



Genetic associations with plasma B12, B6, and folate levels in an ischemic stroke population from the Vitamin Intervention for Stroke Prevention (VISP) trial

Keith L. Keene^{1,2}, Wei-Min Chen^{1,3}, Fang Chen¹, Stephen R. Williams¹, Stacey D. Elkhatib¹, Fang-Chi Hsu⁴, Josyf C. Mychaleckyj^{1,3}, Kimberly F. Doheny⁵, Elizabeth W. Pugh⁵, Hua Ling⁵, Cathy C. Laurie⁶, Stephanie M. Gogarten⁶, Ebony B. Madden⁷, Bradford B. Worrall^{3,8*} and Michele M. Sale^{1,3,9} on behalf of the GARNET Collaborative Research Group

¹ Center for Public Health Genomics, University of Virginia, Charlottesville, VA, USA

² Department of Biology, Center for Health Disparities, East Carolina University, Greenville, NC, USA

³ Department of Public Health Sciences, University of Virginia, Charlottesville, VA, USA

⁴ Department of Biostatistical Sciences, Wake Forest School of Medicine, Winston Salem, NC, USA

⁵ Center for Inherited Disease Research, Johns Hopkins University School of Medicine, Baltimore, MD, USA

⁶ Department of Biostatistics, University of Washington, Seattle, WA, USA

⁷ National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

⁸ Department of Neurology, University of Virginia, Charlottesville, VA, USA

⁹ Department of Biochemistry & Molecular Genetics, University of Virginia, Charlottesville, VA, USA

Edited by:

Zhigang Li, Dartmouth College, USA

Reviewed by:

Konstantin G. Arbeev, Duke University, USA

Raghib Ali, University of Oxford, UK
Jinyoung Byun, Dartmouth College, USA

*Correspondence:

Bradford B. Worrall, Department of Neurology, University of Virginia Health System, Charlottesville, PO Box 800394, VA 22908, USA
e-mail: bbw9r@virginia.edu

Background: B vitamins play an important role in homocysteine metabolism, with vitamin deficiencies resulting in increased levels of homocysteine and increased risk for stroke. We performed a genome-wide association study (GWAS) in 2,100 stroke patients from the Vitamin Intervention for Stroke Prevention (VISP) trial, a clinical trial designed to determine whether the daily intake of high-dose folic acid, vitamins B₆, and B₁₂ reduce recurrent cerebral infarction.

Methods: Extensive quality control (QC) measures resulted in a total of 737,081 SNPs for analysis. Genome-wide association analyses for baseline quantitative measures of folate, Vitamins B₁₂, and B₆ were completed using linear regression approaches, implemented in PLINK.

Results: Six associations met or exceeded genome-wide significance ($P \leq 5 \times 10^{-08}$). For baseline Vitamin B₁₂, the strongest association was observed with a non-synonymous SNP (nsSNP) located in the *CUBN* gene ($P = 1.76 \times 10^{-13}$). Two additional *CUBN* intronic SNPs demonstrated strong associations with B₁₂ ($P = 2.92 \times 10^{-10}$ and 4.11×10^{-10}), while a second nsSNP, located in the *TCN1* gene, also reached genome-wide significance ($P = 5.14 \times 10^{-11}$). For baseline measures of Vitamin B₆, we identified genome-wide significant associations for SNPs at the *ALPL* locus (rs1697421; $P = 7.06 \times 10^{-10}$ and rs1780316; $P = 2.25 \times 10^{-08}$). In addition to the six genome-wide significant associations, nine SNPs (two for Vitamin B₆, six for Vitamin B₁₂, and one for folate measures) provided suggestive evidence for association ($P \leq 10^{-07}$).

Conclusion: Our GWAS study has identified six genome-wide significant associations, nine suggestive associations, and successfully replicated 5 of 16 SNPs previously reported to be associated with measures of B vitamins. The six genome-wide significant associations are located in gene regions that have shown previous associations with measures of B vitamins; however, four of the nine suggestive associations represent novel finding and warrant further investigation in additional populations.

