Research Article

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Higher circulating vitamin B12 is associated with lower levels of inflammatory markers in individuals at high cardiovascular risk and in naturally aged mice

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Abstract

BACKGROUND: Vitamin B12 is an essential nutrient that is involved in numerous physiological processes, and its deficiency can lead to various complications, including neurological and haematological disorders. Some studies have suggested that vitamin B12 may have anti-inflammatory effects, but the mechanisms underlying this relationship are not yet fully understood. We investigated the relationship between circulating vitamin B12 and inflammatory markers interleukin (IL)-6 and C-reactive protein (CRP). The association of peripheral levels of vitamin B12 with IL-6 and CRP was assessed in 136 human samples from a high cardiovascular risk population. To corroborate the results from the human trial, the analysis was replicated in naturally aged mice.

RESULTS: Individuals with higher serum levels of vitamin B12 showed lower concentrations of IL-6 and CRP after adjustment for potential confounders, and an inverse association was also found between serum IL-6 and vitamin B12 levels in naturally aged mice.

CONCLUSION: Circulating vitamin B12 was inversely associated with IL-6 and CRP in humans and with IL-6 in mice, suggesting that it may exert an anti-inflammatory effect through modulation of these pro-inflammatory molecules.

Supporting information may be found in the online version of this article.

Keywords: micronutrients; bioactive compounds; inflammation; intereukin-6; C-reactive protein; PREDIMED trial

INTRODUCTION

Vitamin B12 or cobalamin is known for its role in essential biological processes[1] because there are two key vitamin B12-dependent enzymes: methionine synthase and methylmalonyl-CoA mutase.[2] Methionine synthase is involved in the conversion of homocysteine to methionine, an important amino acid that is required for protein

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synthesis and many other physiological processes.[3] This enzyme also generates *S*-adenosylmethionine, a vital molecule that donates methyl groups for DNA, RNA and other reactions, impacting gene expression and metabolism.[4] Methylmalonyl-CoA mutase plays a key role in the breakdown of certain amino acids, including valine, isoleucine, methionine and threonine, which otherwise could not be properly metabolized.[5]

Clinical vitamin B12 deficiency is prevalent, affecting a significant proportion of the population, with estimates ranging from 1.5% to 15%, particularly among the elderly.[6] This deficiency can lead to neurological or haematological complications. Despite its essentiality for all eukaryotes, vitamin B12 is only synthesized by some genus of bacteria and some archaea; humans obtain it almost exclusively from dietary animal-derived products, where it is stored within animal proteins.[7] Dietary insufficiency therefore is a major concern for vegan and vegetarian populations, especially when no supplementation or fortified foods are used.[8] However, vitamin B12 deficiency can also be a result of inefficient absorption and digestion because its bioavailability is highly dependent on individual metabolic characteristics.[9] The process of vitamin B12 uptake in mammals is complex and extensively regulated. It starts with salivary enzymes dissociating it from animal proteins, followed by binding with haptocorrin (also known as transcobalamin I) to prevent degradation.[10] In the stomach, digestive enzymes release B12 from haptocorrin, and intrinsic factor binds to it.[10] Intrinsic factor is necessary for uptake in the terminal ileum, where vitamin B12 is processed and bound by transcobalamin II for release into the circulation.[11] The holo-transcobalamin complex (vitamin B12 bound to transcobalamin II) is recognized and taken up by cells via CD320 receptor.[11] Mutations in genes encoding these proteins can cause B12 deficiency.[12] In addition, vitamin B12 requirements increase during pregnancy, lactation and at older ages, with the latter often as a result of decreased absorption.[13]

The Mediterranean diet is well-known for including a wide variety of products and culinary techniques, which minimize the risk of deficiencies of specific nutrients. Although it is mostly characterized by high consumption of plant-derived products, it also includes moderate consumption of both fish and dairy products, and low consumption of meat.[14] Through these animal-derived proteins, the Mediterranean diet provides enough vitamin B12 to meet the nutritional requirements of 4.0 μg day^{-1} proposed by the European Food Safety Authority.[15] The benefits of a Mediterranean diet were assessed in the PREDIMED (PREvención con DIeta MEDiterránea) trial, a multicenter study of over 7000 participants at risk for cardiovascular disease.[16,17] Adhering to a Mediterranean diet regiment supplemented with extra virgin olive oil or nuts conferred a significant decrease in heart attack, stroke or cardiovascular death.[18]

Vitamin B12 or folic acid deficit leads to increased levels of homocysteine. Homocysteine is a sulphur-containing amino acid strongly associated with inflammation because it induces the production of proinflammatory molecules and reactive oxygen species.[19] This is particularly interesting because most common chronic and autoimmune diseases, including diabetes, cancer and atherosclerosis, are closely related to inflammatory processes.[20] We hypothesized that serum vitamin B12 may be associated with lower concentrations of inflammatory biomarkers. Therefore, the present study aimed to assess serum vitamin B12 levels and to evaluate their association with the circulating inflammatory molecules interleukin (IL)-6 and C-reactive protein (CRP) in a cross-sectional subanalysis of participants in the PREDIMED trial. To extend to an experimental system the results observed in humans, the analysis was replicated in an experimental system with naturally aged mice that could be manipulated and followed-up to avoid the chance effect.

