Genome-wide Association Study of Vitamin B6, Vitamin B12, Folate, and Homocysteine Blood Concentrations

Toshiko Tanaka,^{1,2,*} Paul Scheet,³ Betti Giusti,⁴ Stefania Bandinelli,⁵ Maria Grazia Piras,⁶ Gianluca Usala,⁶ Sandra Lai,⁶ Antonella Mulas,⁶ Anna Maria Corsi,⁷ Anna Vestrini,⁴ Francesco Sofi,⁴ Anna Maria Gori,^{4,8} Rosanna Abbate,⁴ Jack Guralnik,⁹ Andrew Singleton,¹⁰ Goncalo R. Abecasis,¹¹ David Schlessinger,¹² Manuela Uda,⁶ and Luigi Ferrucci²

The B vitamins are components of one-carbon metabolism (OCM) that contribute to DNA synthesis and methylation. Homocysteine, a by-product of OCM, has been associated with coronary heart disease, stroke and neurological disease. To investigate genetic factors that affect circulating vitamin B6, vitamin B12, folate and homocysteine, a genome-wide association analysis was conducted in the InCHIANTI (N = 1175), SardiNIA (N = 1115), and BLSA (N = 640) studies. The top loci were replicated in an independent sample of 687 participants in the Progetto Nutrizione study. Polymorphisms in the *ALPL* gene (rs4654748, p = 8.30 × 10⁻¹⁸) were associated with vitamin B6 and *FUT2* (rs6022662, p = 2.83×10^{-20}) with vitamin B12 serum levels. The association of *MTHFR*, a gene consistently associated with homocysteine, was confirmed in this meta-analysis. The *ALPL* gene likely influences the catabolism of vitamin B6 while *FUT2* interferes with absorption of vitamin B12. These findings highlight mechanisms that affect vitamin B6, vitamin B12 and homocysteine serum levels.

One-carbon metabolism (OCM) is a process whereby folate transfers one-carbon groups in a range of biological processes including DNA synthesis, methylation, and homocysteine metabolism.^{1,2} Water-soluble B vitamins folate, vitamin B6, and vitamin B12 play key roles as enzyme cofactors or substrates in OCM. Patients with deficiencies in these vitamins can develop anemia (MIM 170900 and 261100) and, in the case of vitamin B12 deficiency, neurological problems. Subclinical deficiencies during pregnancy have been linked with neural tube defects (NTD [MIM 601634]).^{3,4} In adults, subclinical B vitamin deficiency has been associated with increased risk of coronary artery disease (CAD [MIM 607339])^{5–8} and some cancers such as colorectal cancer (CRC [MIM 114500]).^{9,10}

The exact mechanism that links B vitamins with human health is unknown, but is thought to involve the OCM metabolic pathways.^{1,11} One of the main hypotheses for the protective effect of B vitamins on CAD is that folate, vitamin B12, and vitamin B6 deficiencies increase the production and decrease the catabolism of homocysteine, which is an independent risk factor for CAD.^{12–14} Homocysteine affects various proatherogenic processes including inflammation, thrombosis, endothelial dysfunction, and vascular smooth muscle cell proliferation.^{15,16} Folate insufficiency alters gene expression through changes in methylation pattern of DNA and histones.^{17–19}

The study of genetic variants that affect circulating levels of B vitamins is important for understanding the interplay of diet, genetics, and human health. Circulating levels of B vitamins and homocysteine are at least in part genetically determined. The most-studied polymorphism is the $677T \leftarrow C \text{ (rs1801133) in exon 5 of the 5,10-methylenete$ trahydrofolate reductase (MTHFR [MIM 607093]) gene. The 677T variant results in an thermolabile enzyme that is less effective in the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.²⁰ Accordingly, individuals with the 677T variant have higher homocysteine concentrations as well as lower genomic DNA methylation.^{21,22} In addition to MTHFR, genetic variations in other genes have been linked to B vitamin and homocysteine concentrations, although results have been inconsistent.^{1,23} The goal of the present study was to conduct a genome-wide investigation to identify SNPs associated with differential concentrations of folate, vitamin B12, vitamin B6, and homocysteine in the InCHIANTI study from the Chianti region in Tuscany, Italy;^{24,25} SardiNIA study from Ogliastra province of Sardinia, Italy;²⁶ and Baltimore Longitudinal Study of Aging (BLSA) based in the Baltimore-Washington DC area.²⁷ The results from the genome-wide association (GWA) were examined in the Progetto Nutrizione study based in Tuscany, Italy.²⁸

