.8 (6.3)	28.3 (7.6)	27.7 (7.1)
	Level of serum vitamin B6 dosage at baseline					
No.subjects, N	52	54	53	51	53	Total (N = 263)
Quintile, nmol/L	[5–23]	[23-33]	[33–48]	[48–67]	[67–780.2]	-
DASS-42 stress, Mean (±SD)	29.0 (6.6)	27.0 (7.1)	26.6 (7.2)	27.9 (7.3)	28.1 (7.2)	27.7 (7.1)

N numbers vary between level of erythrocyte magnesium and level of serum vitamin B6 dosage at baseline as subjects were only included if viable samples were available.
DASS-42: Depression Anxiety Stress Scale; mITT: modified Intent-To-Treat; SD: standard deviation.

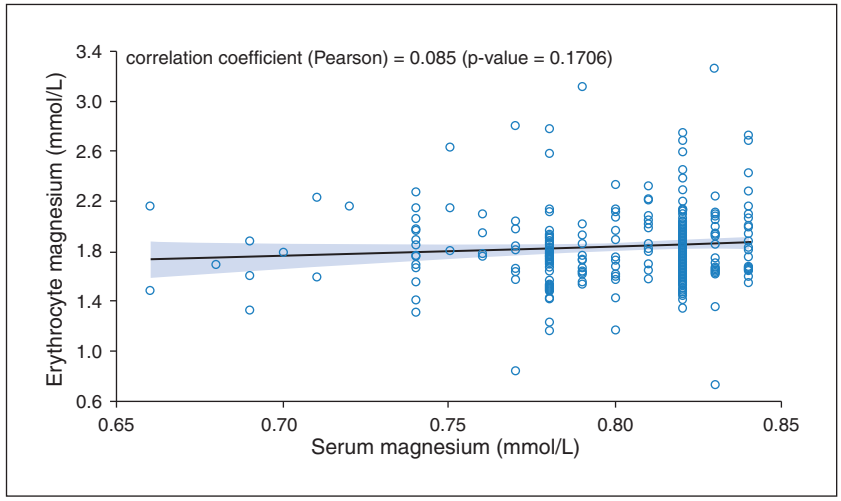

Figure 1. Correlation between serum and erythrocyte magnesium levels (mITT) at baseline. mITT: modified Intent-To-Treat.Shaded areas represent 95% confidence intervals. *Significant difference (5% threshold) between magnesium and magnesium-vitamin B6 group. *Significant increase (5% threshold) at week 4 or week 8 within each treatment group.

Blood magnesium levels

There was no correlation between serum and erythrocyte magnesium concentrations at baseline (*figure 1*).

The erythrocyte magnesium level, which better represents body intracellular magnesium, was selected to assess magnesium status during the study. Between baseline and both weeks 4 and 8, there was a slight nonsignificant increase in erythrocyte magnesium levels following treatment with both the magnesium-vitamin B6 combination and magnesium alone in the overall population (*figure 2A*), with no significant difference between the two treatment arms (*table 3*). No change in erythrocyte magnesium was observed between baseline and week 8 for baseline magnesium levels >1.6 mmol/L (*figure 2B*). Interestingly, a significant increase in erythrocyte magnesium levels was observed in subjects with baseline magnesium levels < 1.6 mmol/L (below a normal level of erythrocyte magnesium) in the magnesium group and magnesium-vitamin B6 group at week 4 (0.21 mmol/L [95% confidence interval (CI), 0.10 to 0.31], p = 0.0003; and 0.13 mmol/L [95% CI, 0.02 to 0.23], p = 0.0233, respectively) and week 8 (0.17 mmol/L [95% CI, 0.08 to 0.25], p = 0.0003; and 0.13 mmol/L [95% CI, 0.04 to 0.22], p = 0.0050, respectively). This improvement, as indicated by a statistically significant increase in erythrocyte magnesium, accounted for approximately 17% of the trial population (*figure 2C*). However, no difference was seen between the treatment arms regardless of baseline erythrocyte magnesium levels. In the present study, no correlation was seen in baseline stress levels, as assessed using DASS-42, and the apparent changes in magnesium levels observed following treatment with magnesium-vitamin B6 or magnesium alone, as shown in *table 4*.

