Title: Interaction of nutrition and genetics via DNMT3L-mediated DNA methylation determines cognitive decline

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Abstract

Low homocysteine levels and B vitamin treatment are reported to protect against declining cognitive health. Both B vitamins and homocysteine are involved in the production of S-adenosylmethionine, a universal methyl donor essential for the process of DNA methylation. We investigated the effect of a damaging coding variant within the DNA methyltransferase gene, *DNMT3L* (R278G, A/G) by examining B vitamin intake, homocysteine levels, cognitive performance, and brain atrophy in individuals in the VITACOG study of Mild Cognitive Impairment and the TwinsUK cohort. In the VITACOG study, individuals who received a two-year treatment of B vitamins and carried the G allele, showed better 'visuospatial associative memory' and slower rates of brain atrophy. In the TwinsUK study, improved 'visuospatial associative memory' was evident in individuals who reported regular vitamin intake and were A/A homozygotes. *In silico* modelling indicated that R278G disrupts protein interaction between DNMT3L and DNMT3A, affecting the DNMT3A-3L-H3 complex required for DNA methylation. These findings show that vitamin intake and genetic variation within *DNMT3L* interact to influence cognitive decline.

Key words

DNA methylation; DNMT3L; Mild Cognitive Impairment; memory; epigenetics; B vitamins; homocysteine.

Abbreviations

5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-Carboxylcytosine; DNMT1, DNA (cytosine-5)-methyltransferase 1; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; DNMT3L, DNA methyltransferase 3 like; Hcy, homocysteine; MCI, Mild Cognitive Impairment; PCA, Principal Component Analysis; ROA, Rates of Brain Atrophy; SAM:SAH, *S*-adenosylmethionine:*S*-adenosylhomocysteine (SAM:SAH).

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1. Introduction

Dementia is one of the largest health problems facing medical science, with the worldwide prevalence set to triple within the next 30 years [1]. Efforts to improve understanding of dementia aetiology and to identify early targets for intervention have led to growing focus on a prodromal stage of Alzheimer's disease (AD) known as Mild Cognitive Impairment (MCI). The most commonly studied feature of MCI is the presentation of memory deficits greater than those expected in age-matched controls. Particular emphasis has been placed on visuospatial associative memory deficits, a characteristic feature of AD and MCI that is associated with early stage hippocampal dysfunction [2,3].

One of the most established risk factors for dementia is an elevated level of homocysteine (Hcy), an α-amino acid that is essential to methionine metabolism within the one-carbon cycle (Figure 1A). In addition to the raised levels in individuals diagnosed with AD,

significantly high Hcy levels have also been reported in individuals with early stage MCI [4]. A prominent feature of methionine synthesis and Hcy metabolism in the one-carbon cycle is the involvement of vitamins B6, B12, and B9 (folic acid). Importantly, B vitamin treatment has been shown to reduce Hcy levels in individuals with AD [5,6] and in a recent longitudinal study of MCI (VITACOG), B vitamin treatment was found to suppress regional and global brain atrophy as well as protect against general cognitive and semantic memory decline in individuals with high baseline Hcy [7-9]. Proposed mechanisms by which B vitamins could protect against cognitive decline include mitigation of the neurotoxic effects of Hcy and the maintenance of methyl donation [10,11].

DNA methylation is a covalent chemical modification of DNA associated with the regulation of transcription [12]. This reversible biochemical process is also dependent on products generated during the metabolic cycling of methionine (Figure 1 A). It is now firmly established that the most studied DNA methylation modification, 5-methylcytosine (5mC), can be oxidised into further functionally distinctive modifications (5hmC, 5fC, 5caC) [13-15] and that intermediate modification states have been observed in neuronal cell populations in human adult brain[16,17]. The establishment, interaction, and conversion of DNA methylation modification is performed by a number of methyltransferases, demethylases and DNA interacting proteins referred to as “writers”, “erasers” and “readers”. Proteins from each of these groups have been implicated in various pathologies. For example, coding mutations within two DNA methyltransferase writer genes, *DNMT1* and *DNMT3A,* are known to cause a familial form of dementia and an overgrowth syndrome with intellectual disability respectively [18,19]. Whilst coding variants within a third methyltransferase gene, *DNA methyltransferase 3 like*, *DNMT3L*, have been associated with intelligence scores in childhood and in old age as well as reported to influence global methylation patterns [20,21].

The DNMT3L protein is not a typical methyltransferase as it is catalytically inert. However, by forming a complex with DNMT3A and histone H3, it facilitates the regulation of

methyltransferase activity [22,23]. Although highlighted as important for *de novo* methylation and a role in imprinting during development, our new understanding of the reversible nature of the DNA methylome supports that DNMT3L may be important for DNA methylation throughout adult life. Indeed, DNMT3L is expressed during development and in adulthood in the human cortex, cerebellum, striatum, amygdala, thalamus and hippocampus; is highly expressed in the regions of the Cornu Ammonis within the adult hippocampus, [24,25] and is abundant in neuronal and glial cell types in human adult cerebral cortex [26].

The findings from rodent studies indicate that *de novo* methylation and DNA methyltransferases are required for memory formation and synaptic plasticity. For example, pharmacological inhibition of DNMTs impairs LTP in the hippocampus and amygdala and the consolidation and reconsolidation of memory-associated neural plasticity [27-29]. Similarly, Dnmt1 and Dnmt3a double knockout mice exhibit deficits in long term plasticity and memory as well a significant decreases in 5mC and 5hmC DNA methylation [30,31]. Furthermore, restoring decreased expression levels of DNMT3a2 in the hippocampus of aged mice, rescued age-dependent cognitive impairment and produced a significant increase in global DNA methylation levels[32] whilst in young mice, overexpression of DNMT3a2 induced memory enhancements and increased expression of plasticity related genes[33]. In mid-aged people, a decrease in cognition ability over a ten-year period was found to correlate with 5mC levels in genes associated with neuronal survival [34]. However, how DNA methylation regulates neuronal processes and memory or what other important factors may also influence changes in methylation, is not well understood.