MATERIALS AND METHODS

Clinical trial

Study design

A cross-sectional analysis was carried out using baseline data from the PREDIMED trial, a large, parallel-group, multicenter, randomized, controlled, clinical trial designed to assess the effect of the Mediterranean diet on the primary prevention of cardiovascular disease (http://www.predimed.es). It included 7447 participants at high cardiovascular risk, aged between 55 to 80 years for men and 60–80 years for women, who were recruited in Spain between October 2003 and December 2010. Eligible participants had either type 2 diabetes at baseline or at least three of the following cardiovascular risk factors: current smoking, hypertension, dyslipidemia, overweight/obesity or family history of premature coronary heart disease. A detailed description of methods and participants has been reported previously.[16,17]

In the present study, 136 participants were randomly selected among the participants from the PREDIMED-Hospital Clinic recruitment center (Barcelona) from whom samples were available.

Ethics statement

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on 16 July 2002. All participants provided their written informed consent.

Covariate assessment

A validated, semi-quantitative 137-item food frequency questionnaire was used to determine food consumption within the prior year with the assistance of trained dietitians.[21] Nutrient intakes were calculated from Spanish food composition tables. Trained personnel took anthropometric measures by standard methods, including weight and height, from which body mass index (BMI) (kg m^{-2}) was calculated. Physical activity (metabolic equivalent tasks per minutes per day, METs min day^{-1}) was assessed with a validated Spanish version of the Minnesota physical activity questionnaire.[22]

Inflammatory biomarkers

Circulating inflammatory biomarkers were analysed as described elsewhere.[23] Briefly, commercial enzyme-linked immunosorbent assays (ELISA) kits were used to determine plasma IL-6 (Elast Amplification System; PelkinElmer, Waltham, MA, USA). A technician blinded to group allocation was responsible for processing the samples. As reported previously, high-sensitive CRP was determined in serum by particle-enhanced immunonephelometry.[23]

Serum vitamin B12

Serum vitamin B12 concentrations were measured by an automated electrochemiluminescence immunoassay system (Advia-Centaur; Siemens, Barcelona, Spain) in frozen aliquots kept at −80 °C.

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Statistical analysis

Baseline characteristics of the participants are presented as the mean ± SD for continuous variables and percentages for categorical variables.

We used multivariable adjusted linear regression models to assess the association between serum vitamin B12 and circulating inflammatory biomarkers. Three adjustment models of increasing complexity were used. Model 1 was minimally adjusted for sex and age. Model 2 was further adjusted for educational level, smoking habit, BMI, physical activity, diabetes, hypertension, hypercholesterolemia and aspirin use. Model 3 was additionally adjusted for energy intake, alcohol consumption and Mediterranean diet adherence.

Animal studies

Animal procedures

Animal experimentation at the IRB Barcelona was performed according to protocols approved by the Science Park of Barcelona Ethics Committee for Research and Animal Welfare. C57BL6/J mice ($n = 5$ females, $n = 13$ males, based on availability in our colony) were bred and aged in-house in a specific pathogen-free facility on a 12:12 h light/dark photocycle (lights on 20:00 h). Mice were fed commercially available diets manufactured by SAFE® Complete Care Competence (Rosenberg, Germany) (SAFE A30 during pregnancy and weaning; SAFE A40 in adulthood) containing 0.02 mg kg^{-1} vitamin B12. Mice were deeply anesthetized in a carbon dioxide chamber, and blood was collected several hours prior to the end of the light cycle, around 17:00 h, by intracardiac puncture, followed by cervical dislocation. Upon collection, blood was allowed to clot at room temperature for 10 min, and then spun at 6000 rpm for 10 min in a microcentrifuge. The supernatant containing serum was removed and snap frozen.

IL-6 determination

Serum IL-6 was analysed by ELISA using the Mouse IL-6 ELISA Kit from Sigma-Aldrich (St Louis, MO, USA). Antibody levels higher than 2 pg mL^{-1} were considered positive in accordance with the manufacturer's guidelines. It was not technically possible to measure CRP in mice because the sensitivity of the current assays for CRP is not sufficient to detect the low levels found in mice.

Vitamin B12 determination

Serum was diluted 1:20 in PBS. HoloTC was measured using an ADVIA Centaur Immunoassay System (Siemens) with ADVIA Centaur Vitamin B12 Test Packs (Ref 07847260) in accordance with the manufacturer's instructions.

Statistical analysis

Graphs were generated using Prism, version 9 (GraphPad Software Inc., San Diego, CA, USA) and statistical analysis was performed using simple linear regression. We found two mice outliers with IL-6 levels greater than 2 SD from the mean, potentially suggesting illness or infection, and one mouse with vitamin B12 levels greater than 2 SD, indicative of hematologic or hepatic disease.[24] We removed these three mice from our dataset, interpreting the subset that remained as the mice that were likely to be healthy in their natural aging. Linear regressions were used to assess the association between vitamin B12 and IL-6. We performed further analyses to describe the relationship of age with vitamin B12 and IL-6.