Vitamin B6 serum levels

No change in serum vitamin B6 levels was observed in the magnesium alone arm from baseline to week 4 (4.45 nmol/L [95% CI, -16.60 to 25.50]) or week 8 (-0.39 nmol/L [95% CI, -20.73 to 19.94]). From week 4, a significant (p < 0.0001) increase in circulating vitamin B6 levels was observed following magnesium-vitamin B6 treatment compared with magnesium alone (*table 3*). Serum vitamin B6 levels increased by 6.5 times in the magnesium-vitamin B6 arm (306.08 nmol/L [95% CI, 285.01 to 327.14]) between baseline and week 4, and 6.6 times (314.96 nmol/L [95% CI, 294.61 to 335.31]) between baseline and week 8. The change from baseline compared with magnesium alone was

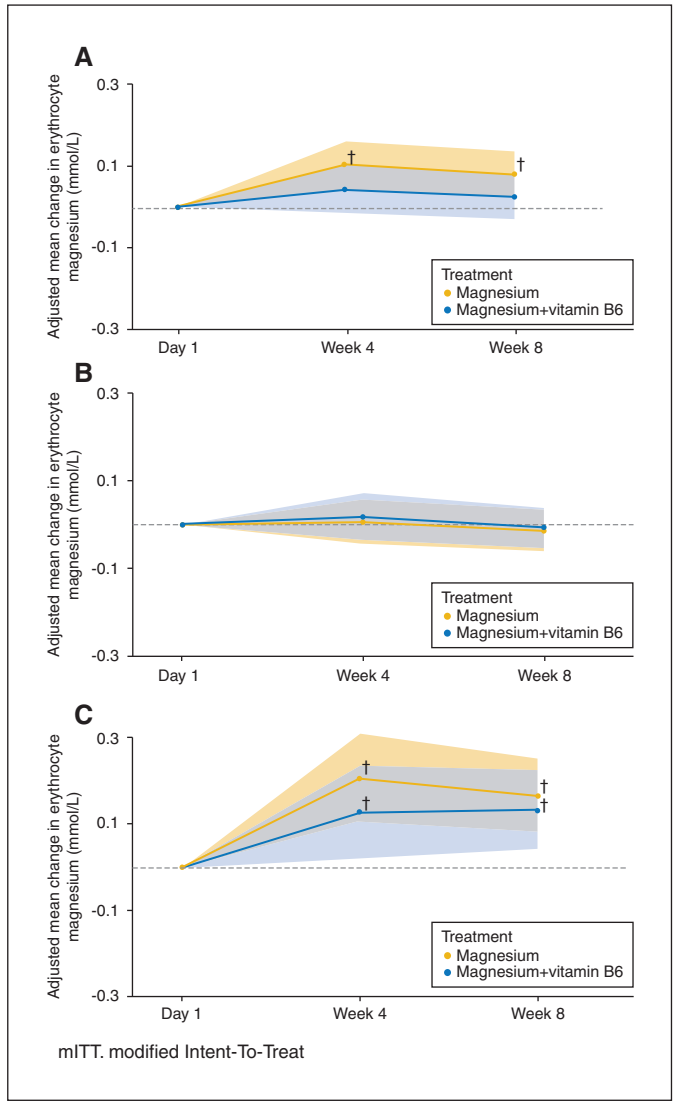

Figure 2. Change in erythrocyte magnesium levels from baseline to week 8 in the total trial population (A), and by baseline magnesium level; ≥1.6 mmol/L (B) and <1.6 mmol/L (C) (mITT population). Shaded areas represent 95% confidence intervals.
*Significant difference (5% threshold) between magnesium and magnesium-vitamin B6 group.
†Significant increase (5% threshold) at week 4 or week 8 within each treatment group.
mITT: modified Intent-To-Treat.

statistically significant at both week 4 and week 8 (-301.63 nmol/L [95% CI, -331.41 to -271.84] and -315.36 nmol/L [95% CI, -344.13 to -286.59], respectively; $p < 0.0001$ for both).

As assessed using DASS-42, the perceived level of stress reported in subjects at baseline had no impact on the change in vitamin B6 levels observed following treatment with magnesium-vitamin B6 or magnesium alone (*table 4*).

Discussion

The results from the primary randomized controlled trial by Pouteau *et al.* [15] corroborate

Table 3. Change in erythrocyte magnesium and serum vitamin B6 levels from baseline to weeks 4 and 8 following magnesium-vitamin B6 or magnesium treatment alone (mITT population).

