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Transcobalamin 766G Homozygosity, Raised Homocysteine, Raised Methylmalonic Acid and High Creatinine: A Dementia-Predisposing Phenotype? Implications for Dementia and Alzheimer's Disease

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Abstract

Raised homocysteine may be a weak, nutritionally modifiable risk factor for Alzheimer's disease, an etiologically complex disorder likely consisting of subtypes. To further understanding of homocysteine in dementia, we evaluated a set of variables known to reflect/affect the metabolism of vitamin B12 as risk factors for dementia development in a pilot, exploratory nested case/control study. These included raised homocysteine, raised methylmalonic acid, high creatinine and the C766G alleles of transcobalamin II (TCN2), the major blood transporter of B12. High creatinine reflects kidney dysfunction, and can affect levels of homocysteine and methylmalonic acid. In the absence of kidney dysfunction, abnormally high homocysteine is a marker of B12 and/or folate deficiency while raised methylmalonic acid reflects B12 deficiency. Transcobalamin produced from 766G may differ functionally from that produced from 766C. Participants were stringently-selected, community volunteers who at study entry were >age 65 and had high cognitive function. Among those who developed dementia within 6.39 years, *all* with raised serum homocysteine (>15μmol/L) at study entry also had co-occurring raised methylmalonic acid (>350nmol/L), high creatinine (>87μmol/L) and transcobalamin II 766G homozygosity. The odds ratios of TCN2 766G homozygosity plus raised homocysteine, raised methylmalonic acid, or high creatinine at study entry for dementia were high compared to factors singly. We have tentatively dubbed this four-factor cluster "the TCN2 dementia-predisposing phenotype, TDPP". These original findings warrant investigation in larger samples. New questions to ask are if TDPP heralds a distinct subtype of impending Alzheimer's and if TDPP might distinguish a group to target with nutritional intervention before onset of cognitive impairment.

Keywords: B12; Creatinine; Dementia; Folate; Homocysteine; Kidney; Methylmalonic acid; Transcobalamin

Abbreviations

Genetic terms

A: Adenine; C: Cytosine; G; Guanine; T: Thymine; APOE: Apolipoprotein E Gene Symbol; e2,e3,e4: the three most common alleles of APOE; MTHFR: Methylenetetrahydrofolate Reductase Gene Symbol; MTHFR C677T: C-to-T Substitution at Position 677 of the Gene; 677C/C: MTHFR 677C Homozygote (having 2 C alleles); 677C/T: MTHFR 677C/T Heterozygote (having 1 C and 1 T allele); 677T/T: MTHFR 677T Homozygote (having 2 T alleles); MTHFR A1298C: A-to-C Substitution at Position 1298 of the Messenger Ribonucleic Acid (mRNA); 1298A/A: MTHFR 1298A Homozygote (having 2 A alleles); 1298A/C: MTHFR 1298A/C Heterozygote (having 1 A and 1 C allele); 1298C/C: MTHFR C Homozygote (having 2 C alleles); TCN2: Transcobalamin II Gene Symbol; TCN2 C766G: C-to-G Substitution at Position 766 in Relation to the first Nucleotide (+1) of the ATG Translation Initiation Codon of TCN2; 766C/C: TCN2 766C Homozygote (having 2 C alleles); 766C/G: TCN2 766G Heterozygote (having 1 C and 1 G allele); 766G/G: TCN2 766G Homozygote (i.e., having 2 G alleles)

Enzymes

MTHFR: Methylenetetrahydrofolate Reductase [NAD(P)H]; MTR: 5-Methyltetrahydrofolate-Homocysteine Methyltransferase (alternative name, Methionine Synthase: MS); MS: Methionine Synthase (see MTR); MUT: Methylmalonyl-CoA Mutase

Enzyme substrates

MethyleneTHF: 5,10-Methylenetetrahydrofolate; MethylTHF: 5-Methyltetrahydrofolate; THF: Tetrahydrofolate

Vitamins and co-factors

B2: Vitamin B2 (Riboflavin); B6: Vitamin B6 (Pyridoxine); B12: Vitamin B12 (Cobalamin); CoA: co-enzyme A; FAD: Flavin Adenine Dinucleotide, a derivative of B2; NADPH: Reduced form of Nicotinamide Adenine Dinucleotide Phosphate

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Indicators of homocysteine metabolism

Cr: Creatinine; HCY: Homocysteine; MMA: Methylmalonic Acid

Miscellaneous

α: Alpha - the probability of rejecting the null hypothesis given that it is true; AD: Alzheimer's Disease; C: Centigrade; CI: Confidence Interval; °: Degree; ID#: Participant Identification Number; L: Liter; μmol: Micromole; nmol: Nanomol; pmol: Picomol; MCI: Mild Cognitive Impairment; MMSE: Mini-Mental State Examination; n.d: Not Determined; n.s: Not Significant; OR: Odds Ratio; P: Probability of observing an effect given that the null hypothesis is true; PCR: Polymerase Chain Reaction; RBC: Red Blood Cell; RFLP; Restriction Fragment Length Polymorphism; SAS: Statistical Analysis System; sec: Seconds

Introduction

Overview of dementia, Alzheimer's disease and homocysteinemia

Dementia is the most serious form of memory disorder affecting the elderly. Late-onset Alzheimer's disease (AD) occurring after age 65 is the most common form of dementia. As of 2015, diagnostic and intervention efforts still focus on "prototype AD", though variability in AD clinical presentation, progression and pathology point to the existence of AD subtypes [1]. Understanding the genetic nature of this variability is expected to be important in the application of "personalized medicine" to AD and other forms of dementia [1]. In this approach, an individual's genetic profile along with other information will be used to guide decisions made in regard to dementia prevention, diagnosis, and treatment.