As DNA methylation is dependent upon methionine metabolism, we hypothesised that vitamin B intake and low Hcy levels might modulate cognitive decline by altering DNA methylation, and that particular coding mutations within DNA methylation genes may influence this interaction. Since many reported pathogenic mutations in DNMTS are ultra-rare and segregate with disease in individual pedigrees, we choose to examine a common

(MAF > 0.10) missense variant located in *DNMT3L* (R278G; rs7354779) which has previously been linked to intelligence across the lifespan[21]. Three approaches were adopted. We examined the association between *DNMT3L* (R278G), Hcy levels, and cognitive performance and rates of whole brain atrophy in the VITACOG B vitamin treatment study of MCI. A follow-up study was conducted using a large non-MCI general population cohort, the TwinsUK cohort, which included self-reported vitamin intake and biochemical measurement of Hcy levels. Finally, we applied an *in silico* modelling approach to predict the functional impact of the *DNMT3L* R278G variant and other clinically relevant DNMT mutations, providing insight into the molecular mechanisms that link this variant with cognitive decline.

2. Methods

2.1 Subjects

The VITACOG study, as part of the wider Oxford Project to Investigate Memory and Aging study, collected data from multiple cognitive tests, biochemical measurements, and magnetic resonance imaging in order to evaluate the impact of vitamin B treatment on MCI progression over a two year period. The treatment consisted of 0·8mg folic acid, 0·5mg cyanocobalamin (vitamin B12), and 20mg pyridoxine (vitamin B6) in contrast to a placebo [9]. Participants were assessed when visited once at baseline and once after the 24 month treatment period was complete. The TwinsUK cohort is a longitudinal registry of British twins who have been continually assessed for a wide range of health and lifestyle factors. Baseline measurements were taken between 1992 and 2004 followed by multiple surveying sweeps and clinical visits.

2.2 Phenotypic variables

Demographic information used in the analysis of both cohorts is presented in Table 1. VITACOG cognitive test output measures were Hopkins Verbal Learning Test-Revised Delayed Recall Total score, Category Fluency Fruit & Vegetables Total score, Graded Naming Test Total score, Mini Mental State Examination Summary score, and Paired

Associates Learning Total Errors score. As VITACOG participants were measured twice during the study period, once at baseline and once after 24 months, change (Δ) in cognitive performance was taken as the difference between the baseline and 24 month scores. TwinsUK cognitive test output measures were Paired Associates Learning Total Errors score, Delayed Matching to Sample Total Correct score, Pattern Recognition Memory Total Correct score, and Spatial Span Length score. Plasma (VITACOG) or serum (TwinsUK) homocysteine was divided into lower quartile/lower middle or upper middle/upper quartile Hcy values.

An annual rate of whole brain atrophy (ROA) was obtained for 156 individuals in the VITACOG cohort. In the original VITACOG study, high-resolution structural T1-weighted images were acquired at baseline and after 24 months and optimised FSL-VBM (voxel-based morphometry) analysis was used to assess regional grey matter change across the duration of the study. The annualized ROA estimated from the baseline and 24 month total brain volume measurements, as reported by Smith *et al.,* 2010 [9], was recoded into volume measured in mL and provided as a precalculated variable in the VITACOG dataset. Only participants with full imaging data were included in the atrophy modelling.

2.3 Genotype data

DNMT3L rs7354779 is an amino acid substitution from Arginine (codon AGG) to Glycine (codon GGG). In the forward strand the variant is A/G. However, in some genomic databases the bases on the reverse strand are listed (T/C) which explains some discrepancy in allele bases reported for this variant in the literature[21]. Genotyping of rs7354779 in the VITACOG cohort was conducted using Kompetitive Allele Specific Polymerase Chain Reaction (KASP) following the manufacturer's recommendations. Primers were designed by LGC Genomics (extra material). Genotyping data was validated by Sanger sequencing. Next generation sequencing data for the TwinsUK cohort was accessed from the European Genome-phenome Archive (EGA; EGAD00001000194 & EGAD00001000741) following a

data access agreement with the UK10K project. Only one individual *per* twin pair was assessed to avoid a genetic twinning effect, i.e. using non-independent related samples. BAM files were visualised using IGV to confirm sequencing read depth quality over the variant region.

The Minor allele frequency (MAF) of rs7354779 in a large European (Non-Finnish) general population is 0.26 [35]. Owing to the minor allele frequency of the rarer G allele in the current VITACOG study (MAF: 0·24) and TwinsUK study (MAF: 0·25), individuals identified as carrying either one (heterozygous, A/G) or two (homozygous, G/G) copies of the *DNMT3L* R278G minor allele were grouped as G carriers.

2.4 Statistical analysis

Principal Component Analysis (PCA) was used to identify the major sources of variance within the select performance outcome variables. PCA was applied to ∆cognitive scores in VITACOG and to cognitive data in TwinsUK. Components with an eigenvalue > 1 were retained. This led to the identification of two derived factors from VITACOG, reflective of 'visuospatial associative memory' and 'verbal semantic memory'. A factor reflecting 'visuospatial associative memory' was also identified in TwinsUK alongside a second derived factor reflecting 'visual scanning' performance. As this 'visual scanning' factor was not identified in VITACOG and hence not consistent with the VITACOG study performance data, this derived factor was not included in subsequent analyses. Correlation coefficient matrices for the cognitive tests and derived factors are presented in extra material.