RESULTS

General characteristics of the participants in the clinical trial

The general characteristics of the participants in the trial with available data on IL-6 and vitamin B12 included in this substudy are shown in Table 1, according to tertiles of serum vitamin B12. The average mean concentrations of vitamin B12 in serum in each of the three groups were 0.3 ± 0.2, 0.4 ± 0.0 and 0.7 ± 0.2 ng mL^{-1}. The mean age of the participants was 68.3 ± 6.0 years and 56% were women. All the groups were well balanced in terms of age, sex and BMI. As a result of the study design, all participants were

Table 1. General characteristics of the participants by tertiles of serum vitamin B12

Characteristics	All participants ($n = 136$)	T1 ($n = 46$)	T2 ($n = 45$)	T3 ($n = 45$)	*P*-value
Age (years)	68.3 ± 6.0	69.3 ± 5.6	67.8 ± 5.5	67.6 ± 6.7	0.357
Women (%)	55.9	50.0	57.8	60.0	0.600
BMI (kg m^{-2})	29.1 ± 4.3	29.1 ± 3.4	29.2 ± 3.3	28.9 ± 3.6	0.883
Diabetes (%)	65.4	69.6	71.1	55.6	0.231
Hypertension (%)	73.5	82.6	71.1	66.7	0.205
Hypercholesterolemia (%)	72.8	58.7	77.8	82.2	0.027
Current smokers (%)	17.7	21.7	15.6	15.6	0.064
Medium and high educational level (%)	30.9	37.0	37.8	17.8	0.067
Physical activity (METS-min day^{-1})	281 ± 259	324 ± 265	195 ± 181	324 ± 305	0.022
Energy intake (kcal day^{-1})	2425 ± 556	2533 ± 599	2310 ± 593	2428 ± 451	0.161
Vitamin supplementation (%)	5.2	2.2	8.9	4.4	0.338
Mediterranean diet adherence[a]	8 ± 2	9 ± 2	8 ± 2	8 ± 2	0.784
Serum vitamin B12 (ng mL^{-1})	0.47 ± 0.20	0.29 ± 0.07	0.45 ± 0.04	0.68 ± 0.20	< 0.001

Note: Continuous variables are shown as the mean ± SD and categorical variables are shown as percentages. A *t*-test was used for continuous variables and a chi-square test was used for categorical variables.
Abbreviations: BMI, body mass index; METS, metabolic equivalents; T, tertile.
[a] Mediterranean diet adherence was assessed with a 14-item score.

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Table 2. Multivariable-adjusted regression that evaluate the association between serum B12 levels and circulating IL-6 and CRP

Inflammatory molecules	Mean (pg mL^{-1})		β (95% CI)	*P*-value
IL-6	0.73	Model 1	−0.20 (−0.52 to 0.13)	0.232
		Model 2	−0.35 (−0.70 to 0.00)	0.049
		Model 3	−0.39 (−0.76 to −0.03)	0.035
	Mean (mg/dL)		**β (95% CI)**	***P*-value**
CRP	0.50	Model 1	−0.14 (−0.41 to 0.12)	0.290
		Model 2	−0.29 (−0.54 to −0.03)	0.028
		Model 3	−0.34 (−0.63 to −0.05)	0.020

Note: Model 1: adjusted for sex and age. Model 2: additionally adjusted for education level, smoking habit, body mass index, physical activity, diabetes, hypertension, hypercholesterolemia and aspirin medication. Model 3: additionally adjusted for energy intake, alcohol consumption and Mediterranean diet adherence.
Abbreviations: ß, difference between groups; CI, confidence interval; CRP, C reactive protein; IL-6, interleukin 6.

overweight or obese and harboured a high load of cardiovascular risk factors: 65.4% had type-2 diabetes, 73.5% had hypertension and 72.8% had hypercholesterolemia. A higher percentage of participants in the highest tertile of vitamin B12 had hypercholesterolemia ($P = 0.027$). The mean physical activity was 281.2 ± 258.7 METS-min day^{-1}, and participants in the second tertile were less physically active ($P = 0.022$). Total energy intake and Mediterranean diet adherence were comparable among groups.

The nutrient and food consumption of participants by tertiles of serum vitamin B12 are shown in the Supporting information (Table S1). The three groups of serum vitamin B12 were well-balanced in all nutrients and food groups.

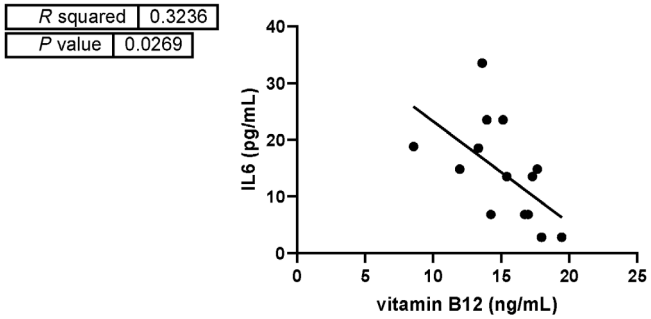

Figure 2. Association between vitamin B12 and IL-6 in mice. IL-6, interleukin-6.