Keywords: VISP, association, GWAS, one-carbon metabolism, B12, B6, folate

INTRODUCTION

The B vitamins constitute a group of water-soluble vitamins that play an important role in human health and cellular functions including growth and development (1). Vitamins B₆ (pyridoxine), B₉ (folic acid or folate), and B₁₂ (cobalamin) have garnered

extensive attention for their putative impacts on human health and diseases, ranging from cardiovascular disease and stroke to neurocognitive function and depression. Specifically, these B vitamins are critical for the maintenance of red blood cells (2), components of the nervous (3), and immune systems (4). Vitamin B₆

deficiency, most common in the elderly, has been associated with conditions such as anemia, and neurological abnormalities such as depression, cognitive dysfunction, and neuropathy (5). Vitamin B₁₂ deficiency can result in irreversible brain and nervous system damage and may be responsible for common symptoms such as fatigue and poor memory (6, 7). Folate (Vitamin B₉) is critical for fetal growth and brain development, therefore folate deficiencies during pregnancy can result in neural tube defects in babies (8). In addition, Vitamins B₆, B₉, and B₁₂ serve as important factors in homocysteine metabolism, with vitamin deficiencies resulting in increased levels of homocysteine (9, 10). Although controversial, elevated homocysteine levels are thought to increase risk for stroke (11) and vascular disease (12, 13).

Multiple factors contribute to variations in B vitamin levels in humans. A balanced diet is one approach to help minimize the detrimental effects of B vitamin deficiency. In January 1998, the United States Food and Drug Administration required manufacturers to fortify bread and grain products with folic acid to help prevent neural tube defects due to Vitamin B₉ deficiency. These efforts have proven somewhat successful, with estimates from the 2002–2006 National Health and Nutrition Examination Survey (NHANES) reporting that most Americans are receiving adequate amounts of folate (14). In contrast, for Vitamin B₁₂, data suggest that 5–15% of elderly patients are Vitamin B₁₂ deficient, including data from the Centers for Disease Control and Prevention (CDC) and the NHANES study (15–17). Poor dietary intake, malabsorption from food, and genetic predisposition may all cause vitamin deficiencies. Polymorphisms in genes involved in B vitamin metabolism and processing, transport, absorption, and excretion are logical candidate genes that can influence B vitamin levels. Two such examples include human conditions Imerslund–Grasbeck syndrome (IGS) and megaloblastic anemia-1. IGS, a rare autosomal recessive disorder caused by mutations in cubilin (*CUBN*) and/or amnionless (*AMN*), was first characterized in the 1960s (18, 19) and results in megaloblastic anemia during childhood as a result of selective malabsorption of Vitamin B₁₂. Additionally, genetic variants in the *CUBN* and *AMN* genes are responsible for the Finnish and Norwegian types of megaloblastic anemia-1, respectively (20, 21).

Understanding the genetic factors contributing to vitamin deficiencies offers opportunities for screening and identification of high-risk individuals before the presentation of any clinical manifestations. To date, several large-scale genome-wide association studies (GWAS) testing for association with Vitamin B₆, B₁₂, and folate have been published, resulting in more than 10 confirmed loci for these traits (22–25). Our group has conducted a GWAS for Vitamin B₆, B₁₂, and folate in an effort both to identify novel associations and replicate previously reported associations for these traits in a population of ischemic stroke patients from the Vitamin Intervention for Stroke Prevention (VISP) clinical trial, an NIH-funded, multi-center, double-blind, randomized, controlled clinical trial designed to determine whether the daily intake of high-dose folic acid, Vitamins B₆, and B₁₂ reduced recurrent cerebral infarction and a combined vascular endpoint. Unlike the previous GWAS, the VISP study population represents an ethnically diverse population of older patients that present with elevated baseline homocysteine levels in the top quartile, have suffered a

stroke, and thus, more closely represent the elderly population that is most prone to vitamin B deficiency and stroke.

MATERIALS AND METHODS

SUBJECTS

The VISP trial was a multi-center, double-blind, randomized, and controlled clinical trial that enrolled patients aged 35 or older with homocysteine levels above the 25th percentile at screening and a non-disabling cerebral infarction (NDCI) within 120 days of randomization (26, 27). NDCI was defined as an ischemic brain infarction not due to embolism from a cardiac source, characterized by the sudden onset of a neurological deficit. The deficit must have persisted for at least 24 h, or if not, an infarction in the part of the brain corresponding to the symptoms must have been demonstrated by CT or MRI imaging. The trial was designed to determine if daily intake of a multivitamin tablet with high-dose folic acid, vitamin B₆, and vitamin B₁₂ reduced recurrent cerebral infarction and non-fatal myocardial infarction (MI) or mortality. Subjects were randomly assigned to receive daily doses of the high-dose formulation ($n = 1,827$), containing 25 mg pyridoxine (B₆), 0.4 mg cobalamin (B₁₂), and 2.5 mg folic acid; or the low-dose formulation ($n = 1,853$), containing 200 μg pyridoxine, 6 μg cobalamin, and 20 μg folic acid. Enrollment in VISP began in August 1997, and was completed in December 2002, with 3,680 participants enrolled from 55 clinic sites across the U.S. and Canada and one site in Scotland. All human research was approved by the relevant institutional review boards (IRBs), and conducted according to the Declaration of Helsinki. The VISP study protocol was approved by the IRBs of Wake Forest School of Medicine (coordinating center) and the University of North Carolina at Chapel Hill School of Medicine (statistical center). The local IRB for each of the individual recruiting sites approved the VISP protocol and all participants provided written, informed consent. VISP data analysis by the Genomics and Randomized Trial Network (GARNET) was approved by University of Virginia School of Medicine IRB.