Parameter	Magnesium-vitamin B6 (n = 132)	Magnesium (n = 132)	Variation**	p*-value
Erythrocyte magnesium (mmol/L)				
Baseline, adjusted* mean (95% CI)	1.84 (1.79 to 1.90)	1.86 (1.80 to 1.91)	0.01 (-0.08 to 0.11)	1.000
Week 4, adjusted* mean (95% CI)	1.88 (1.82 to 1.94)	1.90 (1.84 to 1.96)	0.02 (-0.08 to 0.12)	1.000
Week 8, adjusted* mean (95% CI)	1.86 (1.81 to 1.91)	1.88 (1.82 to 1.93)	0.02 (-0.07 to 0.10)	1.000
Week 4–Baseline, difference* in adjusted means (95% CI)	0.03 (-0.01 to 0.08)	0.04 (-0.01 to 0.09)	0.01 (-0.06 to 0.07)	0.859
Week 8–Baseline, difference* in adjusted means (95% CI)	0.01 (-0.03 to 0.06)	0.02 (-0.03 to 0.06)	0.00 (-0.06 to 0.07)	0.912
Serum vitamin B6 levels (nmol/L)				
Baseline, adjusted† mean (95% CI)	55.89 (46.41 to 65.38)	51.86 (42.43 to 61.29)	-4.04 (-19.33 to 11.26)	1.000
Week 4, adjusted† mean (95% CI)	361.97 (339.55 to 384.40)	56.31 (33.90 to 78.72)	-305.66 (-344.01 to -267.31)	<0.0001
Week 8, adjusted† mean (95% CI)	370.86 (348.58 to 393.14)	51.47 (29.20 to 73.73)	-319.39 (-357.49 to -281.30)	<0.0001
Week 4–Baseline, difference† in adjusted means (95% CI)	306.08 (285.01 to 327.14)	4.45 (-16.60 to 25.50)	-301.63 (-331.41 to-271.84)	<0.0001
Week 8–Baseline, difference† in adjusted means (95% CI)	314.96 (294.61 to 335.31)	-0.39 (-20.73 to 19.94)	-315.36 (-344.13 to -286.59)	<0.0001

Data of serum vitamin B6 and baseline for one subject is missing and not imputed in the model. Missing data were imputed by the LOCF method. Multiplicity of tests was taken into account by Bonferroni adjustment.
*ANCOVA analysis adjusted based on sex. **Variation between magnesium and magnesium-vitamin B6. †ANCOVA analysis adjusted on the baseline serum vitamin B6 levels (nmol/L), sex, visit and interaction between visit and treatment.
CI: confidence interval; mITT: modified Intent-To-Treat.

Table 4. Change from baseline to weeks 4 and 8 in erythrocyte magnesium and serum vitamin B6 levels by ANCOVA analysis adjusted by sex and baseline DASS-42 stress scores (mITT population).

Parameter	Magnesium-vitamin B6 (n = 132)	Magnesium (n = 132)	Variation**	p*-value
Erythrocyte magnesium (mmol/L)				
Week 4–Baseline, difference* in adjusted means (95% CI)	0.03 (-0.02 to 0.08)	0.04 (-0.01 to -0.09)	0.01 (-0.06 to 0.08)	1.000
Week 8–Baseline, difference* in adjusted means (95% CI)	0.01 (-0.04 to 0.06)	0.01 (-0.03 to 0.06)	0.01 (-0.06 to 0.07)	1.000
Serum vitamin B6 (nmol/L)				
Week 4–Baseline, difference* in adjusted means (95% CI)	309.21 (286.81 to 336.61)	6.62 (-15.78 to -29.02)	-302.59 (-332.50 to -272.68)	<0.0001
Week 8–Baseline, difference* in adjusted means (95% CI)	317.79 (296.03 to 339.54)	1.78 (-19.98 to 23.53)	-316.01 (-344.96 to -287.07)	<0.0001

*ANCOVA analysis adjusted on sex and level of baseline stress DASS-42 (normal to moderate *vs.* severe or extremely severe). **Variation between magnesium and magnesium-vitamin B6. Missing data were imputed by the LOCF method and multiplicity of tests was taken into account by Bonferroni adjustment.
CI: confidence interval; erythrocyte: red blood cell; mITT: modified intent-to-treat.

previously reported findings indicating potential synergistic effects of vitamin B6 with magnesium on stress and anxiety symptoms [18, 19]. The exploratory post-hoc analyses reported in this publication describe the effect of the magnesium-vitamin B6 combination with that of magnesium alone on erythrocyte magnesium and serum vitamin B6 levels in stressed adults with suboptimal serum magnesium levels, and explore whether these findings may explain the benefits on stress levels observed after treatment with the magnesium-vitamin B6 combination in severely stressed subjects [15].