Association between vitamin B12 and IL-6 and CRP in humans

The association between serum concentrations of vitamin B12 and circulating concentrations of IL-6 is presented in Table 2. In the minimally adjusted model, which accounted for sex and age, the association between IL-6 and vitamin B12 presented an inverse, albeit not statistically significant, relationship. Further adjustment for other potential confounders in the multivariable models 2 revealed a significant inverse association between IL-6 and vitamin B12 (−0.39 pg mL^{-1}; 95% confidence interval = −0.76 to

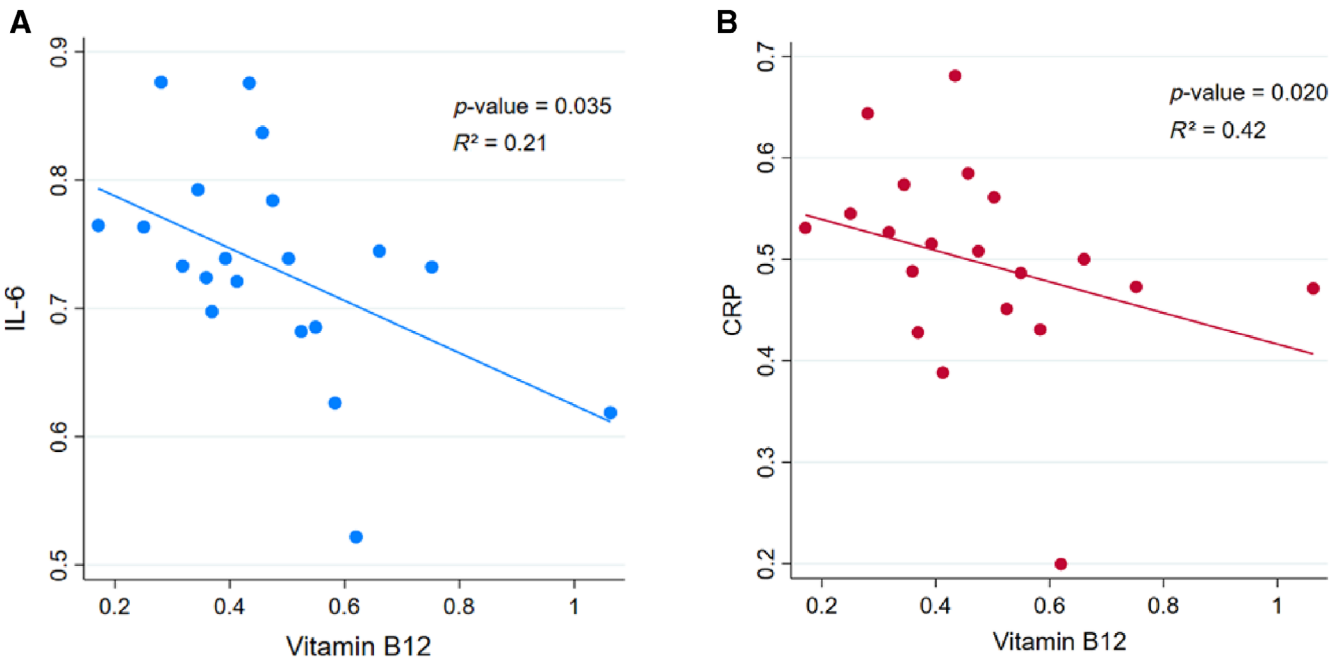

Figure 1. Binned scatterplot of the relationship between vitamin B12 and IL-6 (A) and CRP (B) in humans adjusted for sex, age, education level, smoking habit, BMI, physical activity, diabetes, hypertension, hypercholesterolemia, aspirin, medication, energy intake, alcohol consumption and Mediterranean diet adherence. IL-6, interleukin-6. CRP, C-reactive protein.

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−0.03, $P = 0.031$). Similar results were obtained for CRP. In the minimally adjusted model, no significant association was found. After adjustment for models 2 and 3, a significant inverse association was found between CRP and vitamin B12 (−0.34 pg mL^{-1}; 95% confidence interval = −0.63 to −0.05, $P = 0.020$). Figure 1 illustrates that participants with higher concentrations of vitamin B12 had lower concentrations of IL-6 (A) and CRP (B) in the fully adjusted model.

Serum vitamin B12 levels are negatively correlated with IL-6 in naturally aged mice

We next wanted to extend our observation in an experimental animal model of natural aging, independent from cardiovascular disease. Although circulating IL-6 levels are generally low in healthy, unstressed laboratory mice, it has been reported that IL-6 levels increase with natural aging.[25] To assess biologically available vitamin B12 levels, we measured holotranscobalamin in the serum of mice. Following the exclusion of three outliers (defined as > 2 SD above the mean or *z*-score = 2; for details, see Materials and methods), two mice displaying IL-6 levels likely indicative of illness or infection, along with one mouse exhibiting elevated levels of vitamin B12, suggestive of hematologic or hepatic disease, there was a significantly negative correlation between serum IL-6 and vitamin B12 levels in naturally aged mice ($P = 0.027$) (Fig. 2). Taken together, our results indicate a negative association between serum IL-6 and vitamin B12 levels in healthy, naturally aged wild-type mice. When aging was considered on each variable separately, IL-6 and vitamin B12, there was no correlation between age and vitamin B12 levels, whereas IL-6 levels showed a modest but insignificant increase with age (see Supporting information, Fig. S1).

