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# Combined dietary folate, vitamin B-12, and vitamin B-6 intake influences plasma docosahexaenoic acid concentration in rats

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## Abstract

**Background:** Folate, vitamin B-12, and vitamin B-6 are essential nutritional components in one-carbon metabolism and are required for methylation capacity. The availability of these vitamins may therefore modify methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) by PE-N-methyltransferase (PEMT) in the liver. It has been suggested that PC synthesis by PEMT plays an important role in the transport of polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA) from the liver to plasma and possibly other tissues. We hypothesized that if B-vitamin supplementation enhances PEMT activity, then supplementation could also increase the concentration of plasma levels of PUFAs such as DHA. To test this hypothesis, we determined the effect of varying the combined dietary intake of these three B-vitamins on plasma DHA concentration in rats.

**Methods:** In a first experiment, plasma DHA and plasma homocysteine concentrations were measured in rats that had consumed a B-vitamin-poor diet for 4 weeks after which they were either continued on the B-vitamin-poor diet or switched to a B-vitamin-enriched diet for another 4 weeks. In a second experiment, plasma DHA and plasma homocysteine concentrations were measured in rats after feeding them one of four diets with varying levels of B-vitamins for 4 weeks. The diets provided 0% (poor), 100% (normal), 400% (enriched), and 1600% (high) of the laboratory rodent requirements for each of the three B-vitamins.

**Results:** Plasma DHA concentration was higher in rats fed the B-vitamin-enriched diet than in rats that were continued on the B-vitamin-poor diet ( $P = 0.005$ ; experiment A). Varying dietary B-vitamin intake from deficient to supra-physiologic resulted in a non-linear dose-dependent trend for increasing plasma DHA ( $P = 0.027$ ; experiment B). Plasma DHA was lowest in rats consuming the B-vitamin-poor diet ( $P > 0.05$  vs. normal,  $P < 0.05$  vs. enriched and high) and highest in rats consuming the B-vitamin-high diet ( $P < 0.05$  vs. poor and normal,  $P > 0.05$  vs. enriched). B-vitamin deficiency significantly increased plasma total homocysteine but increasing intake above normal did not significantly reduce it. Nevertheless, in both experiments plasma DHA was inversely correlated with plasma total homocysteine.

**Conclusion:** These data demonstrate that dietary folate, vitamin B-12, and vitamin B-6 intake can influence plasma concentration of DHA.

**Keywords:** B-vitamins, Plasma DHA, Plasma homocysteine, Methylation capacity, Rats

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## Background

Several clinical studies in different populations have found a negative correlation between serum, plasma or erythrocyte content of docosahexaenoic acid (DHA) and markers of B-vitamin deficiency such as plasma levels of homocysteine and/or *S*-adenosylhomocysteine (SAH) [1-3]. In line with these observations, dietary deficiency studies in rats have shown that deficiencies of folate, vitamin B-12, or vitamin B-6 may reduce peripheral DHA levels [4-6]. Although these data indicate a link between dietary B-vitamin intake and DHA status, it has not been studied whether concurrently varying the dietary intake of folic acid, vitamin B-12, and vitamin B-6 could influence plasma DHA concentration.

Hypothetically, dietary B-vitamin availability might influence plasma DHA by influencing synthesis of phosphatidylcholine (PC) in the liver. Hepatic PC can be synthesized by two different metabolic pathways, the cytidine diphosphate (CDP)-choline pathway (Kennedy cycle) and the phosphatidylethanolamine-*N*-methyltransferase (PEMT) pathway. The CDP-choline pathway utilizes 1,2-diacylglycerol and CDP-choline for the synthesis of PC [7], whereas PEMT catalyzes the sequential methylation of phosphatidylethanolamine (PE) to PC [8]. It has been suggested that the methylation of PE to PC by PEMT plays an important role in the transport of polyunsaturated fatty acids (PUFAs) like DHA from the liver to the plasma and other tissues [3,9,10]. Most likely, two mechanisms are involved. First, PC synthesis is required for normal secretion of very low density lipoprotein (VLDL) from liver cells [11]. An impairment of the PEMT pathway results in a diminished PC synthesis and therefore limits hepatic secretion of VLDL [12]. Because VLDL is the main carrier of endogenous triglycerides, phospholipids, and cholesterol esters, impairment of the PEMT pathway directly affects the transport of these components from the liver to peripheral tissues [13]. Second, PC synthesized by the PEMT pathway contains more PUFAs such as DHA than does PC synthesized by the CDP-choline pathway because PEMT prefers species of PE containing PUFAs [10,14,15]. Hence, PEMT activity can influence both VLDL secretion and the rate of synthesis of PUFA-rich PC species. Therefore, a factor that influences hepatic PEMT activity could potentially affect the availability of PUFAs such as DHA in plasma [9] and even their transport to the brain [16].