Homocysteinemia (raised levels of homocysteine in the serum or plasma) is considered to be one nutritionally modifiable risk factor that if treated may delay the onset of AD. However, intervention efforts in AD patients with B vitamin supplementation have been disappointing, and prospective studies of factors associated with homocysteinemia in the dementia field are warranted. In order to further our understanding of the role of homocysteinemia in dementia, we undertook a pilot study to evaluate a set of candidate "risk factors" known to reflect or affect the metabolism of vitamin B12 (cobalamin) and homocysteine metabolism. A brief review of information relevant to this study is presented next. Following this is a description of the study design, hypotheses, and reasons for choosing particular variables as candidate risk factors. [Note: Risk factors are variables *associated* with increased risk of disease; statistically significant associations should not be equated with causality.]

The enigmatic relation of homocysteine with dementia

Homocysteine (HCY) is a non-protein, sulfur-containing amino acid that is the major metabolite of the essential amino acid methionine. It is found in small quantities in all cells and plays an important role in human health [2-4]. Serum or plasma concentrations of HCY are determined in large part by the body's status of folate, B12 and B6, because these B vitamins are cofactors or substrates for particular enzymes involved in HCY metabolism [5,6]. However, particular polymorphisms of various enzymes [7-10], kidney dysfunction [11,12], and other factors [13-15] also can affect HCY concentrations. High dose supplements of folic acid and vitamins B12 and B6 can reduce homocysteine levels even in the absence of vitamin deficiency [6,16].

High levels of circulating HCY (denoted as *homocysteinemia or hyperhomocysteinemia*) precede and/or co-occur not only with heart disease and vascular disease [17,18], but also with cognitive deterioration in aging, elderly adults [5], dementia [19], AD (the most common form of dementia) [20,21], and vascular dementia [22], as well as with other neurological disorders [23,24]. However, such findings have not been found consistently [25]. Because elevated HCY is associated with deficiency of B12, B6 and/or folate [5,6], and even normal levels of HCY can be lowered with B vitamin supplementation [6,16], deficiencies of B vitamins are suspected of being causal or contributing factors in these disorders.

With respect to cardiovascular and neurological diseases, it still is not clear if homocysteinemia is a causal or contributing factor, a marker of underlying vitamin deficiency, or the result of some other factor or factors [20,21,26-27]. One reason for controversy is that clinical trials with B vitamin supplementation to alleviate homocysteinemia have had no or few clinical effects aside from HCY lowering [16,28]. However, one clinical trial involving participants with mild cognitive impairment (MCI), a precursor to dementia, B vitamin therapy was shown to reduce the rate of brain shrinkage which occurs during normal aging and also in persons with MCI and AD [29]. In this particular trial, B vitamins also appeared to slow cognitive and clinical decline in the MCI participants, particularly in those with elevated HCY [30,31]. The success of the latter study may be related to the fact that the trial involved participants with pre-dementia (i.e., MCI) rather than relatively established disease.

Relationship between B12 deficiency, raised homocysteine and raised methylmalonic acid

B12 deficiency results in raised HCY and raised methylmalonic acid (MMA). Only two enzymatic reactions are dependent upon B12. The first involves the methylation of HCY to methionine by methionine synthase [MTR] which uses methylcobalamin as a cofactor [32]. The reaction catalyzed by MTR converts 5-methyltetrahydrofolate (methylTHF) into tetrahydrofolate (THF) while transferring a methyl group to HCY to form methionine. (MethylTHF is produced from 5,10-methylenetetrahydrofolate via the enzyme, methylenetetrahydrofolate reductase (MTHFR) which contains a bound flavin cofactor and uses NADP(H) as the reducing agent.) B12 is thus crucial for the cycling of folate in the folate cycle as well as for generating the methionine needed for synthesis of S-adenosylmethionine, the source of active methyl groups for methylation reactions [5,6]. Interference with the MTR reaction from deficiency of methylcobalamin or methylTHF will result in buildup of HCY [5,6]. The second B12 dependent reaction catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA by methylmalonyl-CoA mutase (MUT); this requires 5-deoxyadenosylcobalamin [33]. (Succinate is an intermediate of the energy generating Krebs cycle.) When B12 deficiency interferes with MUT, methylmalonyl-CoA accumulates, leading to buildup of methylmalonic acid (MMA). Buildup of methylmalonyl-CoA results in buildup of propionyl-CoA, the precursor of methylmalonyl-CoA. Through the catalytic action of citrate synthase, excessive propionyl-CoA results in the buildup of 2-methylcitric acid via condensation

with oxaloacetate. The MUT reaction mediates the catabolism of propionyl-CoA derived from odd chain fatty acids and some branched chain amino acids [5,6].