In the present analysis, subjects with a <0.85 mmol/L magnesium serum level were selected, which is classified as suboptimal and may already reflect chronic, latent magnesium deficiency [20]. Subjects with magnesium levels <0.85 mmol/L represented approximately 44% of the screened population selected for stress (not including those with the presence of exclusion criteria). While serum magnesium remains the most common and readily available method to assess magnesium status in the body, it has been challenged as being poorly representative of actual magnesium status [9, 21, 22] due to the poor correlation between levels of magnesium in the serum and tissue, especially for latent deficiency [9, 21].Less than 1% of the body's total magnesium is found in blood [22], with only 0.3% in the serum [21] as levels are tightly controlled through homeostatic regulation. This makes evaluation difficult, since serum magnesium reflects the extracellular concentration and only weakly correlates to total body magnesium or specific tissue levels [9, 21]. Instead, the highest levels of magnesium within the blood have been found to reside in erythrocytes [22]. Thus, erythrocyte magnesium, representing intracellular magnesium concentration, has been suggested to better reflect the body's magnesium status [23,24], and changes in erythrocyte magnesium levels might better correlate with clinical signs of magnesium deficiency [25,26]. Analyses performed in the current study found no correlation between serum and erythrocyte magnesium levels at baseline, and the range of values was not sufficient to see a correlation in mild deficiency. Nevertheless, magnesium levels, as measured in erythrocytes, support that the selected population had low magnesium status. Indeed, depending on the reference range of the laboratory where the test is carried out [27], between 20–40% of the studied population could be considered as below the lower cut-off point (1.65 or 1.74 mmol/L). This figure compares favorably with the findings by Hermes Sales *et al.* [28], in which 17% of participants (healthy Brazilian university students) were shown to have an erythrocyte magnesium level below the lower reference range. Stress level was not evaluated in the latter study, but the student population is generally considered to be particularly exposed to a "stressful" way of life, due to sleep deprivation, malnutrition, and lack of physical activity [29]. This may explain the similarly high numbers of subjects with low magnesium levels in both the present study and the Hermes Sales *et al.* study. Furthermore, this may support the potential relationship already established in the literature between stress and low magnesium levels [1]. In the current analysis, although we did observe a higher DASS-42 stress score for subjects in the lowest erythrocyte magnesium quintile, stress levels were not statistically different in the different erythrocyte magnesium quintiles of the studied population. However, it is important to note that all study subjects presented with low levels of magnesium, restricting the analysis to this subgroup, and the study was not specifically powered for subgroup analyses, which limits the interpretation of the results.

In the present study, erythrocyte magnesium levels were used to evaluate the mid-term effect of magnesium supplementation following magnesium-vitamin B6 or magnesium alone on the magnesium status of the subjects. No significant differences were observed between the groups; however, a small and significant ($p = 0.0001$) increase in magnesium levels, similar to that described by others [9, 26, 30, 31] (weighted mean differences of 0.12–0.16 mmol/L and increases of 1.6% to 6%, depending on the study), was seen after 4 weeks of supplementation in subjects with the lowest erythrocyte magnesium levels at baseline (<1.6 mmol/L), following both supplements. This is also in line with previous data, generally reporting the use of magnesium-based products for a minimal period of 1 month to improve magnesium status and related symptoms [18, 26]. The current data demonstrate that 4 weeks of treatment can be sufficient to significantly improve magnesium status in subjects with low levels of erythrocyte magne-

sium at baseline. Magnesium supplementation of an individual with a very low magnesium status, indicated by an erythrocyte magnesium concentration <1.6 mmol/L, may reduce the risk for stress and improve any resultant stress [3].