With the exception of folic acid, the ranges of B vitamin concentrations were similar in the SardiNIA, InCHIANTI,

*Correspondence: tanakato@mail.nih.gov

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¹Medstar Research Institute, Baltimore, MD 21250, USA; ²Clinical Research Branch, National Institute on Aging, Baltimore, MD 21250, USA; ³Department of Epidemiology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA; ⁴Department of Medical and Surgical Critical Care, University of Florence, 50134 Florence, Italy; ⁵Geriatric Unit, Azienda Sanitaria Firenze (ASF), 50125 Florence, Italy; ⁶Istituto di Neurogenetica e Neurofarmacologia, Consiglio Nazionale delle Ricerche, Monserrato 09042, Cagliari, Italy; ⁷Tuscany Regional Health Agency, 50134 Florence, Italy; ⁸Centro S. Maria agli Ulivi, Fondazione Don Carlo Gnocchi IRCCS, Impruneta, 50134 Florence, Italy; ⁹Laboratory of Epidemiology, Demography and Biometry, National Institute on Aging, Bethesda, MD 20892, USA; ¹⁰Laboratory of Neurogenetics, National Institute on Aging, Bethesda, MD 20892, USA; ¹¹Center on Aging, Baltimore, MD 21250, USA

 Table 1. Descriptive Characteristics of InCHIANTI, SardiNIA, BLSA, and Progetto Nutrizione Study

Studies	InCHIANTI		BLSA		SardiNIA		Progetto Nut	rizione
N	1178		641		1115		686	
Vitamin B6 (ng/mL)	7.5	(7.8)	_		_		12.7	(10.2)
Vitamin B12 (pg/mL)	467.4	(330.4)	487.2	(240.5)	404.1	(278.2)	387.8	(194.8)
Folic acid (ng/mL)	3.3	(2.0)	15.8	(8.6)	3.4	(1.7)	5.9	(2.9)
Homocysteine (µmol/L)	15.2	(6.9)	10.2	(5.3)	11.7	(5.1)	11.0	(5.5)
Age (years)	68.1	(15.5)	68.3	(15.5)	44.7	(18.4)	47.0	(12.7)
BMI (kg/m2)	27.2	(4.2)	27.2	(4.2)	25.5	(4.7)	24.9	(4.3)
% Males (N)	44.4%	(523)	54.0%	(346)	42.2%	(470)	40.0%	(276)
% Smokers (N)	18.9%	(222)	3.7%	(24)	19.4%	(216)	40.2%	(276)

BLSA, and Progetto Nutrizione studies (Table 1). Folic acid concentration was higher in the BLSA (which is U.S. based) compared to the Italian studies, reflecting fortification policies in the United States. For the same reason, homocysteine concentration was lowest in the BLSA even though the individuals in this study, on average, were the oldest in the four studies.

Genome-wide significant associations were observed for vitamin B6, B12, and homocysteine concentrations. Vitamin B6 was only measured in the InCHIANTI study, thus the GWA of 484,115 autosomal SNPs was assessed with a Bonferroni corrected p value threshold of 1×10^{-7} (Figure 1A and Figure S1 available online). Three SNPs that met the threshold were located in a region with high linkage disequilibrium (LD) including the neuroblastoma breakpoint family, member 3 (NBPF3 [MIM 612367]), and ~12-50 kb upstream of the tissue nonspecific alkaline phosphatase (ALPL [MIM 171760]) gene (Figure S2). The most significant SNP was rs4654748 (p = 1.21×10^{-8}), in which the presence of the C allele was associated with 1.38 ng/mL lower vitamin B6 concentrations (Table 2). This association was replicated in the Progetto Nutrizione study (rs4654748; ALPL $p = 2.08 \times 10^{-11}$). A sample-sizeweighted meta-analysis across the two studies resulted in a p value of 8.3 \times 10⁻¹⁸ and 1.45 ng/mL lower vitamin B6 per copy of the C allele.