Design of the present study

The study described in this paper used a nested case/control design. It took advantage of a previously characterized cohort of stringently-selected, community volunteers who at study start had high cognitive function and were >age 65 years. Over an observation window of 6.39 years, it was possible to distinguish those who developed dementia by the study end (the cases) and those who still had normal cognitive function (the controls) from others who became afflicted only with MCI [34,35].

Hypotheses of the present study

Because previous studies indicated that mild homocysteinemia is a relatively weak risk factor for dementia [19], we hypothesized that the association strength of homocysteinemia for dementia might be increased by considering raised HCY in combination with a genetic polymorphism thought to affect B12 and HCY metabolism as a risk factor. Additionally, we assumed that if B vitamin and/or folate abnormalities were contributing to homocysteinemia, then levels of other variables reflecting or affecting HCY status also should be evaluated. The rationales for selecting particular candidate genetic and biochemical risk factors for development of dementia are discussed next.

Genetic factors selected for evaluation in the development of dementia

Genetic factors selected as potential risk factors for development of dementia in the present study included the 766C and 766Gpolymorphisms of transcobalamin II (TCN2) the major blood transporter of vitamin B12 to tissues and cells. The TCN2 C766G polymorphisms were selected for evaluation for two main reasons. First, compared to common polymorphisms of MTHFR [36,37], TCN2 C766G polymorphisms have been understudied in the dementia field. Zetterberg and colleagues reported that 766G was strongly associated with lower levels of holo-transcobalamin (the B12-transcobalamin complex) compared to other 766 genotypes in the cerebrospinal fluid of patients with AD, in spite of normal vitamin levels in the peripheral blood [38]. McCaddon et al. had observed that "proportionately fewer 776C homozygotes appear to develop AD at any given age [than other TCN2 genotypes], but this will require confirmation in a longitudinal study" [39]. Second, as detailed below, other evidence is suggestive that the 766C and 766G alleles may produce transcobalamins that do not function identically, making them plausible candidates for evaluation.

Although a number of TCN2 alleles have been described, the C766G polymorphisms occur most commonly in the general population, though their frequencies and relative proportion vary markedly from one geographical region to another and among different ethnic groups [40]. The 766G allele contains a cytosine (C) to guanine (G) substitution at nucleotide position 766 (in relation to the first nucleotide (+1) of the ATG translation initiation codon of TCN2. This nucleotide substitution converts a proline to an arginine residue at position 259 of the transcobalamin protein [41,42]. While no basic research studies have been published to indicate that the proline and arginine variant transcobalamins are functionally different, this is implied from various clinical studies distinct from those described above. For example, Miller and colleagues concluded that the TCN2 genotype may influence susceptibility to B12 deficiency [43]. Subjects homozygous for the proline form of transcobalamin [i.e., who were TCN2 766C homozygotes] had a significantly higher mean concentration of bound B12 than persons homozygous or heterozygous for the arginine form. As well, persons homozygous or heterozygous for the proline form of transcobalamin [i.e., who were TCN2 766C homozygotes or 766C/G heterozygotes] had a significantly lower mean level of methylmalonic acid (MMA, a metabolite that becomes elevated in B12 deficiency), than those homozygous for the arginine form [43]. Conversely, TCN2 776G homozygosity has been associated with higher MMA than other 776 genotypes [44]. Although the majority of studies have failed to find 766G genotype effects on serum or plasma HCY [44], HCY concentrations were higher in TCN2 776G homozygotes with evidence of B12 deficiency than in individuals with other genotypes [9]. One working explanation for such genotype influences is that B12 may have a higher affinity for the proline form of transcobalamin than the arginine form [43]. Another is that the proline form may be more efficient in delivery of B12 to cells and tissues [9].

Biochemical factors at study entry selected for evaluation in development of dementia

Because TCN2 766G influences may manifest in combination with vitamin B12 deficiency (see above), we evaluated biochemical measures commonly used to probe B12 and folate status as risk factors for the development of dementia in our participants. Of the battery of standard and specialized tests already completed for the cohort study [34], we selected measures of serum HCY, B12, MMA and red cell folates for evaluation. High HCY and MMA are used as surrogate markers of B12 deficiency [45]. Folate deficiency [7] and high folate in the presence of B12 deficiency [46] are associated with high HCY. Because high creatinine, an indicator of kidney dysfunction, is often associated with high HCY and MMA [11,47], complicating the use of high MMA or HCY as B12 deficiency indicators [12], serum creatinine also was selected as a candidate risk factor.