Folate, vitamin B-12, and vitamin B-6 availability are important determinants of methionine and *S*-adenosylmethionine (SAM) synthesis and of SAH and homocysteine clearance, and therefore of methylation capacity. Thus, B-vitamin availability can be hypothesized to directly modify liver PEMT activity and PEMT-dependent PUFA secretion. This in turn, is predicted to influence

plasma PUFA concentration and thus tissue availability. If so, then adequate dietary intake of these three vitamins would be necessary to maintain normal plasma DHA concentrations, and increasing dietary intake above the normal range might be expected to increase plasma DHA. The aim of the present study was to determine whether dietary enrichment with the combination of these three B-vitamins could increase plasma DHA concentration in rats. In a first experiment, plasma DHA concentration was measured in rats that had consumed a B-vitamin-poor diet for 4 weeks, after which they were either continued on the B-vitamin-poor diet or switched to a B-vitamin-enriched diet for another 4 weeks. In a second experiment, the dependency of plasma DHA on dietary B-vitamin content was determined by feeding rats for 4 weeks one of four diets containing varying levels of the B-vitamins across the range from inadequate to supra-physiological.

## Methods

Two experiments were conducted to investigate the effects of varying dietary levels of folate, vitamin B-12, and vitamin B-6 on plasma DHA concentration. Experiment A was conducted at the Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology (Cambridge, MA, USA). Experiment B was conducted at the Centrum Kleine Proefdieren, Wageningen University (Wageningen, The Netherlands).

## Animals

A total of sixty-four male Sprague–Dawley rats (CrI:CD (SD)) were obtained from either Charles River, Wilmington, MA, USA (experiment A;  $n=16$ ) or Charles River, Sulzfeld, Germany (experiment B;  $n=48$ ). Animals aged 6–8 weeks on arrival were housed in groups in a temperature- and light-controlled room, under 12 h light–12 h dark cycles. Rats had free access to food and water. Body weight was registered once a week. All animal experimental protocols were conducted in accordance with international and national laws and institutional guidelines and approved by the local ethics committee, i.e. the Committee on Animal Care at Massachusetts Institute of Technology, Cambridge, MA, USA (experiment A) and DEC Consult, Bilthoven, The Netherlands (experiment B).

## Diets

Four different diets with increasing folate, vitamin B-12, and vitamin B-6 contents were used: 1) B-vitamin-poor; 2) B-vitamin-normal; 3) B-vitamin-enriched; and 4) B-vitamin-high. Diets were AIN-93 M based [17], isoenergetic, and identical with respect to their protein, carbohydrate, fat, fiber, and mineral contents. All diets were devoid of any measurable amounts of DHA. The vitamin mix (AIN-93-VX) [17] was prepared without folic acid,

cyanocobalamin, and pyridoxine; these vitamins were subsequently supplemented accordingly. Diets were formulated with vitamin-free, ethanol-precipitated casein (Harlan Teklad, Madison, WI, USA) and were manufactured by Research Diet Services, Wijk bij Duurstede, The Netherlands (experiment A) and Ssniff Spezialdiäten, Soest, Germany (experiment B).

The B-vitamin-poor diet contained low amounts of folate (<0.1 mg/kg), vitamin B-12 (<1.0 µg/kg), and vitamin B-6 (<0.6 mg/kg). No sulfathiazole drugs were added to the diet and therefore a limited amount of folate was still expected to be provided by the gut flora. Vitamin B-12 deficiency in the rat is difficult to achieve because of considerable endogenous storage of this vitamin. To attain a moderate reduction of endogenous vitamin B-12, the B-vitamin-poor was supplemented with 50 g/kg pectin (polygalacturonic acid, high methoxyl, Obipektin<sup>®</sup>, NF/USP Citrus; TEFKO FoodIngredients, Bodegraven, The Netherlands), which binds vitamin B-12 in the intestine, making it less bioavailable [18]. Pectin consequently promotes depletion of endogenous vitamin B-12 through the enterohepatic circulation of the vitamin. Since pectin could affect food intake [19], all four diets were supplemented with pectin to maintain uniform intakes of the diets. Pectin has minimal effects on vitamin B-12 status when the diet contains adequate amounts of this vitamin [18].