GENOME-WIDE ASSOCIATION STUDY IN VISP

A subset of VISP participants provided consent for inclusion in genetic studies. These participants were included in the GWAS component of VISP, supported by the National Human Genome Research Institute (NHGRI), Grant U01 HG005160, as part of the Genomics and Randomized Trials Network (GARNET); dbGaP Study Accession: phs000343.v3.p1. Samples were genotyped at the Johns Hopkins Center for Inherited Disease Research (CIDR), using the Illumina HumanOmni1-Quad_v1-0_B BeadChip (Illumina, San Diego, CA, USA). Individuals were excluded if they were unexpected duplicates or had gender discrepancies. A total of 2,100 individuals were included in the final genetic analyses; summary statistics are provided in **Table 1**. These subjects consisted of 1,725 individuals of European descent, 258 individuals of African descent, and 117 individuals classified as others.

BIOMARKER MEASUREMENTS IN VISP

As previously described (28), basal levels of folate and Vitamin B₁₂ were determined by the central laboratory at Oregon Regional Primate Research Center using single radioassays of folate and Vitamin B₁₂ (Bio Rad Quantaphase II, Bio Rad Diagnostics, Hercules,

Table 1 | Demographic summary statistics.

Number of individuals (EA/AA/other)	2100 (1725/258/117)
Age (years)	
Mean \pm SD	67.2 \pm 10.8
Range	35–89
% Female participants (N)	37.4 (785)
Current smokers (%)	15.6
Hypertension (%)	71.0
Diabetes mellitus (%)	27.1
B Vitamin baseline measures	
Vitamin B ₆ (pm/mL) \pm SD	42.45 \pm 37.38
Median	33.49
Vitamin B ₁₂ (pg/mL) \pm SD	358.79 \pm 181.91
Median	326
Folate (ng/mL) \pm SD	25.86 \pm 15.91
Median	22.67

CA, USA). For measures of Vitamin B₆, EDTA plasma samples were analyzed using a commercially available method for plasma Pyridoxine 5' Phosphate (ALPCO Inc Windham, NH, USA). The principle of the assay is as follows: ³H-tyrosine is decarboxylated by the vitamin B₆ dependent enzyme tyrosine apodecarboxylase to ³H-tyramine. The activity of tyrosine apodecarboxylase is quantitatively dependent on the amount of PLP present in the reaction mixture. The ³H-tyramine thus produced is selectively extracted into the scintillation cocktail and can be measured by liquid scintillation counting. The excess ³H-tyrosine remains in the aqueous phase and is not measured.

STATISTICAL ANALYSES

Extensive quality control (QC) measures were performed, resulting in a total of 737,081 SNPs for analysis. QC measures included filtering SNPs based on missing call rate, Mendelian errors in control trios, deviation from Hardy–Weinberg equilibrium in controls, discordant calls in duplicate samples, sex differences in allele frequency or heterozygosity, and minor allele frequency (MAF) (29). Briefly, samples were clustered and genotypes determined using GenomeStudio (version 2010.2). For initial QC, SNPs meeting one or more of the following criteria were excluded: call rate <85%, more than one replicate HapMap error, or cluster separation <0.2. Genotype calls for individual chromosomes in samples with large chromosomal anomalies (>10 Mb) or missing call rate >5% were filtered out. Furthermore, samples with overall missing call rates >5% and SNPs with call rates <95% and Hardy–Weinberg *P*-values $\leq 10^{-4}$ were excluded from subsequent analyses. Multidimensional Scaling (MDS), utilizing the software KING (30), was performed to address confounders due to population substructure. Genome-wide association analyses for baseline quantitative measures of folate, and Vitamins B₁₂ and B₆ were performed using linear regression approaches, assuming an additive model, as implemented in PLINK; using age, sex, and the top 10 principal components as covariates. Inverse normal transformation was performed for each of the quantitative traits, prior to analysis. Inverse normal transformations were used to maintain ranks of the trait for each individual and minimize the

impact of outliers while also allowing for sufficient power. Regression coefficients (beta), coefficient *T*-statistic (STAT), and *P*-values (asymptotic *P*-value for *T*-statistic) were calculated for the tested (minor) allele. The proportion of total variance explained (h^2) was calculated as $h^2 = \text{Beta}^2 \times 2 \times \text{MAF} \times (1 - \text{MAF})$. Our GWAS scan results showed no evidence for inflation (GC lambda ≤ 1.013 in all scans.).