Methods

Approach

This study involved: inspection of data and graphical analysis to search for trends; use of contingency table analysis and odds ratio (OR) analysis to determine association strengths of putative risk factors for dementia singly and in combination; and other analyses such as comparison of means. Results of statistical analyses were interpreted at the 95% level of confidence ($\alpha = 0.05$). Bonferroni corrections to reduce the chance of obtaining spurious results through multiple comparisons were not applied since the study was pilot and exploratory in nature. As noted, the candidate risk factors selected for evaluation are related in that they all affect the metabolism of HCY. Hence the obtaining of multiple, positive results should not be surprising.

Participants

Study participants and ethics approval for involvement of human subjects have been described previously [34,35]. In brief, participants

(N=281) were Caucasian, community volunteers. At study entry they were: >65 years old (average, 72.6); free of cerebrovascular, neurological and chronic kidney disease (participants with serum creatinine >146μmol/L were excluded); had a Mini-Mental State Examination (MMSE) score >25; and were not taking excessively high dose B12 supplement or getting B12 vitamin injections. After study entry, they had had up to three follow-up visits for assessment of cognitive function and health status over a period of 6.39 years. At the study end, 26 (15 females, 11 males) were classified as: having developed dementia (the cases), 192 (147 females, 45 males) as being still cognitively normal (the controls) while 22 had developed only MCI. Of this sample, a random subgroup of 15/26 cases (7 females, 8 males) and 171/192 controls (110 females, 36 males) underwent genetic analysis (see Genotyping).

The study began at approximately the same time that folic acid fortification of grain products was mandated in Canada [34,35] -January 1999, and continued until mid-2005. For information, B2 (riboflavin) fortification of certain foods has been mandatory in North America for more than half a century [48].

Genotyping

Preparation of genomic DNA from peripheral blood was described previously [35]. The focus was on TCN2 C766G polymorphisms but for comparison MTHFR typing also was done. Genotypes resulting from the TCN2 766C and G alleles and the MTHR 677C and T alleles were identified using the RFLP-PCR procedures of Miller et al. [43], and Bottiglierri et al. [49], respectively. Genotypes resulting from the MTHFR 1298A and C alleles were identified using a TaqMan allelic discrimination assay. Briefly, labeled allele-specific probes (1298C_FAM: ACACTTGCTTCACTGGT, 1298A_VIC: AGACACTTTCTTCACTGGT) flanked by common amplification primers (F: AGAGCAAGTCCCCCAAGGA, R: CTTTGTGACCATTCCGGTTTG), were used in an amplification reaction on a GeneAmp 9700 (Life Technologies) with an initial incubation at 95^{O}C for 105 sec, followed by 35 cycles of 95^{O}C for 15 sec and 60^{O}C for 60 sec, and endpoint quantitative analysis on a 7900HT Fast Genetic Analyzer (Life Technologies). TCN2 677C-G and MTHFR 677C-T typing was done in the Neurogenetics Laboratory at Surrey Place Centre, Toronto. MTHFR 1298A-C typing was done by M.J.S. and L.P. in the University of Alberta department of Medical Genetics.

Biochemical tests

Blood samples for the standard and specialized tests were non-fasting and taken by a single venipuncture at study entry. Total serum cobalamin (B12), red blood cell (RBC) folates, and serum creatinine were determined using standard procedures by the Kingston General Hospital Laboratory (Kingston, ON) [34,50]. Levels of HCY and MMA were measured in once-frozen serum samples using specialized procedures by Metabolite Laboratories, Inc. (Denver, CO) [5,34,50]. Serum preparation involved clotting at room temperature for 30 minutes prior to centrifugation [50]. Reference ranges for the study period were: cobalamin (B12), 165-740pmol/L; RBC folates, 750-1800nmol/L; total HCY, 5.1-13.9μmol/L; MMA, 73-271nmol/L; creatinine, 50-110μmol/L [34]. HCY levels are to some extent methodology dependent and measures in serum and plasma are not directly comparable [51]. We used serum samples prepared in the same way [50] to control for possible influences of environmental factors (e.g., ambient temperature, diurnal variation, centrifugation parameters) on biochemical variables within individuals [52].

Statistical analyses

We asked if particular genetic polymorphisms or genotypes or abnormal levels of biochemical markers of HCY metabolism at study entry were risk factors for dementia that developed by the study end (i.e., over the observation window of 6.39 years). The statistical methods that were used have mostly been described [35]. These include: comparison of the frequencies of candidate risk factors in the case and control groups using Fisher's exact test (two-tailed); comparison of means using the independent samples *t*-test (two-tailed, unequal variance); determination of odds ratios (ORs) and their 95% confidence intervals (CIs) to denote the strength of association between potential risk factors and outcomes; and post-hoc power analysis. MedCalc software was used for OR determinations [53]. (This software generates ORs identical to those produced by SAS.) For comparison of means, continuous variables were logarithmically transformed to reduce skewness. Analyses were conducted on up to 218 individuals whose blood work had been completed and on up to 186 individuals who also underwent genotyping (see Participants). Levels of red cell folates at study entry correlated strongly and positively with duration of exposure to mandatory folic acid fortification prior to study entry in persons who developed dementia and who retained normal cognitive function at study end, but levels of HCY, MMA, B12 or creatinine did not (data not shown).