Univariate and repeated general linear models were used for the analysis of demographic, biochemical and cognitive data, with *post-hoc* Bonferroni correction for multiple comparisons and student's *t*-test where applicable. Missing data was omitted from statistical modelling by specifying missing values within SPSS. In the VITACOG cohort, a significant interaction between age and performance in 'visuospatial associative memory' was observed and hence age was included as a covariate in subsequent analyses. Linear regression was

performed on the rate of whole brain atrophy. Covariates used in the atrophy modelling were age, baseline brain volume, baseline Hcy, baseline creatinine, and treatment group. Logarithmic transformation was applied to variables that did not demonstrate a normal distribution and geometric means presented. Cohen's *d* estimates of effect size were included for group comparisons and r^2 estimates of effect size were included for the atrophy modelling.

2.5 In silico modelling

Protein Data Bank files for the methyltransferases DNMT1, DNMT3A, and DNMT3L were accessed from the online Protein Data Bank repository or created from the canonical amino acid sequence using the RaptorX Structure Prediction tool (Table 2). *In silico* mutagenesis of amino acid residues was performed using PyMOL version 1.3, (Schrödinger, LLC). Default hydrogen, backbone, and rotamer options were retained to allow for consistent comparison of secondary structure changes such as hydrogen bond dynamics. The influence of clinically and non-clinically relevant genetic variants (*DNMT1* Y495C, *DNMT3A* R749C, *DNMT3L* R271Q, *DNMT3L* H313Y, and *DNMT3L* R278G) on thermodynamic stability was measured using FoldX version 3.0 [36]. Models of mutant and wild-type (WT) variants were generated and changes in free energy ($\Delta\Delta G$) between the mutant and WT structures were calculated. Variant influence on electrostatic surface potential was estimated using Adaptive Poisson-Boltzmann Solver.

2.6 Role of funding source

The research was funded by the University of Nottingham. The funding source had no involvement with the study design, analysis and interpretation of data, the writing of, or decision to submit, the report for publication.

3. Results

3.1 PCA-derived cognitive factors

To assess domains of cognition that may be relevant to dementia progression, we performed PCA on the VITACOG and TwinsUK cognitive test outcome measures. The emergence of two derived factors reflective of 'visuospatial associative memory' and 'verbal semantic memory' provides particular clinical sensitivity to our analysis (Figure 1B). For instance, combined performance on the visuospatial associative PAL and GNT tests has been identified as the most accurate predictor of progression from questionable dementia to AD [37,38], whilst performance on the verbal semantic HVLT-R and CF tests has been used to differentiate between amnestic MCI and non-amnestic MCI [39,40].

3.2 B vitamins associated with reduced Hcy levels and slower brain atrophy

We first sought to confirm the expected association of B vitamin treatment with Hcy levels and rates of brain atrophy (ROA). In line with previous work using the VITACOG cohort [9], B vitamin treatment significantly reduced Hcy levels by an average of 24.5% ($p < 0{\cdot}001$) (Figure 1C) and ROA by an average of 28% ($p = 0{\cdot}003$). This effect was particularly strong in those with upper quartile baseline Hcy, showing an average of 53·7% difference in ROA between treatment and placebo groups (Figure 1D). However, B vitamin treatment was found to have no effect on 'visuospatial associative memory' or 'verbal semantic memory' performance regardless of baseline Hcy level. This finding contrasts with the significant association between B vitamin treatment and individual memory task performance in subjects with high Hcy previously reported in the VITACOG study, underlining the distinction between our cognitive factors and the original cognitive tests [7].

3.3 Influence of DNMT3L R278G, B vitamin treatment, and Hcy on cognitive performance in the VITACOG MCI cohort

We then investigated the relationship between the *DNMT3L* R278G genotype, Hcy levels, and visuospatial associative and 'verbal semantic memory' performance. No influence of

DNMT3L R278G on Hcy levels or ROA was found. Performance in 'visuospatial associative memory' and 'verbal semantic memory' also did not differ between the *DNMT3L* R278G A/A homozygotes or G carriers.

After inclusion of B vitamin treatment, the *DNMT3L* R278G genotype groups showed differences in 'visuospatial associative memory' and 'verbal semantic memory' performance. In the B vitamin treatment group, G carriers showed a trend towards improved 'visuospatial associative memory' compared to A/A homozygotes (A/A = -0·12, G carriers = 0·19, d = 0·33, p = 0·06). The opposite was found for 'verbal semantic memory', with A/A homozygotes showing a marginally significant improvement compared to G carriers (A/A = 0·16, G carriers = -0·19, d = 0·36, p = 0·043). In the placebo group, performance on both factors remained unaffected by genotype (Figure 2A). These findings demonstrate that B vitamin treatment had an influence on cognitive performance which was only evident with the *DNMT3L* R278G genotype.

As individuals with the highest baseline Hcy levels gained the most benefit from the B vitamin treatment, we incorporated these Hcy measurements into the analysis of 'visuospatial associative memory' and 'verbal semantic memory'. Stratification by baseline Hcy revealed that the improved 'visuospatial associative memory' performance seen in treated G carriers became significant in those with upper quartile baseline Hcy (p = 0·014) (Figure 2B). No significant effects were seen for 'verbal semantic memory'. This indicates that B vitamin treatment was associated with significantly improved cognitive performance in individuals with MCI, high levels of baseline Hcy, and the *DNMT3L* R278G minor allele.