RESULTS

We identified six associations that meet or exceed genome-wide significance ($P \leq 5 \times 10^{-8}$; **Table 2**; Figure S1 in Supplementary Material). Of these six associations, four were for Vitamin B₁₂, while the remaining two were for Vitamin B₆. The strongest evidence of association was observed for baseline Vitamin B₁₂ ($P = 1.76 \times 10^{-13}$; beta = -0.22) with a non-synonymous SNP (nsSNP), located on chromosome 10 in the *CUBN* gene. Two additional *CUBN* intronic SNPs (**Figure 1**) were also strongly associated with Vitamin B₁₂ ($P = 2.92 \times 10^{-10}$; beta = -0.19 and 4.11×10^{-10} ; beta = -0.18). A second nsSNP, located on chromosome 11 in the transcobalamin 1 (*TCN1*) gene (**Figure 2**), was also associated with baseline measures of Vitamin B₁₂ ($P = 5.14 \times 10^{-11}$; beta = -0.29). The two genome-wide significant associations for Vitamin B₆ measures (**Figure 3**) were located in the alkaline phosphatase (*ALPL*), liver/bone/kidney gene region (rs1697421; $P = 7.06 \times 10^{-10}$, beta = 0.173 and rs1780316; $P = 2.25 \times 10^{-08}$; beta = -0.325). Although not reaching the genome-wide significance threshold, our GWAS study has identified nine additional SNPs with suggestive ($P \leq 10^{-07}$) evidence of association, two for measures of Vitamin B₆, six for measures of Vitamin B₁₂, and one for measures of folate (**Table 2**). A cluster of suggestive associations for Vitamin B₁₂ was observed on chromosome 19, near the fucosyltransferase 2 (*FUT2*) gene. *P*-Values for the four associated SNPs ranged from 9.33×10^{-07} to 2.67×10^{-07} . Individually, each associated SNP explains only a small amount of the variance (h^2) for each trait (ranging from 0.009 to 0.021).

In addition to our genome-wide analyses, we performed a look-up of 16 SNPs previously associated with measures of vitamin B₆ ($n = 1$) (22), vitamin B₁₂ ($n = 12$) (22–24, 31), and folate ($n = 3$) (22, 23, 25). Of the 16 SNPs previously reported in the literature, genotype data were available for 10 of the reported SNPs, while surrogate SNPs in high linkage disequilibrium (LD) ($r^2 > 0.9$) or the most significant SNP within 100 kb of the reported SNP were reported for the remaining six SNPs. Accounting for multiple testing ($P = 0.05/16$), five of the 16 SNPs, located in *ALPL*, *MS4A3*, *TCN1*, *CUBN*, and *FUT2*, were successfully replicated ($P \leq 0.003$) in our study (**Table 3**; Table S1 in Supplementary Material). For comparison, association results for the untransformed B vitamin measures are reported in Table S2 in Supplementary Material.