Cut-off values for biochemical variables

Measures of HCY were denoted as raised if they exceeded 15μmol/L (the upper limit for "healthy" adults and in the range for "mild homocysteinemia" [51,54]); MMA levels were denoted as raised if they exceeded 350nmol/L (considered "elevated" in some previous studies [55]). Measures of creatinine were denoted as raised if they exceeded the upper lab limit of 110μmol/L. Measures of B12 were denoted as deficient if they were less than the lower lab limit of 165pmol/L. We also considered: creatinine measures within the top 15% of values for participants (>87μmol/L, denoted as "high"); and B12 levels within the lower 25% for participants (<210pmol/L, denoted as "low").

Results and Discussion

Results are summarized in Tables 1-5.

Among those who developed dementia during the study, raised homocysteine, raised methylmalonic acid and high creatinine at study entry are associated with TCN2 766G homozygosity.

Inspection of Table 1 revealed substantial representation of TCN2 766G homozygosity among the 15 persons who developed dementia: 7 of the 15 (47%) had this marker. Of the TCN2 766G homozygotes, 5of 6 whose blood results were complete had MMA >350nmol/L at study entry. Four of the 5 with raised MMA had HCY >15μmol/L, and these same 4 had serum creatinine >87μmol/L) at study entry, possibly indicative of mild kidney dysfunction. Of those with raised HCY, raised MMA and high creatinine, one person had B12 <165pmol/L, two had B12 <210pmol/L plus low folate <750nmol.L) and one had raised folate >1800nmol/L.

Table 1: Characteristics of individual participants who developed dementia by the study end. The ranges for the biochemicals at study entry are the upper and lower limits specified by the collaborating laboratories. The levels of folate are significantly correlated with the duration of folate fortification since January 1999, but levels of B12, HCY, MMA and creatinine are not (see text).

ID#	Age at entry (years)	TCN2 766 type	Serum B12 165-740 pmol/L	Serum HCY 5.1-13.9 µmol/L	Serum MMA 73-271 nmol/L	Serum Creatinine 50-110 µmol/L	Red Cell Folates 750-1800 nmol/L
Females							
1	73	C/G	330	8.3	93	54.3	919
2	76	C/C	180	10.3	244	68.5	1695
3	79	G/G	362	16.8	366	142	1909
4	82	C/G	142	11.7	242	61.4	633
5	77	C/G	653	7.7	103	69.4	1545
6	67	G/G	335	7.8	105	70.3	894
7	81	G/G	205	23.1	720	113	457
Males							
1	78	G/G	100	11.8	333	70.3	1382
2	75	G/G	209	16.6	562	93.5	724
3	77	C/C	321	11.3	168	82.8	1030
4	81	C/G	395	7.1	160	77.4	n.d.
5	83	G/G	574	n.d.	n.d.	69.4	2368
6	69	C/G	324	10.5	269	89.9	1120
7	80	G/G	119	16.6	379	87.2	831
8	77	C/G	467	8.6	194	73.9	1144

Abbreviations: B12: Vitamin B12; HCY: Homocysteine; L: Liter; ID#: Participants Identification Number; MMA: Methylmalonic Acid; µmol: Micromole; nmole: Nanomole; pmol: Picomole; n.d.: Not Determined;TCN2: Transcobalamin II Gene Symbol; TCN2 766C/C: TCN2 766C Homozygosity (having 2 C alleles); TCN2 766C/G: TCN2 766C/G Heterozygosity (having 1 C and 1 G allele); TCN2 766G/G: TCN2 766G Homozygosity (having 2 G alleles).

Table 2: TCN2 genotype effects on abnormal levels of HCY, MMA, creatinine and B12 at the study start among those who developed dementia. Note that a cluster of four factors: raised homocysteine, raised methylmalonic acid, high creatinine, plus TCN2 766G homozygosity occurs at the study start in persons who developed dementia by the end of the study but not in those who retained normal cognitive function. This cluster of factors has been tentatively dubbed as "the TCN2 dementia-predisposing phenotype, TDPP".

TCN2 Genotype	Serum Biochemical Abnormality at Study Start	Developed Dementia by Study End (Cases)	Still Cognitively Normal by Study End (Controls)	P
766G/G		N=6	N=28	
		Prevalence (%)		
	HCY>15µmol/L	66.7	3.57	0.00150
	MMA>350nmol/L	66.7	7.14	0.00430
	Cr>87µmol/L	66.7	14.3	0.0180
	B12<210pmol/L	66.7	21.4	0.0477
766C/G		N=6	N=64	
		Prevalence (%)		
	HCY>15µmol/L	0	4.69	n.s.
	MMA>350nmol/L	0	9.38	n.s.
	Cr>87µmol/L	16.7	14.1	n.s.
	B12<210pmol/L	16.7	21.9	n.s.
766C/C		N=2	N=42	
		Prevalence (%)		
	HCY>15µmol/L	0	4.76	n.s.
	MMA>350nmol/L	0	4.76	n.s.
	Cr>87µmol/L	0	9.52	n.s.
	B12<210pmol/L	50	21.4	n.s.