DISCUSSION

The present study aimed to characterize the relationship of circulating vitamin B12 with IL-6 and CRP. The analysis of the PREDIMED trial samples revealed that serum vitamin B12 is associated with lower levels of inflammatory molecules IL-6 and CRP. The data from naturally aged, healthy, wild-type mice provided supporting evidence by also showing an inverse relationship between serum vitamin B12 and IL-6.

Evidence suggests that vitamin B12 deficiency is associated with inflammation and metabolic complications.[26,27] Tripathi *et al.*[28] showed that dietary vitamin B12, together with folate, decreased inflammation in a mouse model of non-alcoholic steatohepatitis (NASH) through reduction of hyperhomocysteinemia. Vitamin B12 deficiency promoted NASH through activation of proinflammatory pathways with an imbalanced release of pro-inflammatory IL-1β and anti-inflammatory IL-10 in animals with colitis.[29] In humans, it was found that vitamin B12 may have a positive effect on inflammation and oxidative damage by improving the antioxidant capacity of plasma.[30] In a cross-sectional study that included middle-aged participants, a negative association was observed between tumor necrosis factor-α and serum vitamin B12.[31]

There are some potential explanations for these findings. Deficiency of vitamin B12 leads to elevated levels of homocysteine because this molecule cannot be converted into methionine via the methionine cycle because methionine synthase, which requires B12 as a cofactor, cannot function properly.[32] Hyperhomocysteinemia is a pathology characterized by homocysteine accumulation that leads to proinflammatory, cytotoxic and proatherogenic effects.[28] It has been reported that it is associated with pro-inflammatory cytokines, including IL-6, and is related to neuroinflammation.[33] Hyperhomocysteinemia also causes endothelial damage, reduces the arterial compliance and alters the process of endothelial homeostasis.[34] This condition is a potential risk for cardiovascular diseases such as atherosclerosis or coronary arterial disease.[35] Another hypothesis to explain the inverse relationship between vitamin B12 and inflammation suggests that the vitamin supresses the production of cytokines in T lymphocytes. Yamashiki *et al.*[36] demonstrated that the *in vitro* synthesis of cytokines including IL-6, interferon-γ and IL-1β was reduced when methyl B12 was added in culture. Overall, the findings suggest that vitamin B12 plays an important role in modulating inflammation.

The specific relationship between vitamin B12 and the inflammatory biomarkers IL-6 and CRP has been scarcely investigated. Scalabrino *et al.*[37] reported that vitamin B12 regulated IL-6 levels in rat cerebrospinal fluid, independently of other regulators of IL-6 production such as vasoactive intestinal peptide or somatostatin. An *in vitro* study found increased gene expression of IL-6 and other interleukins in adipocytes cultured in low vitamin B12 conditions.[38] These results are in line with the findings of the present study because we have observed that higher levels of vitamin B12 relate to lower concentrations of IL-6 in mice. Evidence from observational studies and clinical trials support these results. Ma *et al.*[39] found that supplementation of combined folate and vitamin B12 in elders with mild cognitive impairment reduced inflammatory cytokines, including IL-6, in association with lowering homocysteine levels. In patients with Alzheimer's disease, higher levels of IL-6 were detected in peripheral blood mononuclear cells when serum vitamin B12 concentrations were lower.[40] These findings are consistent with our results because we found an inverse association of vitamin B12 with inflammatory biomarkers. It is worth mentioning that the populations of these studies were similar to ours in that elderly adults were included. However, in the PREDIMED trial, participants with cognitive impairment or dementia were not included. A clinical trial with 285 patients with transient ischemic attack or stroke found that supplementation with 0.5 mg of vitamin B12, 2 mg of folic acid and 25 mg of B6 for 6 months did not reduce blood concentrations of IL-6 or CRP.[41] Nevertheless, the biological concentrations of vitamin B12 were not assessed, and thus it was not possible to determine whether the supplements were sufficient to increase blood levels of the vitamin. Previous studies have failed to establish a connection between vitamin B12 and CRP levels. Two clinical trials reported no CRP changes after an intervention with vitamin B12.[42,43] Young *et al.*[44] found a weak inverse correlation between vitamin B12 and CRP in a cross-sectional analysis. A large Finnish observational study failed to find any significant association between serum vitamin B12 and CRP levels.[45] Further studies of the clinical use of vitamin B12 as an anti-inflammatory and to reduce the risk of cardiac disease are warranted.