3.4 DNMT3L R278G influences rate of brain atrophy

To further substantiate the interaction between *DNMT3L* R278G and 'visuospatial associative memory' or 'verbal semantic memory', the relationship between these factors and yearly ROA measurements was investigated. A significant negative correlation between

'visuospatial associative memory' performance and ROA was observed for G carriers which increased after covariate adjustment (r^2 = 0.420, p < 0·001) whilst this relationship remained absent in A/A homozygotes (r^2 = 0·011, p = 0·336). In addition, a significant negative correlation between 'verbal semantic memory' performance and ROA was observed in A/A homozygotes which increased after covariate adjustment (r^2 = 0·294, p < 0·001). This relationship remained absent in G carriers (r^2 = 0·003, p = 0·652) (Figure 2C).

These findings indicate that the *DNMT3L* R278G G carriers who showed improved 'visuospatial associative memory' performance following B vitamin treatment had corresponding reductions in ROA. Similarly, A/A homozygotes who showed improved 'verbal semantic memory' performance following B vitamin treatment had analogous reductions in ROA (Figure 2C). Based on neurophysiology relevant to cognitive processing, it is expected that these findings would be driven by a slowing of hippocampal ROA for improved 'visuospatial associative memory' in G carriers, and a slowing of frontal ROA for improved 'verbal semantic memory' in A/A homozygotes. Previous region-specific imaging analysis using the VITACOG cohort supports our prediction about hippocampal ROA [8].

3.5 Follow-up in TwinsUK cohort

After establishing a relationship between the *DNMT3L* R278G genotype, one-carbon cycle components, and cognitive factors in the VITACOG cohort of MCI, we investigated this relationship in the TwinsUK non-MCI general population cohort. We have previously reported that self-reported regular vitamin intake (p < 0·001) and high serum B vitamin levels (p = 0·002) were associated with significantly lower levels of Hcy in the TwinsUK cohort. We also found that, whilst serum vitamin B12 and folate levels had no influence on cognition, self-reported regular vitamin intake was associated with significantly better 'visuospatial associative memory' performance[41].

In line with the present VITACOG results, we found no association between the *DNMT3L* R278G genotype and Hcy levels. However, both A/A homozygotes and G carriers who self-reported regular vitamin intake performed better on 'visuospatial associative memory', significantly so in the A/A homozygotes (Regular = 0·21, Not reported = -0·54, d = 0·76, p = 0·001) (Figure 2D). This relationship between A/A homozygotes and 'visuospatial associative memory' is analogous to the relationship between G carriers and 'visuospatial associative memory' in the VITACOG cohort. However, as the allele associated with improved cognition is reversed, this indicates an allele-specific difference between the MCI and general population cohorts.

Stratification by Hcy levels did not reveal any significant differences between A/A homozygotes and G carriers in the TwinsUK cohort, contrasting with the modulating role of Hcy in the VITACOG cohort. As the influence of B vitamin treatment was most significant in those with the highest levels of Hcy, we initially predicted that a critical level of Hcy must be reached before effects on cognition could be observed. However, the Hcy levels in the highest quartile were similar between VITACOG (17·1, SD = 3·4) and TwinsUK (16·8, SD = 4). Thus, the genotype-dependent relationship between vitamin intake and Hcy levels for 'visuospatial associative memory' appears to be more prominent in those with MCI disease compared to general population controls.

3.6 In silico modelling of DNMT3L R278G

Amino acid substitution prediction tools such as SIFT, PMUT, and MutationTaster characterised the *DNMT3L* R278G variant as 'damaging' and 'disease causing'. To better understand the functional impact of the R278G variant, *in silico* modelling tools were used to investigate structural, thermodynamic, and electrostatic changes in the DNMT3L protein associated with this variant. To provide a clinical context to the modelling, we also assessed two methyltransferase variants reported to cause a neurodegenerative phenotype (*DNMT1* Y495C) and intellectual disability (*DNMT3A* R749C) respectively [18,19] as well as a

neighbouring variant within *DNMT3L* known to affect global methylation patterns (*DNMT3L* R271Q) and a control variant with no known clinical importance (*DNMT3L* H313Y) (Figure 3A) [20].

DNMT3L forms a complex with DNMT3A and histone H3 in order to stabilise the methylation machinery and direct the addition of methyl groups to DNA. From structural modelling we discovered that the *DNMT3L* R278G variant resulted in the disruption of hydrogen bonds adjacent to one of the DNMT3A-3L interaction sites (Figure 3B & C). Similar disruption of secondary structure was also seen for the clinically associated *DNMT1* Y495C and *DNMT3A* R749C variants. No disruption was seen for the nearby *DNMT3L* R271Q variant or the negative control variant. We quantified these observations by assessing changes in free energy ($\Delta\Delta G$) across available WT and variant protein models. Both *DNMT3L* R278G and the nearby R271Q resulted in a highly destabilising $\Delta\Delta G$ in the DNMT3A-3L-H3 complex model. These values were similar to the $\Delta\Delta G$ calculated for the clinically associated variants, whilst the negative control variant showed neutral $\Delta\Delta G$ (Supplementary 3).

Examination of electrostatic surface potential indicated that the *DNMT3L* R278G variant resulted in a clear transition from positive to negative electrostatic potential stretching over the DNMT3A-3L interaction sites (Figure 3D). Similar patterns were seen for the clinically associated variants and the nearby *DNMT3L* R271Q variant. No observable change was seen for the non-clinically associated control variant. In combination, the *in silico* analyses supports that both structural and electrostatic perturbations may be caused by the *DNMT3L* R278G variant. Moreover, the proximity of these disruptions to the DNMT3A-3L interaction sites indicates a potential impact on the DNMT3A-3L protein complex.