Abbreviations: B12: Vitamin B12; Cr: Creatinine; HCY: Homocysteine; L: liter; MMA: Methylmalonic Acid; µmol: Mmicromole; nmol: Nanomole; pmol: Picomole; N: Number of Participants in the Case or Control Groups; %: percent; P: Probability that the Prevalence of a Particular Biochemical Abnormality at the Study Start Differs for the Cases Compared to the Controls;TCN2: Transcobalamin II Gene Symbol; 766G/G: TCN2 766G Homozhgosity (having 2 G alleles); 766C/G: TCN2 766C/G Heterozygosity (having 1 C and 1 G allele); 767C/C: TCN2 766C Homozygosity (having 2 C alleles).

To determine if some or all of the biochemical abnormalities at study entry among those who developed dementia by the study end were potential dementia-predisposing effects associated with TCN2 766G, we stratified persons according to their outcome at the study end (i.e., having dementia or still cognitively normal) by TCN2 genotype, and compared the prevalences of abnormalities at study start in these two groups (Table 2). Among those with TCN2 766G homozygosity (but not other TCN2 genotypes), development of dementia was significantly associated with raised HCY, raised MMA and high creatinine at the study start. TCN2 766G homozygosity also

Table 3: Evaluation of involvement of specific allelic polymorphisms of TCN2 and MTHFR in dementia development. The whole numbers in this table indicate the number of individuals with given genotypes or the number of particular alleles in the categories denoted in column 1. The percentages indicate the fraction in each group with the denoted allele. The ratio of TCN2 allele G to C is significantly higher in the cases (those who developed dementia by the study end) compared to the controls (those who retained normal cognitive function) (P = 0.0200). The allelic ratios of MTHFR 677T: C and 1298C: A were not different in the case and control groups. The "wild type" alleles are TCN2 766C, MTHFR 677C and MTHFR 1298A.

	Genotype			Alleles	
TCN2 766 C-G allele involvement					
	766C/C	766C/G	766G/G	Allele C	Allele G
All genotyped participants	50	76	36	176 (54.3%)	148 (45.7%)
Still normal by study end (control group)	46	65	28	157 (56.5%)	121 (45.5%)
Developed dementia by study end (case group)	2	6	7	10 (33.3%)	20 (66.7%*)
Developed mild cognitive impairment by study end	2	5	1	9 (56.3%)	7 (43.8%)
MTHFR 677 C-T allele involvement					
	677C/C	677C/T	677T/T	Allele C	Allele T
All genotyped participants	79	67	16	225 (69.4%)	99 (30.6%)
Still normal by study end (control group)	69	57	13	195 (70.1%)	83 (29.9%)
Developed dementia by study end (case group)	6	5	3	17 (60.7%)	11 (39.3%)
Developed mild cognitive impairment by study end	4	5	0	13 (72.2%)	5 (27.8%)
MTHFR 1298 A-C allele involvement					
	1298A/A	1298A/C	1298C/C	Allele A	Allele C
All genotyped participants	64	68	16	196 (66.2%)	100 (33.8%)
Still normal by study end (control group)	51	60	17	162 (63.3%)	94 (36.7%)
Developed dementia by study end (case group)	7	6	0	20 (76.9%)	6 (23.1%)
Developed mild cognitive impairment by study end	6	2	0	14 (87.5%)	2 (12.5%)

Abbreviations: MTHFR: Methylenetetrahydrofolate Reductase Gene Symbol; 677C/C: MTHFR 677C Homozygosity (having 2 677C alleles; 677C/T: MTHFR 677C/T Heterozygosity (having 1 C and 1 T allele); 677TT: MTHFR 677 T Homozygosity (having 2 677T alleles); 1298A/A; MTHFR 1298A Homozygosity (having 2 A alleles); 1298A/C: MTHFR 1298A/C Heterozygosity (having 1 A and 1 C allele); 1298C/C: MTHFR 1298c Homozygosity (having 2 C alleles); TCN2: Transcobalamin II Gene Symbol; 766C/C: TCN2 766C Homozygosity (having 2 C alleles); 766C/G: TCN2 766C/G Heterozygosity (having 1 C and 1 G allele); 766G/G: TCN2 766G Homozygosity (having 2 G alleles).

was associated with low B12, although this significance approached the borderline.

That TCN2 766G was associated with dementia was independently established from comparisons of the 766C and G allele frequencies in the cases and the controls (Table 3). There was no analogous involvement of MTHFR polymorphisms at two different loci (Table 3).

The odds ratios characterizing the association of TCN2 766G homozygosity, and abnormal levels of HCY, MMA and creatinine at study entry with dementia development are significant when considered singly.