The inverse association of vitamin B12 and IL-6 may be relevant to inflammatory processes, as IL-6 is involved in chronic and acute inflammation.[46] In acute inflammation, IL-6 promotes the synthesis and release of most proteins involved in the acute phase response to a wide variety of stimuli. One of the acute-phase proteins is CRP,[47] which was also significantly inversely associated with vitamin B12 in our clinical study; we did not measure CRP in mouse serum because values would be below the limits of detection. IL-6 also mediates the transition from acute to chronic inflammation by recruiting monocytes to the area involved.[48] Through inflammatory processes, these molecules play an important role in the development of other diseases. Regarding

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cardiovascular health, IL-6 is systematically increased in patients with obesity due to an increased release from adipocytes.[49] It is also involved in the development of insulin resistance and β-cell dysfunction that lead to type-2 diabetes. Recently, IL-6 has been proposed as a potential target for cancer treatment as a result of its involvement in the proliferation of cancerous cells.[50,51] Strong evidence suggests that CRP is a predictor of arterial thrombotic events. Two meta-analyses have confirmed that CRP is linked to a higher risk of incident cardiovascular events and can also predict future cardiovascular and all-cause mortality in individuals with type-2 diabetes.[52,53] CRP is also a potential biomarker for overall cancer because it plays a role in the occurrence of various types of cancer.[54,55]

The present study has some limitations. First, we had only a modest number of mice and humans in our study, and the human participants were older Mediterranean individuals at high cardiovascular risk; therefore, the results cannot be generalized. Second, we used a single measure of vitamin B12 and inflammatory biomarkers at baseline, which limits the potential to discern temporal and causal relationships. Our study also has important strengths. For the first time, we have assessed the relationship between circulating vitamin B12 and IL-6 both in mice and humans. Another strength is the analysis of biological samples to determine the status of the experimental animals and human participants.

CONCLUSIONS

There was an inverse association between peripheral vitamin B12 levels and IL-6 in a cross-sectional subanalysis of the PREDIMED trial, which involved a Mediterranean population at high cardiovascular risk, as well as in naturally aged mice, indicating that higher levels of vitamin B12 were linked to lower levels of IL-6. These findings support the potential role of vitamin B12 in inflammatory processes and related diseases. Further research is needed to identify the molecular mechanisms linking this vitamin to the production of IL-6 and its potential as a clinical intervention in cardiovascular and other inflammatory diseases. Importantly, extending our observation to naturally aged laboratory mice presents an attractive experimental system for future studies.

AUTHOR CONTRIBUTIONS

RE, MS and RMLR were responsible for conceptualization. IDL, MK, and RC were responsible for formal analysis. IDL and MK were responsible for writing the original draft. ET, MF, ER, RE, MS and RMLR were responsible for reviewing and editing.

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CONFLICTS OF INTEREST

ER reports grants, personal fees, non-financial support and other from the California Walnut Commission when the study was carried out; and grants, personal fees, non-financial support and other from Alexion, outside the submitted work. RML-R reports personal fees from Cerveceros de España, personal fees and others from Adventia, Wine in Moderation, Ecoveritas S.A., outside the submitted work. RE reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain) and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Alburquerque, USA), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain) and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Kräutler B, Biochemistry of B12-cofactors in human metabolism. *Subcell Biochem* **56**:323–346 (2012).
2 Marsh E, Coenzyme B12 (cobalamin)-dependent enzymes. *Essays Biochem* **34**:139–154 (1999).
3 Banerjee R, B12 trafficking in mammals: a for coenzyme escort service. *ACS Chem Biol* **1**:149–159 (2006).
4 Froese DS, Fowler B and Baumgartner MR, Vitamin B12, folate, and the methionine remethylation cycle—biochemistry, pathways, and regulation. *J Inherit Metab Dis* **42**:673–685 (2019).
5 Takahashi-Iñiguez T, García-Hernandez E, Arreguín-Espinosa R and Flores ME, Role of vitamin B12 on methylmalonyl-CoA mutase activity. *J Zhejiang Univ Sci B* **13**:423–437 (2012).