Mutations in the ALPL gene cause hypophosphotasia (MIM 241510, 231500, and 146300), an inborn error of metabolism characterized by low or complete absence of alkaline phosphatase (ALP) activity. The clinical manifestation of hypophosphatasia is highly variable, ranging from a lethal perinatal form to a more moderate adult form presenting with bone abnormalities.^{29,30} One characteristic of hypophosphatasia is the accumulation of phospho compounds including vitamin B6. All of the hypophophatasia mutations identified, many of which are missense mutations, lie within the ALPL gene.³⁰ In a recent metaanalysis that included the InCHIANTI study, rs1780324 within the ALPL gene region was the top signal associated with ALP concentration.³¹ This SNP was one of the topthree SNPs in the vitamin B6 analysis in the current study and is in LD with the most significant SNP, rs4654748 $(r^2 = 0.6)$. When the regression model was conditioned

on rs4654748, rs1780324 was no longer significantly associated with vitamin B6 (p = 0.358), indicating that these two SNPs represent the same locus. In addition, when ALP levels are included in the regression model, the associations of both rs4654748 (p = 0.0331) and rs1780324 (p = 0.019) with vitamin B6 were no longer significant. This indicates that the association of the two *ALPL* gene SNPs with vitamin B6 is mediated by ALP. Mechanistically, ALP is the major enzyme involved in the clearance of vitamin B6,³² and therefore the lower vitamin B6 in C allele carriers most likely results from more efficient clearance of the vitamin.

Vitamin B12, folate, and homocysteine concentrations were available for GWA in InCHIANTI, SardiNIA, and BLSA. To conduct a meta-analysis of the results from these three studies, which were genotyped with different platforms (Affymetrix and Illumina), we imputed ~2.5 million SNPs with MACH using the HapMap CEPH sample as reference.³³ The SNPs that passed quality control (MAF > 1%, $r^2hat > 0.3$) were used to conduct a GWA analysis in each study. For the meta-analysis, an arbitrary reference allele is selected and a z statistic summarizing the magnitude and direction of effect relative to the reference allele is weighted by the square root of the sample size of each study. A fixed-effects inverse-variance method was used for calculating the effect sizes. For the meta-analysis, a Bonferroni corrected p value threshold of 5 \times 10⁻⁸ was considered to be genome-wide significant.

One locus reached genome-wide significance in the meta-analysis of vitamin B12 concentrations (Figure 1B; Figure S1; Table 2). The top SNP in this locus, rs6022662 ($p_{meta} = 2.43 \times 10^{-12}$), mapped to exon 2 of the fucosyl-transferase 2 (*FUT2*) gene (Figure S2A). The presence of the A allele was associated with 44.2 pg/mL higher vitamin B12 concentrations. This is the same SNP reported in a recent GWA analysis in 2717 women in the Cancer Genetic Markers of Susceptibility projects and the Nurses' Health Study.³⁴ Polymorphisms in *FUT2* determine the human secretor (Se) blood group through the expression of a1,2-fucosyltransferase that mediates the fucosylation of oligosaccharides to form H type 1 and 2 antigens.^{35,36} These H antigens mediate the adhesion of various gastric



Figure 1. Genome-wide Scans of Plasma Vitamin B12 and B6 in the InCHIANTI Study on Aging

(A) Genome-wide associations of plasma vitamin B6 graphed by chromosome position and *—*log10 p value. The most significant variant was in the *ALPL* and *NBPF3* genes on chromosome 1.

(B) Genome-wide associations of plasma vitamin B12 graphed by chromosome position and —log10 p value. The most significant variant was in the FUT2 gene on chromosome 19. The next genes of interested included CUBN gene on chromosome 10 and TCN1 on chromosome 11.

(C) Genome-wide associations of plasma homocysteine graphed by chromosome position and —log10 p value. The most significant variant was in the *MTHFR* gene on chromosome 1. The second significant region was in the SYT9 gene on chromosome 11.

Progetto nutrizione study come from

a similar source population as the

InCHIANTI study; thus it is difficult

pathogens such as *Helicobactor pylori* to the gastric and duodenal mucosal.^{36–38} Overgrowth of gastric bacteria, such as with *H. pylori*, has been associated with vitamin B12 deficiency.^{39,40} Interestingly, rs602662 has been identified in nonsecretor status, or the absence of H antigens, in individuals from Northern Portugal.⁴¹ The reduced activity of the FUT2 enzyme with the A allele may decrease susceptibility to bacterial infection and indirectly lower the risk of vitamin B12 malabsorption, thereby resulting in higher vitamin B12 concentrations in A allele carriers.