We next determined ORs and their 95% CIs to characterize the strengths of association of each of the putative genetic and biochemical risk factors for dementia flagged in the section above (Table 4). ORs for TCN2 766G homozygosity, raised HCY and raised MMA were significant, though the post-hoc power was less than 80% in most cases. Notably, the OR for involvement of "raised" creatinine above the upper lab limit of normal (>110μmol/L) was surprisingly high in females, although that for "high" creatinine in the upper 15% of normal values (i.e., >87μmol/L) was only marginally significant. ORs for B12 deficiency or B12 in the lowest quartile of values for participants were not significant.

TCN2 766G homozygosity plus abnormal levels of HCY, MMA or creatinine at study entry have higher odds ratios for dementia development than factors considered singly.

Because the ORs for involvement of candidate risk factors singly were relatively weak, we next asked if particular combinations of factors at study entry had higher ORs for development of dementia (Table 5). Combinations of TCN2 766G homozygosity with raised HCY, raised MMA, high creatine, and raised creatinine (in females only) all had higher ORs for dementia than factors considered singly. The four-factor cluster of TCN2 766G homozygosity, raised HCY, raised MMA and high creatinine had the same OR for dementia as TCN2 766G homozygosity plus raised HCY. This was similar in magnitude to that of APOE e4 homozygosity (Table 4).

Interpretation

The observations provide support for a model in which raised HCY that precedes the development of dementia is not a simple phenomenon reflecting only vitamin B12 and/or folate deficiency as reflected by measurements using standard laboratory techniques. Rather, raised HCY is a complex indicator that co-occurs with TCN2 766G homozygosity, high creatinine possibly indicative of mild kidney dysfunction, as well as with raised MMA (possibly indicative of functional vitamin B12 deficiency), at least in our study sample.

We propose that homocysteinemia >15μmol/L plus TCN2766G homozygosity with co-occurring MMA >350nmol/L and evidence of mild kidney dysfunction (creatinine >87μmol/L) be tentatively dubbed as the "TCN2 dementia-predisposing phenotype (TDPP)". Because the association strength of TDPP for dementia is similar in magnitude to that for APOE e4 homozygosity in our sample (Table 4; [35]), TDPP may be a major player in the homocysteinemia-dementia controversy which has tended to focus on involvement of MTHFR variant alleles. As well, TDPP may herald development of a distinctive form dementia or that comprises a distinct subtype of AD.

The apparent gender effect on involvement of creatinine in

Table 4: Odds ratios for dementia development associated with single candidate risk factors. The top line of each cell in the Dementia column indicates: number of positives: number of negatives among the cases versus number of positives: number of negatives among the controls. ORs for B12<165 or 210pmol/L were not significant.

Potential Risk Factor	Dementia
HCY >15µmol/L at study entry (all available data)	5:20 vs.8:180; P = 0.0100 OR = 5.63 (95% CIs: 1.68-18.9) Posthoc power = 74.8%
HCY >15µmol/L at study entry (genotyped subset)	4:10 vs.6:131; P = 0.00720 OR = 8.73 (95% CIs: 2.11-36.1) Posthoc power = 79.8%
MMA>350pmol/L at study entry (all available data)	8:17 vs. 17:171; P = 0.00330 OR = 4.73 (95% CIs: 1.78-12.6) Posthoc power = 84.1%
MMA>350pmol/Lat study entry (genotyped subset)	4:10 vs. 11:126; P = 0.0351 OR = 4.58 (95% CIs: 1.23-17.0) Posthoc power = 63.1%
Creatinine>110µmol/L at study entry (all available data, females only)	2:12 vs. 1:143; P = 0.0209 OR = 23.8 (95% CIs: 2.01-282) Posthoc power = 74.3%
Creatinine>110µmol/Lat study entry (genotyped subset, females only)	2:5 vs. 2:107; P = 0.0178 OR = 21.4 (95% CIs: 2.48-185) Posthoc power = 77.3%
Creatinine>87µmol/L at study entry (all available data)	7:18 vs. 25:166; P = 0.0552
TCN2 766Ghomozygositycompared to other TCN2 genotypes	7:8 vs. 28:111; P = 0.0448 OR = 3.47 (95% CIs: 1.16-10.4) Posthoc power = 62.6%
APOE e4 homozygosity	2:13 vs. 0:146; P = 0.00820 OR = 54.3 (95% CIs: 2.48-1190) Posthoc power = 80.1%

Abbreviations: APOE: Apolipoprotein E; APOEe4 Homozygosity: having 2 e4 alleles of APOE; CI: Confidence Interval; HCY: Homocysteine; L: liter; MMA: Methylmalonic Acid; µmol: Micromole; nmol: Nanomole; pmol: Picomole; OR: Odds Ratio Reflecting the Strength of Association between given Risk Factors and Development of Dementia; P: Probability that the Denoted Risk Factor is Significantly Associated with Development of Dementia; TCN2 766G Homozygosity: having 2 766G Alleles.

Table 5: Odds ratios for dementia development associated with TCN2 766G homozygosity in combination with abnormal levels at study entry of homocysteine, methylmalonic acid or creatinine. In the second column, the pair of factors under evaluation is that at the left in combination with TCN2 766G homozygosity. The ratios on line 1 of column 2 are as follows: number of positives for the risk factor pair: number of negatives among the cases versus number of positives for the risk factor pair: number of negatives among the controls.