6 Vitamin B12 Deficiency (2022). Available: https://my.clevelandclinic.org/health/diseases/22831-vitamin-b12-deficiency [13 April 2023].
7 Watanabe F and Bito T, Vitamin B12 sources and microbial interaction. *Exp Biol Med (Maywood)* **243**:148–158 (2018).
8 Zeuschner CL, Hokin BD, Marsh KA, Saunders AV, Reid MA and Ramsay MR, Vitamin B_{12} and vegetarian diets. *Med J Aust* **199**:S27–S32 (2013).
9 Green R, Allen LH, Bjørke-Monsen AL, Brito A, Guéant JL, Miller JW *et al.*, Vitamin B12 deficiency. *Nat Rev Dis Primers* **3**:17040 (2017).
10 Blakeley M, Sobczyńska-Malefora A and Carpenter G, The origins of salivary vitamin a, vitamin B12 and vitamin D-binding proteins. *Nutrients* **12**:1–12 (2020).
11 Green R and Vitamin B, 12 deficiency from the perspective of a practicing hematologist. *Blood* **129**:2603–2612 (2017).
12 Froese DS and Gravel RA, Genetic disorders of vitamin B 12 metabolism: eight complementation groups–eight genes. *Expert Rev Mol Med* **12**:1–20 (2010).
13 Wong CW, Vitamin B12 deficiency in the elderly: is it worth screening? *Hong Kong Med J* **21**:155–164 (2015).
14 Bach-Faig A, Berry EM, Lairon D, Reguant J, Trichopoulou A, Dernini S *et al.*, Mediterranean diet pyramid today. Science and cultural updates. *Public Health Nutr* **14**:2274–2284 (2011).
15 Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline, *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. The National Academies Press, Washington DC (1998).
16 Martínez-González MÁ, Corella D, Salas-salvadó J, Ros E, Covas MI, Fiol M *et al.*, Cohort profile: design and methods of the PREDIMED study. *Int J Epidemiol* **41**:377–385 (2012).
17 Estruch R, Ros E, Salas-Salvadó J, Covas M-I, Corella D, Arós F *et al.*, Primary prevention of cardiovascular disease with a mediterranean diet supplemented with extra-virgin olive oil or nuts. *N Engl J Med* **378**:e34 (2018).
18 Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, Ruiz-Gutierez V, Covas MI *et al.*, Annals of internal medicine article effects of a mediterranean-style diet on cardiovascular risk factors. *Ann Intern Med* **145**:1–11 (2006).
19 Moretti R and Caruso P, The controversial role of homocysteine in neurology: from labs to clinical practice. *Int J Mol Sci* **20**:231 (2019).
20 Del GM and Gangestad SW, Rethinking IL-6 and CRP: why they are more than inflammatory biomarkers, and why it matters. *Brain Behav Immun* **70**:61–75 (2018).
21 Fernández-Ballart JD, Piñol JL, Zazpe I, Corella D, Carrasco P, Toledo E *et al.*, Relative validity of a semi-quantitative food-frequency questionnaire in an elderly mediterranean population of Spain. *Br J Nutr* **103**:1808–1816 (2010).
22 Elosua R, Garcia M, Aguilar A, Molina L, Covas MI and Marrugat J, Validation of the Minnesota leisure time physical activity questionnaire in Spanish women. *Med Sci Sports Exerc* **32**:1431–1437 (2000).
23 Casas R, Sacanella E, Urpí-Sardà M, Chiva-Blanch G, Ros E, Martínez-González M-A *et al.*, The effects of the mediterranean diet on biomarkers of vascular wall inflammation and plaque vulnerability in subjects with high risk for cardiovascular disease. A randomized trial. *PLoS One* **9**:e100084 (2014).
24 Ermens AAM, Vlasveld LT and Lindemans J, Significance of elevated cobalamin (vitamin B12) levels in blood. *Clin Biochem* **36**:585–590 (2003).
25 Jergović M, Thompson HL, Bradshaw CM, Sonar SA, Ashgar A, Mohty N *et al.*, IL-6 can singlehandedly drive many features of frailty in mice. *Geroscience* **43**:539–549 (2021).
26 Guéant JL and Alpers DH, Vitamin B12, a fascinating micronutrient, which influences human health in the very early and later stages of life. *Biochimie* **95**:967–969 (2013).
27 Romain M, Sviri S, Linton DM, Stav I and van Heerden PV, The role of Vitamin B12 in the critically ill–a review. *Anaesth Intensive Care* **44**:447–452 (2016).
28 Tripathi M, Singh BK, Zhou J, Tikno K, Widjaja A, Sandireddy R *et al.*, Vitamin B12 and folate decrease inflammation and fibrosis in NASH by preventing syntaxin 17 homocysteinylation. *J Hepatol* **77**:1246–1255 (2022).
29 Harb Z, Deckert V, Bressenot AM, Christov C, Guéant-Rodriguez RM, Raso J *et al.*, The deficit in folate and vitamin B12 triggers liver macrovesicular steatosis and inflammation in rats with dextran sodium sulfate-induced colitis. *J Nutr Biochem* **84**:108415 (2020).
30 Guest J, Bilgin A, Hokin B, Mori TA, Croft KD and Grant R, Novel relationships between B12, folate and markers of inflammation, oxidative stress and NAD(H) levels, systemically and in the CNS of a healthy human cohort. *Nutr Neurosci* **18**:355–364 (2015).
31 Al-Daghri NM, Rahman S, Sabico S, Yakout S, Wani K, Al-Attas OS *et al.*, Association of vitamin B12 with pro-inflammatory cytokines and biochemical markers related to cardiometabolic risk in Saudi subjects. *Nutrients* **8**:1–8 (2016).
32 van de Lagemaat EE, de Groot LC and van den Heuvel EG, Vitamin B12 in relation to oxidative stress: a systematic review. *Nutrients* **11**:482 (2019).
33 Weekman EM, Sudduth TL, Price BR, Woolums AE, Hawthorne D, Seaks CE *et al.*, Time course of neuropathological events in hyperhomocysteinemic amyloid depositing mice reveals early neuroinflammatory changes that precede amyloid changes and cerebrovascular events. *J Neuroinflammation* **16**:268 (2019).
34 Baszczuk A and Kopczyński Z, Hyperhomocysteinemia in patients with cardiovascular disease. *Postepy Hig Med Dosw* **68**:579–589 (2014).
35 Ganguly P and Alam SF, Role of homocysteine in the development of cardiovascular disease. *Nutr J* **14**:6 (2015).
36 Yamashiki M, Nishimura A and Kosaka Y, Effects of methylcobalamin (vitamin B12) on in vitro cytokine production of peripheral blood mononuclear cells. *J Clin Lab Immunol* **37**:173–182 (1992).
37 Scalabrino G, Corsi MM, Veber D, Buccellato FR, Pravettoni G, Manfridi A *et al.*, Cobalamin (vitamin B12) positively regulates interleukin-6 levels in rat cerebrospinal fluid. *J Neuroimmunol* **127**:37–43 (2002).
38 Samavat J, Adaikalakoteswari A, Boachie J and Saravanan P, Increased pro-inflammatory cytokine production in vitamin B12 deficient adipocytes. *Endocrine Abstracts* **59**:P158 (2018).
39 Ma F, Zhou X, Li Q, Zhao J, Song A, An P *et al.*, Effects of folic acid and Vitamin B12, alone and in combination on cognitive function and inflammatory factors in the elderly with mild cognitive impairment: a single-blind experimental design. *Curr Alzheimer Res* **16**:622–632 (2019).
40 Politis A, Olgiati P, Malitas P, Albani D, Signorini A, Polito L *et al.*, Vitamin B12 levels in Alzheimer's disease: association with clinical features and cytokine production. *J Alzheimers Dis* **19**:481–488 (2010).
41 Dusitanond P, Eikelboom JW, Hankey GJ, Thom J, Gilmore G, Loh K *et al.*, Homocysteine-lowering treatment with folic acid, cobalamin, and pyridoxine does not reduce blood markers of inflammation, endothelial dysfunction, or hypercoagulability in patients with previous transient ischemic attack or stroke: a randomized substudy of the VITATOPS trial. *Stroke* **36**:144–146 (2005).
42 van Dijk SC, Enneman AW, Swart KM, van Wijngaarden JP, Ham AC, de Jonge R *et al.*, Effect of vitamin B12 and folic acid supplementation on biomarkers of endothelial function and inflammation among elderly individuals with hyperhomocysteinemia. *Vasc Med* **21**:91–98 (2016).
43 Christen WG, Cook NR, Van Denburgh M, Zaharris E, Albert CM and Manson JAE, Effect of combined treatment with folic acid, vitamin B6, and vitamin B12 on plasma biomarkers of inflammation and endothelial dysfunction in women. *J Am Heart Assoc* **7**:1–9 (2018).
44 Young MF, Guo J, Williams A, Whitfield KC, Nasrin S, Kancherla V *et al.*, Interpretation of vitamin B-12 and folate concentrations in population-based surveys does not require adjustment for inflammation: biomarkers reflecting inflammation and nutritional determinants of anemia (BRINDA) project. *Am J Clin Nutr* **111**:919–926 (2020).
45 Tuuminen T, Sorsa M, Tornudd M, Poussa T, Antila E and Jaakkola K, The association between high sensitivity C-reactive protein and micronutrient levels: a cross-sectional analysis based on a laboratory database. *Clin Nutr ESPEN* **33**:283–289 (2019).
46 Gabay C, Interleukin-6 and chronic inflammation. *Arthritis Res Ther* **8**:S3 (2006).
47 Calabrese LH and Rose-John S, IL-6 biology: implications for clinical targeting in rheumatic disease. *Nat Rev Rheumatol* **10**:720–727 (2014).
48 Kaplanski G, Marin V, Montero-Julian F, Mantovani A and Farnarier C, IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends Immunol* **24**:25–29 (2003).