The B-vitamin-normal diet, the B-vitamin-enriched diet, and the B-vitamin-high diet provided 100%, 400%, and 1600%, respectively, of the requirements for each of the three vitamins according to the National Research Council report on the nutrient requirements of laboratory animals [20]. The exact dietary levels of the three B-vitamins in each experimental diet are indicated in Table 1.

### Experimental design

In experiment A, B-vitamin paucity was first induced in all rats by feeding them the B-vitamin-poor diet for 4 weeks. Subsequently, animals were either continued on the B-vitamin-poor diet or switched to the B-vitamin-enriched diet for another 4 weeks. In experiment B rats were directly fed one of the four experimental diets for 4 weeks.

### Tissue preparation

After the supplementation period, animals that had been feed-deprived for 3–4 hours were killed by CO<sub>2</sub> gas inhalation (experiment A) or by inhalation of isoflurane vaporized in medicinal air (experiment B) and subsequent decapitation by guillotine. Trunk blood was collected through a funnel into EDTA-containing tubes. After centrifugation at 1750 × g for 10 min, plasma was aspirated and analyzed for plasma DHA and homocysteine.

### Plasma DHA and plasma total homocysteine analysis

Plasma total lipid DHA was detected using GC. Total lipid content was extracted from plasma by adding methanol and dichloromethane. Samples were subsequently centrifuged at 1750 × g for 10 min and the organic phase (dichloromethane and lipids) was collected. 200 µL of the dichloromethane layer was dried using a SpeedVac<sup>®</sup> concentrator. Next, 2.0 mL methanol and 40 µL concentrated sulfuric acid were added to the dried extract. The samples were heated at 100 °C for 60 min, and 2 mL hexane and 0.5 mL 2.5 mol/L sodium hydroxide solution were added. After vortexing and centrifuging the samples for 5 min at 1750 × g, the upper layer was collected and dried using a SpeedVac<sup>®</sup>. Dried samples were subsequently dissolved in 125 µL iso-octane and analyzed by GC using flame ionization detection.

Plasma total homocysteine was determined by fluorometric HPLC as previously described [21]. Briefly, thiol amino acids (free and protein-bound) were reduced with tri-*n*-butylphosphine. After protein precipitation and centrifugation to remove the proteins, thiol groups were derivatized with 7-fluoro-2-oxa-1,3-diazole-4-sulfonamide reagent. The content of the derivatized thiol amino acids was determined by fluorescence detection with excitation at 385 nm and emission at 515 nm.

### Statistical methods

All statistical analyses were performed using SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). Data were expressed as means ± SEM. P-values <0.05 were considered significant. Effects of dietary B-vitamins on body weight were analyzed using repeated-measures ANOVA with dietary B-vitamins as between-subject factor and

**Table 1 Folate, vitamin B-12, and vitamin B-6 content of the experimental diets.**

Diet description	% of recommended levels [20]	Calculated dietary levels		
		Folate (folic acid)	Vitamin B-12 (cyanocobalamin)	Vitamin B-6 (pyridoxine-HCL)
		mg/kg diet		
B-vitamin-poor	~0%	<0.1	<0.001	<0.6
B-vitamin-normal	100%	1.0	0.05	6.0
B-vitamin-enriched	400%	4.0	0.20	24.0
B-vitamin-high	1600%	16.0	0.80	96.0

week as within-subject factor. Plasma DHA and homocysteine concentration were compared between rats fed the diets varying in B-vitamin content using ANOVA and post hoc comparisons were performed when appropriate. Standard Pearson correlation coefficients were calculated for plasma DHA and homocysteine.