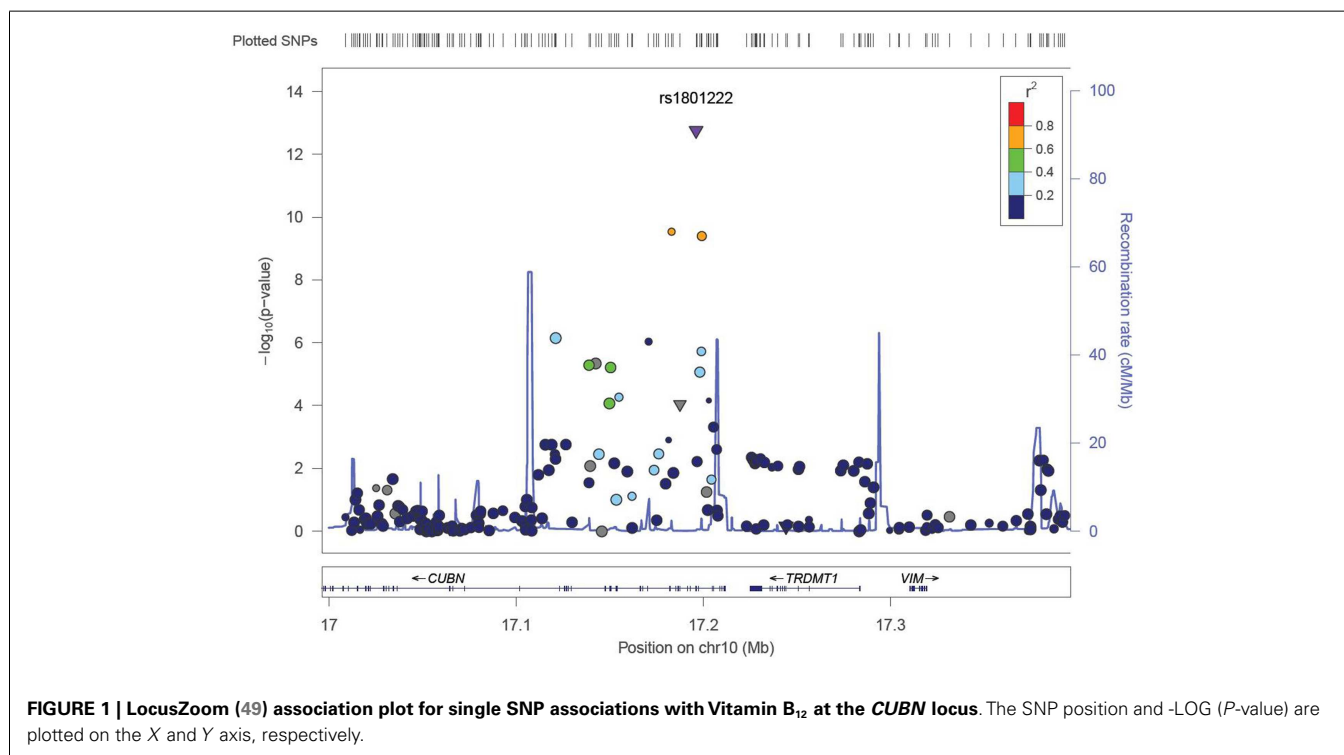
DISCUSSION

We performed a GWAS for measures of Vitamin B₁₂, Vitamin B₆, and folate by evaluating 737,081 SNPs in 2,100 participants from the Vitamin Intervention for Stroke Prevention (VISP) clinical trial. We observed six associations that reached genome-wide significance ($P \leq 10^{-08}$), an additional nine SNPs with suggestive ($P \leq 10^{-07}$) evidence of association, while replicating five

Table 2 | Association results for SNPs with genome-wide ($P \leq 5 \times 10^{-8}$) significance or suggestive evidence ($P \leq 10^{-7}$) for association.