49 Febbraio MA, Rose-John S and Pedersen BK, Is interleukin-6 receptor blockade the holy grail for inflammatory diseases? *Clin Pharmacol* **87**:396–398 (2010).
50 Kumari N, Dwarakanath BS, Das A and Bhatt AN, Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumour Biol* **37**: 11553–11572 (2016).
51 Weber R, Groth C, Lasser S, Arkhypov I, Petrova V, Altevogt P *et al*., IL-6 as a major regulator of MDSC activity and possible target for cancer immunotherapy. *Cell Immunol* **359**:104254 (2021).
52 Tian R, Tian M, Wang L, Qian H, Zhang S, Pang H *et al*., C-reactive protein for predicting cardiovascular and all-cause mortality in type 2 diabetic patients: a meta-analysis. *Cytokine* **117**:59–64 (2019).
53 Yang X, Zhang D, Zhao Y, Liu D, Li Q, Guo C *et al*., Association between serum level of C-reactive protein and risk of cardiovascular events based on cohort studies. *J Hum Hypertens* **35**:1149–1158 (2021).
54 Zhu M, Ma Z, Zhang X, Hang D, Yin R, Feng J *et al*., C-reactive protein and cancer risk: a pan-cancer study of prospective cohort and Mendelian randomization analysis. *BMC Med* **20**:1–13 (2022).
55 Liu T, Zhang Q, Song C, Siyin ST, Chen S, Zhang Q *et al*., C-reactive protein trajectories and the risk of all cancer types: a prospective cohort study. *Int J Cancer* **151**:297–307 (2022).

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