## Results

### Experiment A

After feeding all animals the B-vitamin-poor diet for 4 weeks, they were randomized into the two experimental groups according to their body weight. During the 4 week intervention period body weight was unaffected by the level of dietary B-vitamins ( $F(1,14) = 0.77$ ,  $P = 0.40$ ). After the 4 week intervention period, plasma DHA concentration was 72% higher in rats fed the B-vitamin-enriched diet ( $176.2 \pm 18.3 \mu\text{M}$ ) than in rats that were continued on the B-vitamin-poor diet ( $102.6 \pm 12.5 \mu\text{M}$ ;  $F(1,14) = 10.97$ ,  $P = 0.005$ ; Figure 1A). In addition, plasma total homocysteine concentration was lower in animals receiving the B-vitamin-enriched diet ( $6.1 \pm 0.4 \mu\text{M}$ ) as compared to the B-vitamin-poor group ( $10.7 \pm 0.8 \mu\text{M}$ ) ( $F(1,14) = 29.35$ ,  $P < 0.001$ ; Figure 1B). A significant inverse correlation was observed between plasma concentrations of DHA and homocysteine ( $r = -0.73$ ,  $P = 0.001$ ; Figure 1C).

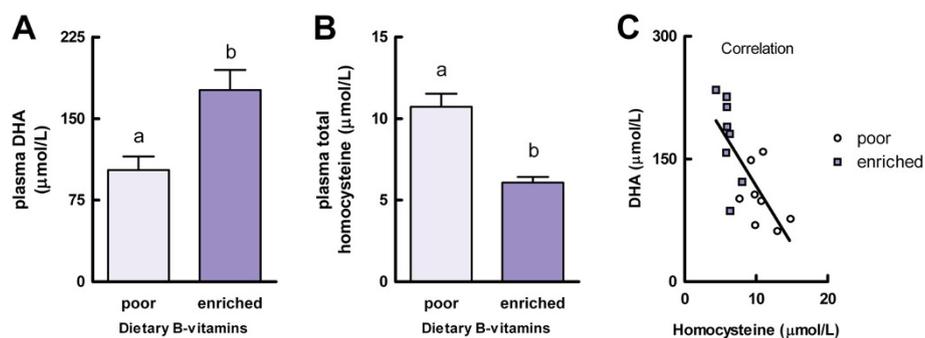
### Experiment B

Animals were randomized into the four experimental groups according to their body weights at the start of the intervention period. Body weight did not differ between the experimental groups fed the four different diets for 4 weeks ( $F(3,44) = 0.16$ ,  $P = 0.92$ ). After the intervention period, plasma DHA concentration was found to be dependent on dietary B-vitamin intake ( $F(3,44) = 3.37$ ,  $P = 0.027$ ; Figure 2A). Plasma DHA showed a non-linear dose-dependent relationship to dietary B-

vitamin intake. Compared with rats consuming B-vitamin-normal diet ( $125.4 \pm 8.0 \mu\text{M}$ ) and B-vitamin-enriched diet ( $143.8 \pm 9.0 \mu\text{M}$ ), plasma DHA was reduced in rats consuming the B-vitamin-poor diet ( $111.9 \pm 10.3 \mu\text{M}$ ,  $P > 0.05$  vs. normal,  $P < 0.05$  vs. enriched) and increased in rats consuming the B-vitamin-high diet ( $157.1 \pm 14.9 \mu\text{M}$ ,  $P < 0.05$  vs. normal,  $P > 0.05$  vs. enriched). Dietary B-vitamin intake also affected plasma total homocysteine ( $F(3,44) = 16.18$ ,  $P < 0.001$ ; Figure 2B). Plasma homocysteine was increased in rats fed the B-vitamin-poor diet ( $11.7 \pm 1.5 \mu\text{M}$ ) as compared with rats fed the B-vitamin-normal diet ( $6.0 \pm 0.5 \mu\text{M}$ ,  $P < 0.05$ ), whereas the B-vitamin-enriched ( $5.0 \pm 0.2 \mu\text{M}$ ) and B-vitamin-high ( $5.1 \pm 0.3 \mu\text{M}$ ) diet further reduced plasma homocysteine as compared with the B-vitamin-normal group although this reduction was not significant ( $P > 0.05$  vs. enriched,  $P > 0.05$  vs. high). Plasma DHA and homocysteine showed a significant inverse correlation ( $r = -0.29$ ,  $P = 0.043$ ; Figure 2C).