Plasma vitamin B6 concentrations (as plasma pyridoxal 5′-phosphate) are generally considered to accurately reflect vitamin B6 intake and status in all general populations [11]. Concentrations in the range of 20–30 nmol/L are believed to correspond to a below optimal vitamin B6 status. Concentrations >30 nmol/L are considered indicative of adequate vitamin B6 status [11]. In the current analysis, about 20% of the population studied had vitamin B6 levels below the lower reference range (approximately 20 nmol/L). In contrast, the values reported for adults aged <50 years and adolescents in the EU show a prevalence of vitamin B6 concentrations <20 nmol/L of 0.5–7% [32, 33]. This could potentially be attributed to the higher stress levels in the population studied here. Indeed, a number of studies have indicated that vitamin B6 possesses antistress properties, including modulating neurotransmitters associated with depression and anxiety, reducing blood pressure, and reducing the physiological impact of corticosteroid secretion [12, 15, 34]. In this study, as expected, there was a marked increase in vitamin B6 levels following treatment with magnesium-vitamin B6, versus no change following magnesium only. Notably, the change in vitamin B6 levels following magnesium-vitamin B6 treatment was not influenced by the level of stress reported in subjects at baseline, with all subjects, regardless of DASS-42 stress score at baseline, having a marked increase in vitamin B6 levels following treatment.

It has been proposed that vitamin B6 facilitates cellular uptake of magnesium. We do not observe in the present paper an increase in erythrocyte magnesium after treatment with magnesium-vitamin B6 vs magnesium alone. This may be explained by moderately affected Mg status of studied subjects and by concomitant supplementation with vit B6 and Mg. However, the additive effect of vitamin B6 had been shown in an experimental Mg deficiency in rats by Iezhitsa et al. [35], which used the same magnesium:vitamin B6 ratio (10:1). Other previous researches investigating and supporting the effects of vitamin B6 on magnesium absorption or distribution in preclinical and clinical studies, however, have most of the time used higher doses of vitamin B6 [13, 18, 36]. Nevertheless, it remains conceivable that vitamin B6 may have an effect on the entry of magnesium into neurons or the distribution of magnesium in the brain, which would be of interest for nervous system function and protection [37, 38].

Another hypothesis is that vitamin B6 could potentially have a direct effect on stress, and may therefore have been responsible for the improved stress levels observed in the severely stressed patient population from the primary findings of this trial. In a murine study, Henrotte *et al.* showed that vitamin B6 decreased the number of stress-induced ulcers in mice exposed to extreme stress, independently from magnesium level [34]. It was subsequently hypothesized that vitamin B6 may antagonize the effect of stress by decreasing brain noradrenaline levels [39] and modulating γ-amino butyric acid (GABA) synthesis [40]. Thus, combining magnesium and vitamin B6, which could both be proposed to have a direct and indirect role in stress relief, may have complementary benefits on reducing stress as observed in our recent clinical trial. The benefit could nonetheless be synergistic or complementary and warrants further investigation to treat stress and adjacent mental health conditions, particularly in subjects with suboptimal serum magnesium levels.

As expected, in the exploratory, post-hoc analyses described here, the baseline stress level (as assessed by DASS-42) had no impact on change in vitamin B6 levels from baseline to week 8. As vitamin B6 treatment enhanced the positive treatment effect of magnesium in severely stressed subjects in the primary analysis [15], further studies assessing the effect of vitamin B6 alone on stress are warranted. In addition, this trial was not originally designed to directly assess the effect of vitamin B6 on stress as shown by the lack of a vitamin B6 only arm; thus, further studies would allow a better assessment of the direct or indirect effects vitamin B6 could have on stress, independent of magnesium.

Conclusion

A significant section (44%) of the screened stressed population in this study could be considered as presenting with chronic latent

magnesium deficiency (<0.85 mmol/L). Compared with baseline levels, magnesium supplementation, with or without vitamin B6, did not significantly increase erythrocyte magnesium levels measured in the overall population and in subjects with erythrocyte magnesium >1.6 mmol/L at baseline. However, magnesium supplementation significantly increased erythrocyte magnesium concentration in subjects with low erythrocyte magnesium <1.6 mmol/L at baseline. No difference was observed between both arms with regards to change in erythrocyte magnesium levels. As expected, supplementation with magnesium-vitamin B6 significantly increased serum vitamin B6 levels.

Based on its proposed antistress properties, and considering the findings on vitamin B6 levels from this post-hoc analysis, it could be hypothesized that vitamin B6 may potentially have a direct, or possibly an additional, effect on stress that is independent from the benefits of magnesium alone. However, additional, more specifically designed studies are required to confirm this hypothesis as there are currently limited data available on this potential interaction.

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Data sharing statement: Qualified researchers may request access to patient level data and related study documents including the clinical study report, study protocol with any amendments, blank case report form, statistical analysis plan, and dataset specifications. Patient level data will be anonymized and study documents will be redacted to protect the privacy of trial participants. Further details on Sanofi's data sharing criteria, eligible studies, and process for requesting access can be found at: https://www.clinicalstudydatarequest.com.

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