The second- and third-top SNPs for vitamin B12 analysis did not reach genome-wide significance but were mapped to genes involved in the absorption and transport of vitamin B12. These were rs11254363 in intron 52 of the intrinsic factor-cobalamin receptor, cubilin (CUBN [MIM 602997]) gene ($p_{meta} = 7.24 \times 10^{-8}$; Table 2, Figure S2) and rs526934 in intron 8 of the transcobalamin I (*TCN1* [MIM 189905]) gene ($p_{meta} = 8.33 \times 10^{-7}$; Table 2, Figure S2). Mutations in CUBN cause a rare autosomalrecessive disorder termed megaloblastic anemia 1 (MGA [OMIM 261100]), characterized by juvenile pernicious anemia.42,43 Mutations in TCN1 result in transcobalamin I deficiency (OMIM 193090), characterized by low vitamin B12. These SNPs, however, were not replicated in the Progetto nutrizione study. There is evidence for heterogeneity among studies for rs11254363 ($\chi^2 = 9.24$, p = 0.03) but not rs526934 ($X^2 = 5.37$, p = 0.15). Subjects in the

to identify any factor contributing to the observed heterogeneity and warrants further investigation.

In the meta-analyses of homocysteine concentrations, rs1801133 reached genome-wide significance ($p_{meta} = 4.36 \times 10^{-13}$; Figure 1C; Figure S1). This SNP is the C677T polymorphism in the *MTHFR* gene (Figure S4). Although this polymorphism was highly associated with homocysteine concentrations in both InCHIANTI ($\beta = 4.2$, $p = 7.24 \times 10^{-12}$) and SardiNIA ($\beta = 2.2$, $p = 7.24 \times 10^{-6}$), there was no evidence of association in BLSA ($\beta = 0.36$, p = 0.819). There was significant evidence of heterogeneity ($\chi^2 = 18.11$, $p = 1.2 \times 10^{-4}$) between the studies. The most likely source of these differences is the higher folate status in the United States due to food fortification. This compensation of $677C \rightarrow T$ effect by folate status has been described in other studies.^{44,45}

The second and third SNPs in the homocysteine analyses were rs11041321 (Synaptotagmin IX (*SYT9* [GeneID 143425]) $p = 2.4 \times 10^{-6}$; Figure S4) and rs2713280 (no nearby genes; $p = 5.41 \times 10^{-6}$), respectively. Synaptotagmins are calcium sensors that regulate exocytosis and have important roles in neurotransmission.^{46,47} Homocysteine has been implicated in the pathogenesis of neurological conditions such as dementia (MIM 600274). In light of the potential role of *SYT9* in neurotransmission, rs11041321 was tested for replication but the association was not confirmed in Progetto nutrizione study.

Table 2. Top SNPs for Vita	min B6, Vita	amin B12	, Folate, and	Homocyste	eine Concent	rations						
						GWAS Meta-a	analysis		Replication		Four Cohort I	Aeta-analysis ^c
Concentrations Chr SNP	Position (bp) Gene ^a	Gene Region	Alleles (+/-	-) Freq $(+)^b$	N Effect	SE	p value	Effect SE	P value	N Effect	SE p value
Vitamin B6 (ng/mL)												
1 rs4654748	21531374	ALPL	5'UTR	C/T	0.50	1178 -1.38	3 0.32	1.21×10^{-8}	-1.668 0.	$56 2.08 \times 10^{-1}$	11 1864 -1.45	$0.28 8.30 \times 10^{-18}$
Vitamin B12 (pg/mL)												
19 rs602662	53898797	FUT2	exon2	A/G	0.53	2927 44.20	8.26	2.43×10^{-12}	58.65 10	.43 2.19 × 10 ⁻	¹⁰ 3613 49.77	6.47 2.83 × 10^{-20}
10 rs11254365	17170699	CUBN	intron52	A/G	0.70	2927 -39.1	.6 9.18	7.24×10^{-8}	3.62 10	.94 0.815	3613 -21.4	9 7.03 1.11 × 10^{-6}
11 rs526934	59390069	TCN1	intron8	A/G	0.67	2927 36.76	10.35	8.33×10^{-7}	12.83 13	.24 0.519	3613 27.62	$8.15 \ 1.51 \times 10^{-6}$
Homocysteine (µmol/L)												
1 rs1801133 ^d	11790644	MTHFR	exon8	A/G	0.47	2965 1.31	0.16	4.36×10^{-13}	•			•
11 rs11041321	. 7310445	SYT9	intron3	T/C	0.10	2965 -0.97	0.25	2.42×10^{-6}	0.30 0.	49 0.280	3651 -0.70	$0.22 \ 1.11 \times 10^{-4}$
Folate (ng/mL)												
1 rs1999594	11893482	MTHFR ^d		A/G	0.34	2931 0.30	0.07	1.12×10^{-7}	•		•	•
3 rs153734	64053049	PRICKLE	2 3'UTR	1/C	0.84	2931 0.35	0.08	1.01×10^{-6}	-0.01 0.	19 0.913	3617 0.29	$0.08 \ 7.20 \times 10^{-6}$
^a Genes positioned \pm 50 kb of :	the SNP (based	i on NCBI b	uild 35).									
^b Frequency is averaged across t	he three GWAS	studies.										
$^{\rm c}$ Analysis of vitamin B6 is from	the InCHIANT	I and replic	cation study on	ly.								
^d rs1999594 is within 100 kb of	MTHFR, $r^2 = 0$.2 with rs1	801133.									