4. Discussion

A meta-analysis of cohort studies supports the hypothesis that there are beneficial effects of B vitamin intake on risk for dementia [42]. However, a role for epigenetic mechanisms as a driving underlying biological mechanism and identification of genetic markers to predict response, are yet unexplored. In this study we report a relationship between one-carbon cycle components, the *DNMT3L* R278G genotype and specific domains of cognitive performance. Following B vitamin treatment, G carriers with MCI in the VITACOG study performed better on 'visuospatial associative memory' whilst A/A homozygotes performed better on 'verbal semantic memory'. These relationships were matched by corresponding changes in whole brain ROA. In the TwinsUK general population cohort, A/A homozygotes with regular vitamin intake performed better on 'visuospatial associative memory'.

Our findings suggest a model in which healthy middle age *DNMT3L* R278G A/A homozygotes who regularly take vitamins demonstrate better 'visuospatial associative memory' performance. However, once individuals decline to MCI levels, B vitamins confer a benefit in 'visuospatial associative memory' for G carriers with high levels of Hcy (Figure 4). The fact that the S-adenosylmethionine:S-adenosylhomocysteine (SAM:SAH) ratio is dependent on Hcy removal in the cycle, and an altered SAM:SAH ratio disrupts methyl donation and thus DNMT activity [43,44], may explain why genotype-dependent cognitive benefit was most striking in MCI individuals with the highest Hcy levels.

DNMT3L differs from classic methyltransferase proteins in that it is catalytically inert. It has a role in direct regulation of methyltransferase activity by forming a complex with DNMT3A, stabilising the active site where DNA binding occurs and attenuating uneven methylation caused by flanking sequence bias [22,45]. DNMT3L has also been reported to interact with histone H3K4 and to co-operate with histone-specific enzymes [23,46]. Our *in silico* modelling provides support for the influence of the *DNMT3L* R278G variant on the interaction of

DNMT3L with DNMT3A and histone H3. The disturbance of the DNMT3A-3L-H3 complex could result in widespread differential 5mC, 5hmC, 5fC and 5caC methylation patterns. In addition, we have shown that the R278G A/G is a CpG dinucleotide site and that the degree of 5mC and 5hmC methylation varies dependent upon the R278G genotype (unpublished data), which might also contribute to changes in gene expression. Base-resolution oxidative methylation and RNA sequencing techniques, possibly in combination with targeted epigenomic CRISPR technologies, will be needed to assess the true impact of this variant on the DNA methylome and RNA transcriptome [47].

Changes to DNA methylation and histone modification patterns are known to occur in the hippocampus during memory formation and consolidation [48,49]. Mnemonic processes can also be disrupted through inhibition of methyltransferase and demethylase proteins[31].
As cellular and neuronal plasticity and adult neurogenesis in the hippocampus have been proposed as a mechanism which contributes to an individual's resilience to cognitive decline and dementia[50,51], it is possible that DNMT3A-3L-H3 complex dynamics in key hippocampal pathways may contribute to such a mechanism. The dynamic and reversible nature of methylation has made it an attractive target for pharmacological intervention, with particular success attributed to the use of methyltransferase inhibiting drugs in the treatment of cancer but also promising effects on hippocampal memory in rodent models [52,53].

The use of cohort studies comes with inherent strengths and limitations. For example, variables are commonly collected during a number of surveys and hence are obtained at different time points in adulthood. In addition, studies exploring the relationship between vitamins and cognition have also highlighted the difficulties in making comparisons between qualitative measures of self-reported vitamin intake and the quantitative measure of serum vitamin levels [54]. It is possible that self-reported vitamin intake data acts instead as a proxy for other environmental factors. For example, individuals who report taking vitamin supplements may be more health-conscious and more likely to exercise regularly and

maintain a good diet - behaviour that is generally agreed to benefit cognition. Future studies to control for potential confounder issues should include longitudinal treatments with vitamins over time, the examination of lifestyle and health factors such as exercise and diet in healthy aged, mild cognitively impaired individuals, and in patients with high homocysteine and dementia.

Our findings support a genotype-environment interaction that impacts upon cognitive function through altered epigenetic regulation. The involvement of DNA methylation and components of the methionine pathway provide a tangible molecular mechanism underlying this genotype-environment relationship. Pharmacological targeting of DNA methyltransferases has led to renewed discussion over the use of dietary supplements, as ingredients capable of methyltransferase inhibition are found in a number of fruits and vegetables, providing further reinforcement for the relationship between diet and cognitive health [55]. These findings may also inform personalised medicine strategies, where combined assessment of genotype and Hcy levels could direct the use of B vitamin treatment in protecting against cognitive decline.

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Contributions

HMK, MF, IAM and ADS designed the study. ADS, and DW collected and managed the phenotypic data from the OPTIMA study. MF, NR, and RW performed the data analysis, genotyping and molecular studies. HMK, IAM, MF, ADS, and DW contributed to the interpretation of the results. HMK, MF and IAM wrote the manuscript.

Declaration of interests

MF, NR, MM, RW, DW, IAM and HMK declare no competing interests. ADS is named as inventor on patents US6008221 and US6127370, with royalties paid to the University of Oxford. ADS are named as inventors on two patent applications pending: PCT/GB2010/051557 and WO2015/140545 A1.