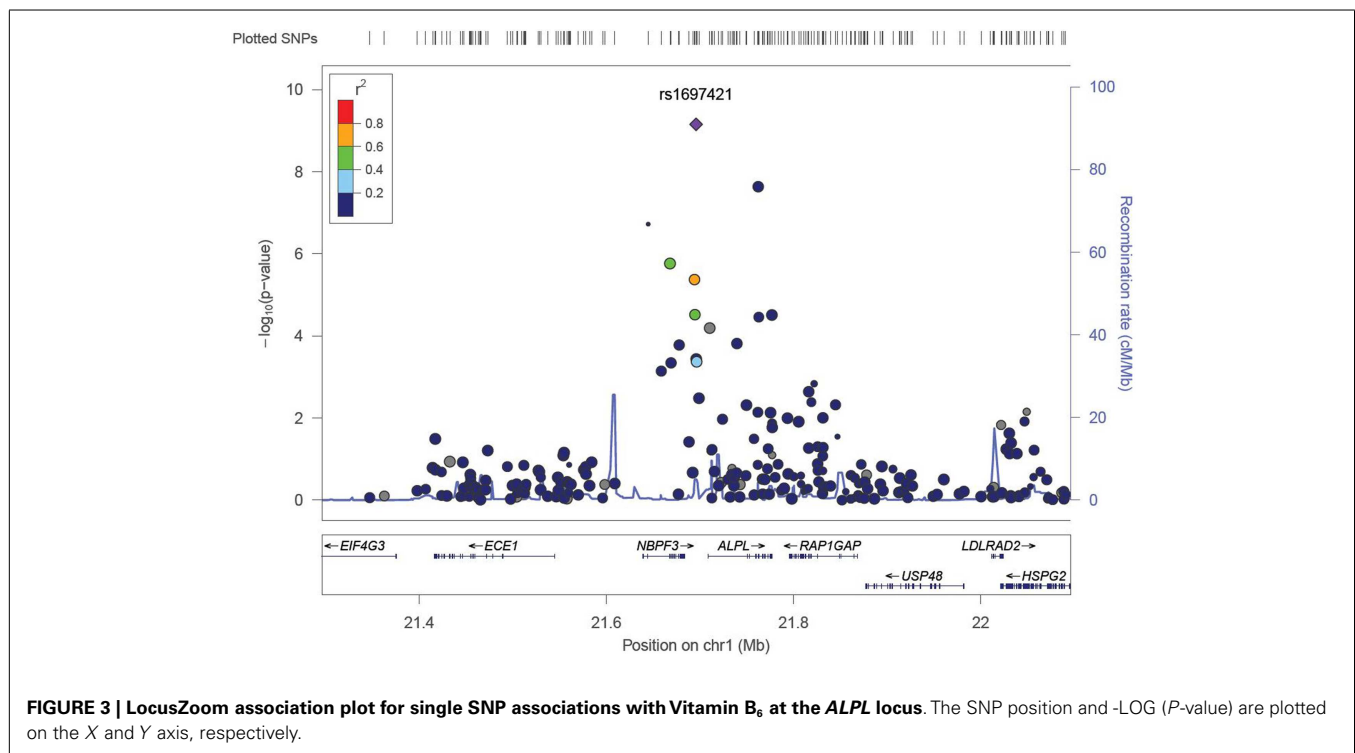
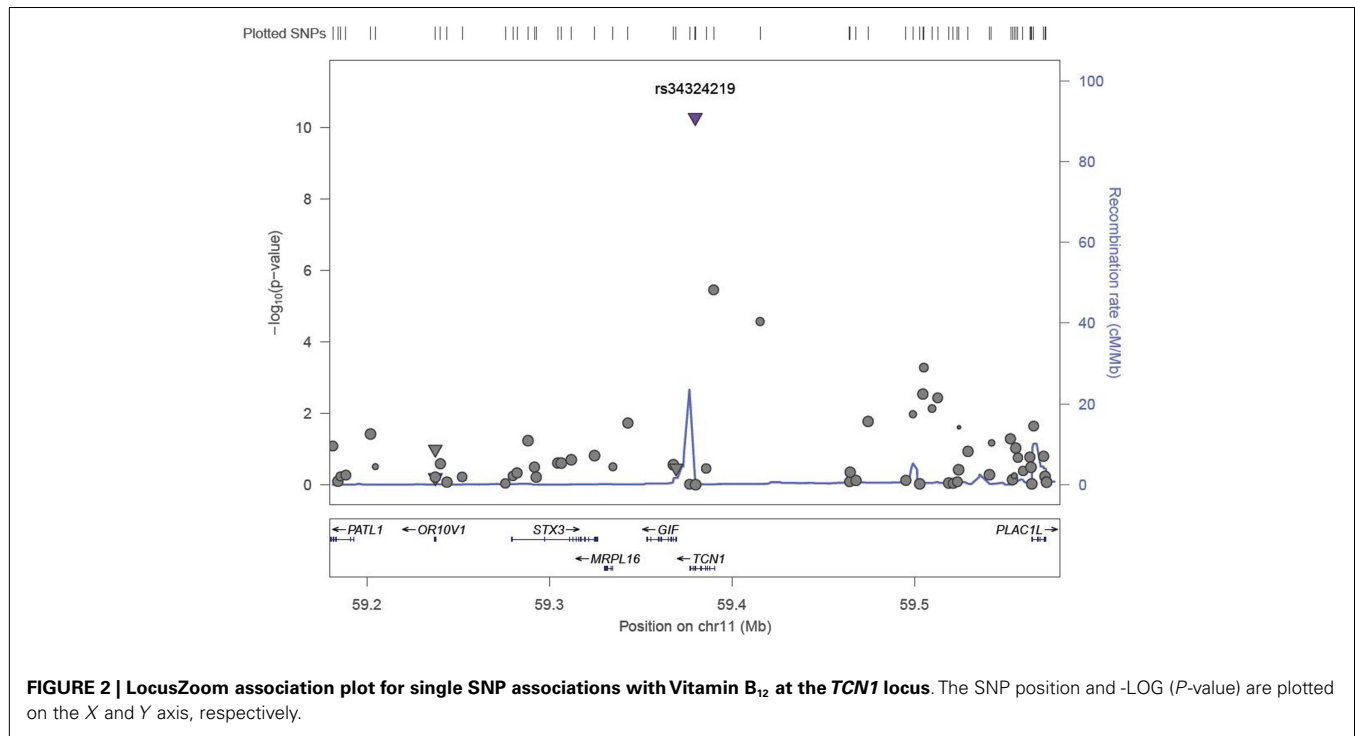
SNP	Chromosome	Location (bp)*	Minor allele	Minor allele frequency	Beta	STAT	Variance explained	P-value (bold indicates $P < 5 \times 10^{-8}$)	Gene (bold indicates novel finding)
VITAMIN B6									
rs12118362	1	21644584	A	0.213	0.172	5.228	0.010	1.91×10^{-07}	NBPF3
rs1697421	1	21695879	T	0.471	0.173	6.197	0.015	7.06×10^{-10}	ALPL (nearest)
rs1780316	1	21762222	T	0.060	-0.325	-5.616	0.012	2.25×10^{-08}	ALPL
rs2267739	7	31103422	G	0.153	-0.241	-4.910	0.015	9.92×10^{-07}	ADCYAP1R1
VITAMIN B12									
rs7893634	10	17121145	A	0.415	0.143	4.972	0.010	7.22×10^{-07}	CUBN
rs11254363	10	17170699	C	0.258	0.155	4.918	0.009	9.48×10^{-07}	CUBN
rs12261966	10	17183006	A	0.311	-0.185	-6.336	0.015	2.92×10^{-10}	CUBN
rs1801222	10	17196157	A	0.316	-0.218	-7.419	0.021	1.76×10^{-13}	CUBN
rs11254375	10	17199198	G	0.312	-0.184	-6.282	0.015	4.11×10^{-10}	CUBN
rs34324219	11	59379954	A	0.104	-0.292	-6.604	0.016	5.14×10^{-11}	TCN1
SNP19-53897957 [‡]	19	53897957	C	0.483	0.142	5.162	0.010	2.69×10^{-07}	FUT2
rs516246	19	53897984	A	0.482	0.142	5.164	0.010	2.67×10^{-07}	FUT2
rs492602	19	53898229	C	0.482	0.143	5.193	0.010	2.29×10^{-07}	FUT2
rs2287921	19	53920084	C	0.471	0.140	4.921	0.010	9.33×10^{-07}	RASIP1
FOLATE									
rs12611820	2	2462633	C	0.245	-0.169	-4.913	0.011	9.75×10^{-07}	MYT1L (nearest)