No SNPs reached genome-wide significance in the metaanalysis of folate concentrations (Figure S1). The most significant rs1999594 ($p_{meta} = 1.06 \times 10^{-7}$) is located ~100 kb from MTHFR (Figure S5). Because this SNP is in partial LD rs1801133 (r2 = 0.2 in the HapMAP CEU sample) and did not reach genome-wide significance, it was tested for replication. The second SNP was rs153734 in the 3' untranslated region of the prickle-like homolog 2 gene, (PRICKLE2 [MIM 608501], Figure S5). In mice, PRICKEL2 homolog is continuously expressed in postmitotic neurons in early embryogenesis and has been implicated in neuron formation during brain development.48 For this observation, PRICKLE2 was a good candidate gene in the context of folate deficiency and NTD. This SNP was tested for replication in the Progetto nutrizione study, but the association was not confirmed.

Several studies have investigated the associations of genes other than MTHFR in the OCM pathway and circulating B vitamins and homocysteine concentrations. We examine the associations of polymorphisms located in ten candidate genes (MTHFR and MTR [MIM 156570], MTRR [MIM 602568], MTHFD1 [MIM 172460], BHMT [MIM 602888], CBS [MIM 236200], TCN2 [MIM 275350], PON1 [MIM 168820], DHFR [MIM 126060], and FOLH1 [MIM 600934]) in the current analyses. None of these genes were associated with vitamin B6 or vitamin B12 (data not shown). Variants in BHMT (rs651852, p = 0.003), FOLH1 (rs202700, p = 0.0008), and CBS (rs2124459, p = 0.0034)showed the greatest evidence for significance association with folate concentrations (Table S1). For homocysteine, MTR (rs12060264, p = 0.0005), MTRR (rs7703033, p = 0.005), and CBS (rs6586282, p = 0.0002) showed the greatest evidence of association (Table S1).

In summary, we report two variants associated with vitamin B6 and vitamin B12 concentrations. In addition to *MTHFR*, several genes in the OCM pathway were associated with circulating folate and homocysteine. These SNPs may be important markers to identify people at risk for lifelong low vitamin and high homocysteine levels. In particular, this may be relevant to persons at risk for vitamin deficiencies such as the elderly in nursing homes. Future studies may investigate the potentially additive nature of multiple polymorphisms of genes in the OCM pathway on homocysteine concentrations and test the hypothesis that polymorphisms in these genes are risk factors for subclinical B vitamin deficiency and its consequences.

Supplemental Data

Supplemental Data include five figures and one table and can be found with this article online at http://www.ajhg.org/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

- EIGENSTRAT software, http://genepath.med.harvard.edu/~reich/ EIGENSTRAT.htm
- GAINQC Package, http://www.sph.umich.edu/csg/abecasis/ GainQC/

Genome browser, http://genome.ucsc.edu/

International HapMap Project, http://www.hapmap.org/

- Markov Chain Haplotyping Package, http://www.sph.umich.edu/ csg/abecasis/mach/ index.html
- MERLIN Package, http://www.sph.umich.edu/csg/abecasis/ Merlin/ index.html

METAL Package, http://www.sph.umich.edu/csg/abecasis/metal/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi. nlm.nih.gov/sites/Omim

The R project for Statistical Computing, http://www.r-project.org/

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