	TCN2 766G Homozygosity (766G/G)
HCY >15µmol/L	4:10 vs. 1:135; P =0.000200 OR = 54.0 (95% CIs: 5.50-530) Posthoc power = 93.1%
MMA >350nmol/L	4:10 vs. 2:134; P = 0.000700 OR = 27.8 (95% CIs: 4.53-171) Posthoc power = 91.1%
Cr >110µmol/L Females only	2:5 vs. 0:105; P = 0.00340 OR = 95.9 (95% CIs: 4.09-2250) Posthoc power = 86.0%
Cr >87µmol/L All	4:11 vs 4:137; P = 0.00320 OR = 12.5 (95% CIs: 2.74-56.7) Posthoc power = 85.3%

Abbreviations: CI: Confidence Interval; Cr: Creatinine; HCY: Homocysteine; L: Liter; MMA: Methylmalonic Acid; µmol: Micromole; nmol: Nanomole; OR: Odds Ratio Reflecting the Strength of Association between the Risk Factor Pair and Development of Dementia; %: Percent; P: Probability of the Factor Combination being Significantly Associated with Dementia Development; TCN2: Transcobalamin II Gene Symbol; TCN2 766G/G: TCN2 766G Homozygosity (having 2 G alleles).

dementia may have resulted from the use of one cutoff value (creatinine >146µmol/L) to exclude volunteers with chronic kidney disease from participating. Because males have higher serum creatinine levels than females as the result of higher muscle mass [54] and kidney function deteriorates more rapidly in males than females [56,57], use of the single cut-off value may have excluded more males with mild kidney dysfunction than females from participation. Previous studies have noted that persons with all stages of chronic kidney disease are at increased risk of developing cognitive impairment or dementia [58,59], but why is not clear. Such involvement might mirror pathological changes occurring in the brain, reflect vascular disease, and/or contribute to dementia development [60,61]. Future studies should consider gender-specific cut-offs for exclusion of persons likely to have chronic kidney disease. That kidney dysfunction might actually be a phenotypic consequence of TCN2 766G homozygosity and possibly result, at least in part, from raised levels of MMA might not be as far-fetched an idea as would seem. Methylmalonic acidemia caused by deficiency of MUT, a mitochondrial enzyme requiring B12 as a co-factor (see Introduction), is often complicated by end stage renal disease because patients are living longer [62,63]. To note is that in a genetically engineered animal model of methylmalonic acidemia, antioxidant administration attenuated damage to proximal renal tubules that occurred in these animals [63]. Future dementia prevention efforts in humans with kidney dysfunction might consider similar antioxidant supplementation. Because kidney dysfunction was not directly established in this study, its involvement in dementia must be qualified as "possible".

Originality

Our findings shed new light on the nature of homocysteinemia in dementia. In the present sample, homocysteinemia that precedes impending dementia appears to be multifactorial in nature.

Specifically, raised HCY appears to be a component of a TCN2 dementia-predisposing phenotype and co-occurs with TCN2 766G homozygosity, raised serum MMA and high serum creatinine. The involvement of TCN2 766G homozygosity with possible co-occurring mild kidneys dysfunction may be two links that have been missed in previous investigations of HCY involvement in dementia development.

Study benefits and limitations

A number of factors may have enhanced the feasibility of obtaining meaningful results in the present study. These include: the stringent exclusion and entry criteria and restriction of age range to those over age 65, and the use of up to four sets of cognitive data generated over a 6-year period which permitted participants who developed dementia or who were still cognitively normal by the study end to be distinguished from those who developed only MCI during the observation window. The main limitation to the study is the low conversion rate to dementia which resulted in a rather small group of cases and post-hoc power less than 80% in some analyses even though these attained significance.

Future research directions

In addition to strategies suggested in Interpretation (above), larger studies to confirm and extend our original and potentially important findings and insights are warranted. Future questions to ask are if TDPP is an early marker of impending dementia that may be a subtype of AD (or not), and if TDPP might distinguish a group to target with appropriate nutritional supplementation *prior* to development of mild cognitive impairment.

Conclusion

This small investigation supports the idea that mild homocysteinemia which precedes impending dementia is part of a cluster of factors including TCN2 766G homozygosity, raised MMA (an indicator of B12 deficiency) and high creatinine (an indicator of mild kidney dysfunction which can raise levels of HCY and MMA). This four-factor cluster has been dubbed "the TCN2 dementia-predisposing phenotype, TDPP". The findings provide new insight about the homocysteinemia-dementia enigma –implicating TCN2 766G homozygosity and possible mild kidney dysfunction as "new players" in the homocysteine-dementia arena. The findings also may aid with delineation of subtypes of Alzheimer's and with the design and interpretation of dementia prevention and intervention trials. Because this study was pilot and exploratory, the important findings warrant replication with larger samples. The findings pertain to a stringently-selected group of Caucasian community volunteers, with an average age of 73 at the study start and 79 at the study end.

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