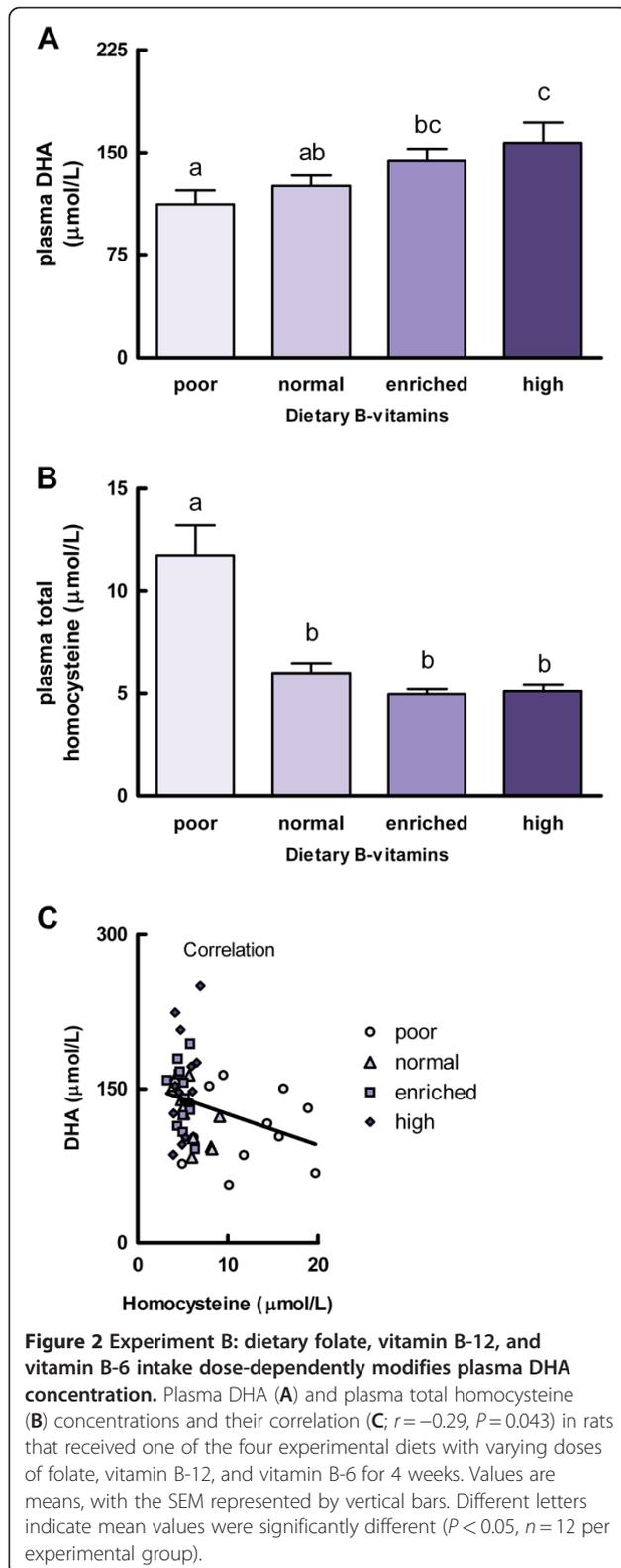
## Discussion

The present results show that concurrently varying the dietary intake of folate, vitamin B-12, and vitamin B-6, across the range from inadequate to supra-physiological supplementation, can dose-dependently modify plasma DHA concentration. Experiment A demonstrates that plasma DHA concentration is dependent on dietary intake of folate, vitamin B-12, and vitamin B-6 in rats. Rats receiving the B-vitamin-enriched diet showed higher plasma DHA and lower plasma homocysteine concentrations as compared to rats that were continued on the B-vitamin-poor diet. In experiment B the observations of experiment A were replicated and extended by showing a positive dose-response relationship between dietary B-vitamins and plasma DHA concentration. Plasma DHA and plasma homocysteine concentrations were



**Figure 1 Experiment A: effects of dietary folate, vitamin B-12, and vitamin B-6 on plasma DHA and homocysteine concentrations.**

Plasma DHA (A) and plasma total homocysteine (B) concentrations and their correlation (C;  $r = -0.73$ ,  $P = 0.001$ ) in rats that received either a B-vitamin-poor diet for 8 weeks (poor) or a B-vitamin-poor diet for 4 weeks followed by a B-vitamin-enriched diet for 4 weeks (enriched). Values are means, with the SEM represented by vertical bars. Different letters indicate mean values were significantly different ( $P < 0.01$ ,  $n = 8$  per experimental group).