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Figures and Tables

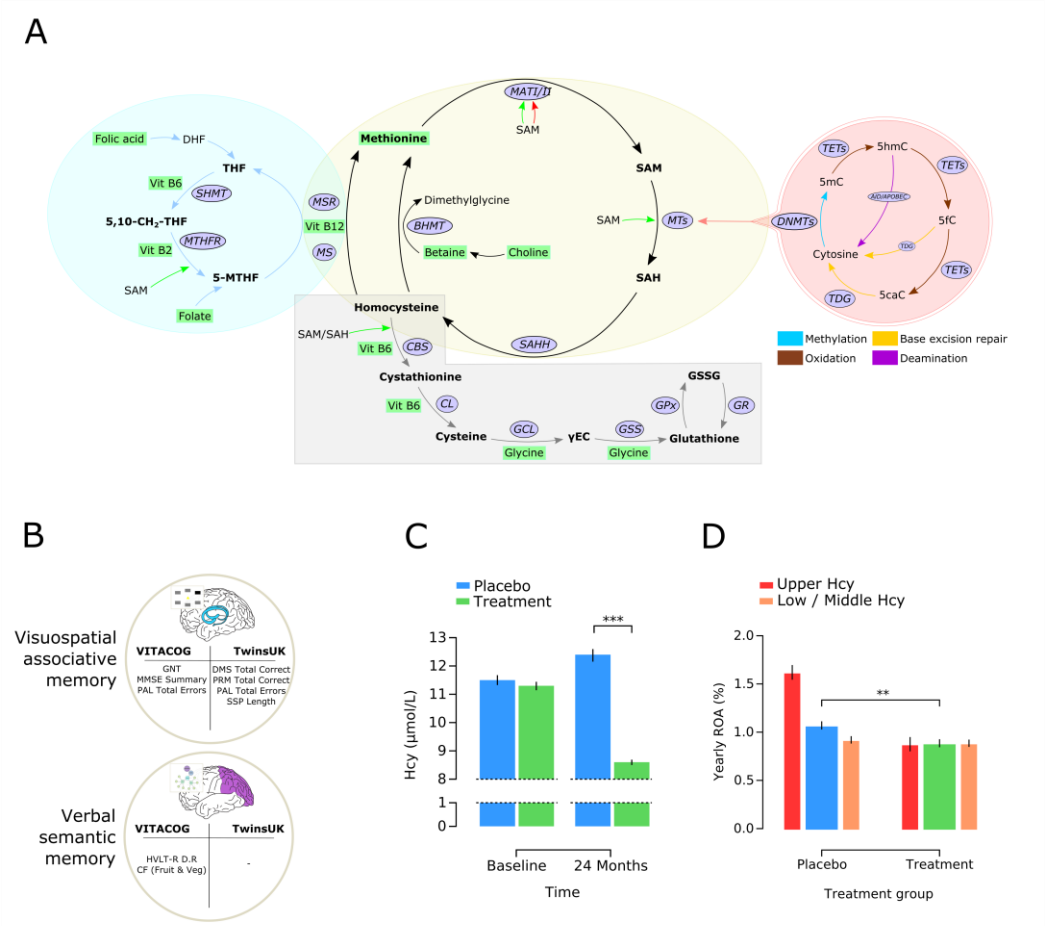


Figure 1. Depiction of the one-carbon cycle, the derived cognitive factors, and the effect of B vitamins on homocysteine (Hcy) and rate of atrophy (ROA) I n the VITACOG study. Error bars indicate 1 standard error.

(A) Diagram of the associated methionine (yellow), folate (blue), and transsulfuration (grey) pathways within the one-carbon cycle, along with the involvement of DNMTs and DNA methylation (red). Areas of dietary influence are highlighted in green.

(B) Principal component analysis resulted in two cognitive factors associated with aspects of cognitive decline, namely ‘visuospatial associative’ and ‘verbal semantic memory’.

(C) In VITACOG, Hcy levels were significantly lower (***; $p < 0{\cdot}001$) in those receiving B vitamin treatment (green) compared to those receiving the placebo (blue).

(D) Significantly reduced ROA was also seen in treated individuals. The reduction in ROA is greatest in those with upper quartile Hcy (red) compared to those with lower and middle Hcy levels (orange).