* Based on hg18

[‡] Corresponds to SNP rs516316

previously reported SNP associations. The most convincing associations were observed for measures of Vitamin B12 at the CUBN and TCN1 loci and the ALPL locus for measures of Vitamin B6.

Although we did not observe any genome-wide significant associations for folate, we did detect suggestive evidence for association ($P = 9.75 \times 10^{-07}$) near the MYT1L gene, located on chromosome



2. Interestingly, genetic variations at this locus have been associated with depression (32) and schizophrenia (33–35). This locus may help explain the recent data positively correlating serum folate levels with cognitive test scores in children (36); suggesting further evaluation of the effects of folate levels in the elderly are warranted.

The most robust associations for Vitamin B₁₂ levels were observed at the *CUBN*, *FUT2*, and *TCN1* loci (Table 2). A cluster of five SNPs spanning the *CUBN* gene provided evidence for association with Vitamin B₁₂ measures (*P*-values ranging from 9.48×10^{-07} to 1.75×10^{-13}). The most significantly associated

Table 3 | Significant associations of 16 previously reported associations of Vitamin B₆, Vitamin B₁₂, and folate.

SNP	Chromosome	Gene	Literature <i>P</i> -value	Reference	Surrogate SNP	VISP <i>P</i> -value
VITAMIN B6						
rs1256335	1	<i>ALPL</i>	1.40×10^{-15}	(22)	–	3.41×10^{-05}
VITAMIN B12						
rs1801222	10	<i>CUBN</i>	2.87×10^{-09}	(22)	–	1.76×10^{-13}
rs526934	11	<i>TCN1</i>	2.25×10^{-10}	(22)	–	3.38×10^{-06}
rs2298585	11	<i>MS4A3</i>	2.64×10^{-15}	(24)	rs7929589	8.67×10^{-04}
rs1047781	19	<i>FUT2</i>	3.62×10^{-36}	(24)	rs516246	2.67×10^{-07}

SNP in this region, rs1801222, was a non-synonymous variant resulting in a missense mutation, Phenylalanine to Serine. These results were not surprising considering rs1801222 was previously associated with Vitamin B₁₂ measures (22) and the protein expressed by *CUBN* forms a receptor complex responsible for Vitamin B₁₂ internalization in the ileum (37). Furthermore, genetic variants in *CUBN* are responsible for the Finnish type of megaloblastic anemia-1 (38) in humans and more broadly for IGS in canines as well (39, 40). A second cluster of suggestive associations near *FUT2* gene were consistent with previously reported associations in this region (24, 25, 41).