**Figure 2 Experiment B: dietary folate, vitamin B-12, and vitamin B-6 intake dose-dependently modifies plasma DHA concentration.** Plasma DHA (A) and plasma total homocysteine (B) concentrations and their correlation (C;  $r = -0.29$ ,  $P = 0.043$ ) in rats that received one of the four experimental diets with varying doses of folate, vitamin B-12, and vitamin B-6 for 4 weeks. Values are means, with the SEM represented by vertical bars. Different letters indicate mean values were significantly different ( $P < 0.05$ ,  $n = 12$  per experimental group).

comparable between animals fed the B-vitamin-poor diet for either 4 weeks (Experiment B) or 8 weeks (Experiment A). In addition, plasma homocysteine concentrations were

only moderately increased by the B-vitamin-poor diet and therefore this diet is considered to induce only a mild B-vitamin deficiency.

It should be noted that the present experiments were conducted in different labs, under somewhat different experimental conditions (e.g. time, animal supplier, diet manufacturer, experimental design), that might have been responsible for small variations in absolute plasma DHA concentration between the two experiments. However, despite the differences in experimental conditions, the results from both experiments are highly consistent and show a robust effect of dietary B-vitamins on plasma DHA concentration.

The methylation of PE to PC by PEMT requires the methyl donor SAM. This reaction is not only influenced by the availability of SAM, but is also inhibited by SAH; the ratio of SAM to SAH therefore affects the activity of PEMT [8]. Moreover, SAH is hydrolyzed to homocysteine via a reversible reaction: thus excess homocysteine will result in increased SAH, thereby inhibiting PEMT [22]. Folate, vitamin B-12, and vitamin B-6 could enhance PEMT activity both by reducing homocysteine levels and by increasing methionine levels, resulting in an increased SAM to SAH ratio, and thus an increased methylation capacity. Both experiments revealed a significant inverse correlation between plasma homocysteine and plasma DHA concentrations. This correlation suggests that the observed effects of B-vitamins on plasma DHA concentration may be explained by the effects of these vitamins on methylation capacity and subsequent enhancement of PC synthesis by the PEMT pathway. Because PC species generated by the PEMT pathway are rich in PUFAs, especially DHA [10,14,15], and because the PEMT pathway influences secretion of VLDL from the liver [12,13], the PEMT pathway may contribute to transport of DHA from the liver to peripheral tissues. This hypothesis is supported by findings from Zeisel *et al.*, who investigated lipid metabolism in PEMT knockout mice and found diminished plasma levels of DHA in adult knockout animals [9]. In a subsequent study, brains from fetuses (embryonic day 17) derived from PEMT knockout dams had lower levels of DHA in several phospholipid species in the brain as compared to wild type controls [16]. In addition, results of a recent human study even suggest that plasma PC-DHA can be used as a marker for *in vivo* hepatic PEMT activity [23].

The present results show that concurrent dietary folate, vitamin B-12, and vitamin B-6 deficiency can lower plasma DHA concentration, which is in line with three previous experiments investigating effects of deficiencies of single B-vitamins in rats. Durand *et al.* found that dietary folate deficiency reduces relative n3 PUFA levels of the plasma total lipid fraction in rats [4]. In an early

study by Peifer and Lewis, PUFA levels in PC and PE of rat liver and brain were changed by long term (20 weeks) dietary vitamin B-12 deficiency [5]. Dietary vitamin B-6 deficiency was also found to decrease the proportion of PUFAs in the phospholipid fractions of various rat tissues [6]. In addition to the observations on the effects of B-vitamin deficiency, the present results indicate that plasma DHA concentrations can be enhanced by combined supplementation with high levels of these vitamins. Pita and Delgado previously also found a rise in the proportion of DHA in various lipid fractions in plasma and other tissues after daily folate administration in rats for 15 days [24]. In that study, folate was administered intramuscularly (500 µg/kg body weight as 5-methyltetrahydrofolate). Notwithstanding the different route of administration, this intramuscular dose falls in the dietary supplementation range used in the present study, i.e. approximately 200 and 800 µg folate per kg body weight per day for, respectively, the B-vitamin-enriched and B-vitamin-high diet.