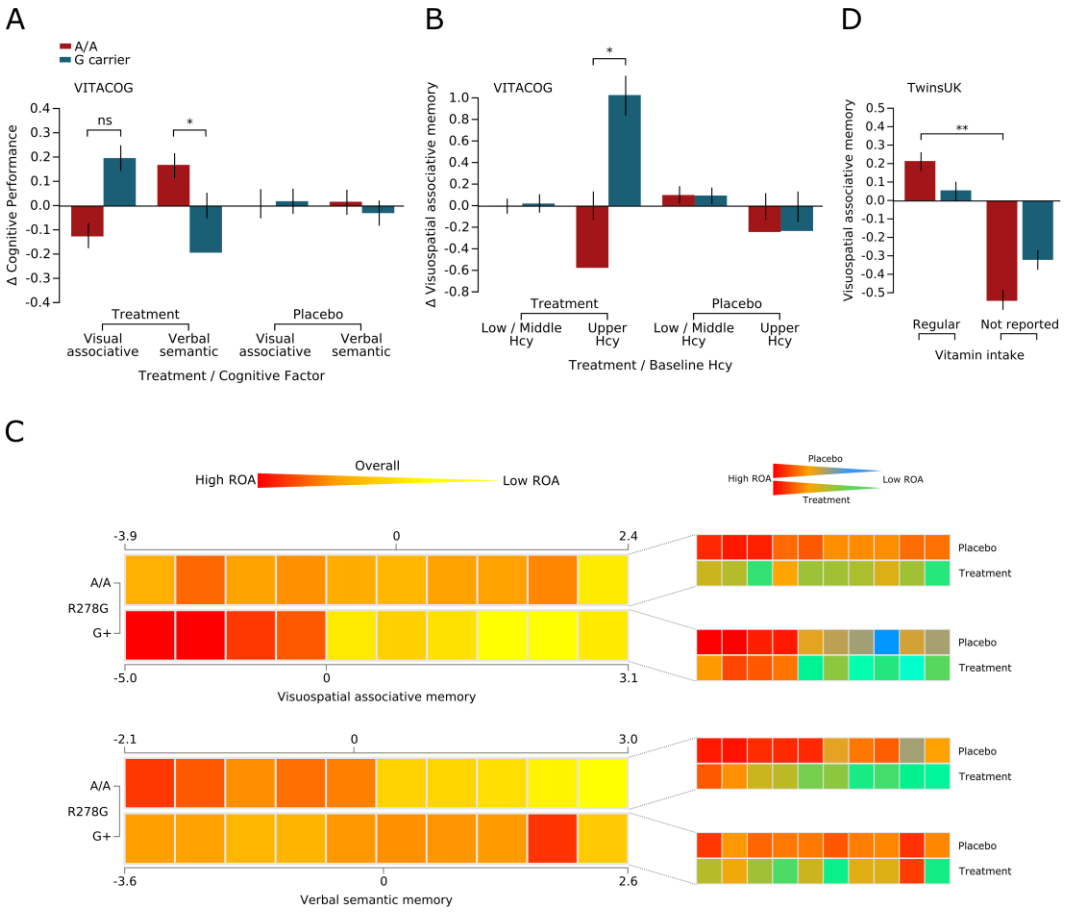


Figure 2. Influence of *DNMT3L* R278G variant on cognition and ROA in the VITACOG study and on cognition performance in the TwinsUK cohort. Error bars indicate 1 standard error.

(A) In VITACOG, B vitamin treatment resulted in *DNMT3L* R278G genotype-specific changes in 'visuospatial associative' (ns; $p = 0{\cdot}06$) and 'verbal semantic memory' (*; $p = 0{\cdot}043$).

(B) Vitamin B treated G carriers with upper quartile baseline Hcy showed significant improvement in 'visuospatial associative memory' (*; $p = 0{\cdot}014$).

(C) Heatmaps portraying greater (red) and slower (yellow) ROA for *DNMT3L* R278G genotypes. G carriers present a significant negative correlation between 'visuospatial associative memory' and ROA whilst A/A homozygotes show a significant negative correlation between verbal semantic memory and ROA. Separating slower ROA by placebo (blue) or treatment (green) confirms that these genotype-dependent relationships are more prominent in the treated individuals.

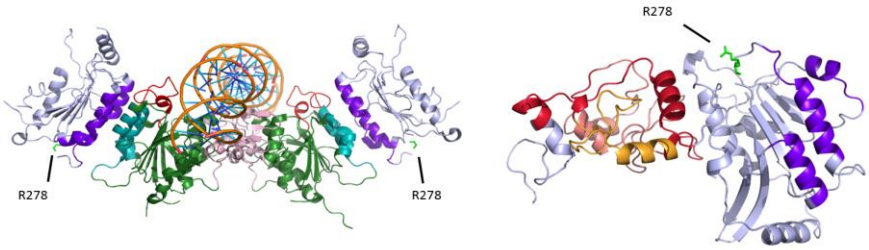
(D) In the TwinsUK, A/A homozygotes who regularly took vitamin supplements showed significantly better 'visuospatial associative memory' performance (**; $p = 0{\cdot}001$).

A

Protein	Variant	RS number	Clinical relevance	Publication	Structural analysis	Free energy change (ΔΔG)*	Electrostatic surface potential
DNMT3L	R278G	rs7354779	Yes	Haggarty et al., 2010	H-bond disruption	Destabilising	Strong + to - change
	R271Q	rs113593938	No	El Maarri et al., 2009	No change	Destabilising	Slight + to - change
	H313Y	-	No	-	H-bond creation	Neutral	No change
DNMT3A	R749C	-	Yes	Tatton-Brown et al., 2014	H-bond disruption	Destabilising	Strong + to - change
DNMT1	Y495C	-	Yes	Klein et al., 2011	H-bond disruption	Highly destabilising	Strong + to - change

*Dependent upon PDB model used. See Supplementary 3.

B



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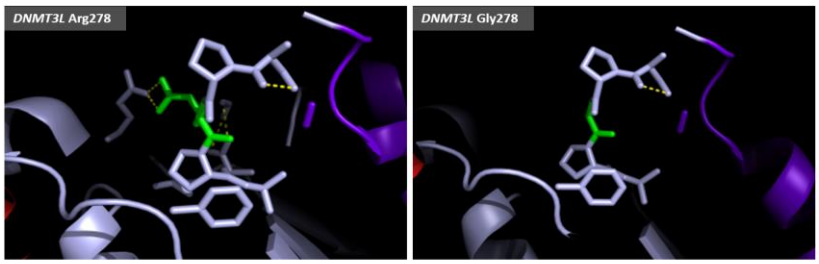

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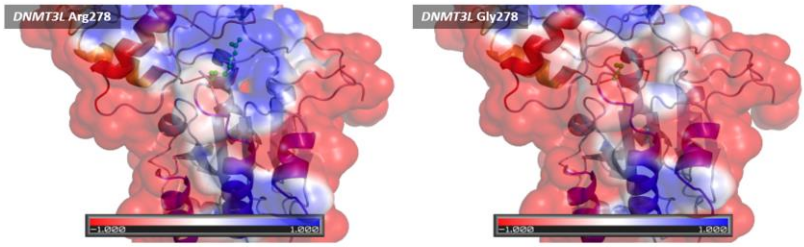

Figure 3. *In silico* analysis of the methyltransferase coding variants and DNMT3A-3L interaction modelling.

(A) Summary of the variants examined and the results from the *in silico* analyses.

(B) PBD models of the DNMT3A-3L complex with DNA *in situ* (left) and the DNMT3L protein (right). The R278 position is annotated (green) for both models. The interaction sites (purple for DNMT3L, teal for DNMT3A) for this complex are also highlighted.