A second missense mutation (rs34324219), located in the Vitamin B₁₂ binding protein, *TCN1* gene was associated with baseline measures of Vitamin B₁₂ ($P = 5.148 \times 10^{-11}$). The nsSNP, rs34324219, results in an Aspartic acid to Tyrosine substitution and represents the second most significant association in our study. In the same VISP population, our group previously detected associations between genetic variants of the related gene, *TCN2*, and recurrent stroke risk (42). Although *TCN1* is a logical candidate gene influencing Vitamin B₁₂ measures in this region, associations with variants in the nearby (~200 kb) *MS4A3* gene (24) suggest that multiple genes in this region may impact Vitamin B₁₂ levels. In an attempt to replicate the associations observed in *MS4A3* by Lin et al. (24) (rs2298585), we detected modest evidence of association for the surrogate SNP, rs7929589 ($r^2 = 0.39$; $P = 8.67 \times 10^{-04}$; **Table 3**). The protein encoded by *MS4A3* has been proposed to function as a hematopoietic cell cycle regulator (43), another potential link to the anemia observed in individuals with Vitamin B₁₂ deficiency (44).

For measures of Vitamin B₆, associations at the *ALPL* locus were most robust. Two variants at this locus reached genome-wide significance (rs1697421; $P = 7.06 \times 10^{-10}$ and rs1780316; $P = 2.25 \times 10^{-08}$). GWAS associations for variants near *ALPL* have been reported for Vitamin B₆ (22). In addition, this region also harbors GWAS associations with traits ranging from TNF α response in patients with rheumatoid arthritis (45) to hematologic traits (46). While the physiological function of ALPLs are unknown, and no direct correlations have been made between *ALPL* variants and cognitive function, tissue non-specific ALPL is increased in Alzheimer's disease patients (47). Furthermore, Alzheimer's disease patients have an increased risk of suffering a stroke (48).

The data were collected as part of a randomized clinical trial in a systematic and standardized fashion, which is a major strength of the study. VISP used centralized laboratory analysis on all

samples that complied with strict quality standards. The study population all had ischemic stroke and had elevated measures of serum homocysteine, which might limit generalizability. However, we replicated a substantial proportion of the previously identified genetic variants from studies using a more “general population.” All participants in the VISP clinical trial were 35 years of age or older and suffered a stroke within 120 days of enrollment. This study population also represents an older group of individuals (mean age 67.2 years) that is most prone to vitamin deficiency and subsequent public health concerns including dementia and stroke. We are unable to make any comparisons in normal, healthy individuals, or assess the relation of such associations on stroke risk and other vascular disorders; however, collectively, our finding may provide some insight into the genetic factors influencing measures of B vitamins, in a vulnerable population. Although some dietary measures were collected as part of the VISP trial, we were not able to incorporate dietary “exposure” as a covariate in our analyses. Thus, we cannot identify gene by environmental interactions.

In summary, we performed a GWAS for measures of Vitamin B₆, B₁₂, and folate observing six genome-wide significant associations, nine suggestive associations, and successfully replicating 5 of 16 SNPs previously reported in the literature. Our study is the first of its kind evaluating genetic contributors for measurements of B vitamins in a stroke population. Additionally, this knowledge could lead to genetic screening approaches, which could identify pre-symptomatic individuals that could benefit from interventions such as enhanced vitamin supplementation prior to clinical manifestations.

AUTHORS CONTRIBUTION

Keith L. Keene – performed locus specific analyses, drafted manuscript, and constructed primary tables and figures. Wei-Min Chen – lead VISP statistical analyst, reviewed and edited manuscript. Fang Chen – performed initial GWAS analyses under the supervision of Wei-Min Chen. Stephen R. Williams – assisted with figures and summary statistics, reviewed and edited manuscript. Stacey D. Elkhatib – conducted initial review of literature for GWAS of B vitamin phenotypes, ran analyses of several candidates prior to GWAS data, reviewed and edited manuscript. Fang-Chi Hsu – contributed to the overall GWAS design and the writing of the manuscript. Josyf C. Mychaleckyj – assisted with statistical analyses, reviewed and edited manuscript. Kimberly F. Doherty – generation of GWAS data and QC of GWAS data, reviewed and edited manuscript. Elizabeth W. Pugh – generation of GWAS data and QC of GWAS data, reviewed and edited manuscript. Hua

Ling – generation of GWAS data and QC of GWAS data, reviewed and edited manuscript. Cathy C. Laurie – quality control of the VISP dataset, assisted with statistical analysis, reviewed and edited manuscript. Stephanie M. Gogarten – quality control of the VISP dataset, reviewed and edited manuscript. Ebony B. Madden – Program Director for the project and made contributions to the writing of the manuscript. Bradford B. Worrall – Co-Principal investigator on GARNET, contributed to the design and analysis plan for paper, and made contributions to the writing of the manuscript. Michele M. Sale – Co-Principal investigator on GARNET, contributed to the design and analysis plan for paper, and made contributions to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fpubh.2014.00112/abstract>

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