The interdependence between B-vitamin intake or status, homocysteine, and DHA status is also suggested by several studies in humans. In a multicenter trial in pregnant women, oral folate supplementation (400 µg/day) was associated with a minimal but significant increase in maternal plasma DHA level in the phospholipid fraction when compared to the control subjects over time [25]. Crowe *et al.*, however, observed no change in relative DHA level in plasma PC after supplementation of folate (1000 µg/day), vitamin B-12 (500 µg/day), and B-6 (10 mg/day) for two years to older individuals [26]. The discrepancy between these results and those of the present preclinical study might be explained by the relative considerable higher dose of folate used in the present study. Additionally, analysis of the percentage of DHA in one phospholipid fraction, as was performed by Crowe *et al.*, is an essentially different parameter than the absolute DHA concentration of the total lipid fraction measured in the present experiment. In accordance with the present results, several clinical studies have shown a negative correlation between plasma, serum, or erythrocyte phospholipid DHA content and plasma levels of homocysteine [1-3], SAH [3], or SAM:SAH ratio [23]. Moreover, two clinical studies in men indicated a positive correlation either between plasma DHA concentration and erythrocyte folate level [27] or plasma phospholipid DHA concentration and serum vitamin B-12 level [1]. In the latter study, plasma phospholipid DHA did not correlate with serum folate [1].

All three B-vitamins are necessary for methylation capacity. Folate (as 5-methyltetrahydrofolate) and vitamin B-12 are cofactors in the remethylation of homocysteine to methionine, from which SAM is subsequently regenerated. Vitamin B-6 is involved in

facilitating the reversible conversion of serine to glycine, which ultimately can generate 5-methyltetrahydrofolate, i.e. the methyl donor for the remethylation of homocysteine to methionine. Vitamin B-6 is also the cofactor for the transsulfuration reaction responsible for the irreversible conversion of homocysteine to cysteine, and hence the clearance of homocysteine. It can be speculated that the effects of the three B-vitamins acting together have a greater impact on PC metabolism via PEMT than each B-vitamin individually. Nevertheless, it should also be noted that the exact mechanism(s) by which the three B-vitamins could have increased plasma DHA concentration is not fully understood and that the relative contribution of PEMT activity to total plasma DHA concentration is unknown. To elucidate this, additional experiments are required in which additional parameters could be measured, e.g. plasma and tissue levels of B-vitamins, SAM, SAH, and phospholipid species.

Recently, we demonstrated that rat plasma choline concentration is also dependent on dietary intake of the three B-vitamins, possibly mediated by enhancing methylation capacity and hence PC synthesis [28]. Because levels of DHA [29-31] and choline [32,33] in the brain are affected by their plasma concentrations, supplemental dietary B-vitamins could ultimately sustain brain DHA and choline levels. This may be relevant in conditions such as Alzheimer's disease, that are associated with lower plasma and/or brain levels of DHA [34,35], choline [36], and concurrent B-vitamin deficiencies [37,38].

## Conclusion

The present study shows that concurrently varying the dietary intake of folic acid, vitamin B-12, and vitamin B-6 can influence plasma concentration of DHA. Poor dietary intake of the three B-vitamins causes plasma DHA to decrease and this decrease can be abolished through dietary supplementation with these vitamins. Furthermore, plasma DHA concentrations can be enhanced by supplemental intake of these B-vitamins exceeding normal dietary recommendations.

## Abbreviations

CDP: Cytidine Di Phosphate; DHA: Docosahexaenoic Acid; PC: Phosphatidyl Choline; PE: Phosphatidyl Ethanolamine; PEMT: Phosphatidyl Ethanolamine-N-Methyl Transferase; PUFA: Poly Unsaturated Fatty Acid; SAH: S-Adenosyl Homocysteine; SAM: S-Adenosyl Methionine; VLDL: Very Low Density Lipoprotein.

## Competing interests

The research as described in this paper was funded by Nutricia Advanced Medical Nutrition, Danone Research, Centre for Specialised Nutrition. Author disclosures. NvW, RJH, JWCS, PJGHK and LMB are all employees of Danone Research, Centre for Specialised Nutrition. RJW is a scientific consultant of Danone Research, Centre for Specialised Nutrition. CJW has no competing interests.

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### Authors' contributions

The contribution of each author to the present paper was as follows: NvW, RJJH, JWCS, PJGHK, RJW and LMB were responsible for the research design; NvW and CJW conducted research; NvW performed data analysis and statistical analysis; and NvW and LMB prepared the manuscript. All co-authors read and approved the final manuscript.

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