(C) *DNMT3L* R278G (green) results in the disruption of hydrogen bonds (yellow dashes) in proximity to the DNMT3A-3L interaction sites (purple).

(C) The R278G variant (green) leads to a change from positive (blue) to negative (red) electrostatic surface potential over the DNMT3A-3L interaction sites (purple helices).

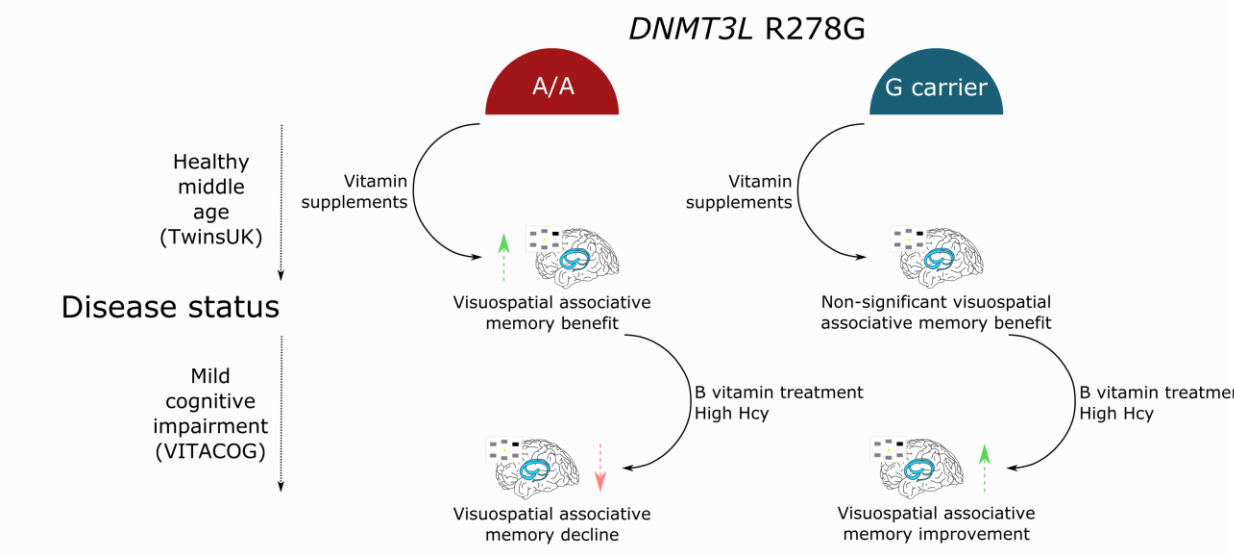

Figure 4. Model of the relationship between disease status, methionine pathway components, and the *DNMT3L* R278G variant with respect to cognitive performance. Beneficial (green arrow) or detrimental (red arrow) cognitive outcomes in 'visuospatial associative memory' are associated with interactions between vitamin intake, Hcy risk and the *DNMT3L* R278G genotype.

	VITACOG	TwinsUK
Number	271	1870
Sex:		
Female	169	1870
Male	96	0
Age at baseline	76·8 ± 4.9	*
***DNMT3L* R278G:**		
A/A	150	996
G carrier	119	832
***ApoE4*:**		
Non-carriers	183	-
Carriers	87	-
Hcy levels (μmol/L)	12 ± 3·8	11·8 ± 4.1
Vitamin treatment:		
Treatment	132	-
Placebo	133	-
Left study prematurely	6	-
Vitamin supplement intake:		
Regular:	-	941
B vitamins, yes?	-	295
B vitamins, no?	-	580
Not recorded	-	559
Vitamin levels:		
Vitamin B12 (ng/L)	-	593·5 ± 289·2
Folate (ng/mL)	-	12·7 ± 6.2
Cognitive scores:		
HVLT-R Delayed Recall	7·6 ± 3·1	
CF (Fruit & Vegetables)	20 ± 5.0	-
GNT	23·1 ± 4·2	-
MMSE Summary	28·2 ± 1·7	-
*PAL Total Errors***	12·5 ± 10·9	19·8 ± 16·9
DMS Total Correct	-	17·2 ± 1·8
PRM Total Correct	-	21 ± 2·3
SSP Length	-	5·6 ± 1·1

Table 1. Demographic information for VITACOG and TwinsUK study cohorts. Where appropriate values are presented as mean ± 1 SD.

Hcy, Homocysteine; Hopkins Verbal Learning Test – Revised; CF, Category Fluency; GNT, Graded Naming Test; MMSE, Mini-Mental State Examination; PAL, Paired Associates Learning; DMS, Delayed Matching to Sample; PMS, Pattern Recognition Memory; SSP, Spatial Span.

*Variables were taken at multiple time points in TwinsUK so there is no baseline age.

**Although the PAL test was used in both cohorts, the PAL Total Errors score was available from VITACOG and the PAL Total Errors (adjusted) was available from TwinsUK accounting for the discrepant scores between the two cohorts.

Protein	PDB name	Source
DNMT3L	-	RaptorX
DNMT3A	-	RaptorX
DNMT3A-3L	4U7P	PDB
DNMT3A-3L C terminus	2QRV	PDB
DNMT3A-3L-H3	4U7T	PDB
DNMT1	-	RaptorX
DNMT1 (351-1600)	4WXX	PDB
DNMT1 replication targeting sequence	3EPZ	PDB

Table 2. Protein models used for the *in silico* analysis. Protein Data Bank (PBD) files for methyltransferases were from the Protein Data Bank repository or created from the canonical amino acid sequence using the RaptorX Structure Prediction tool.