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IMPACT OF HYPERHOMOCYSTEINEMIA TREATMENT ON COGNITIVE DECLINE DUE TO ALZHEIMER'S DISEASE AND RELATED DISORDERS

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Abstract: *Objectives:* Studies have produced conflicting results assessing hyperhomocysteinemia (HYH) treatment with B vitamins in patients with normal cognition, Alzheimer's disease or a related disorder (ADRD). This study examined whether HYH treatment with Cerefolin®/Cerefolin-NAC® (CFLN) influenced cognitive decline. *Design:* Retrospective cohort study of subjects followed longitudinally. *Setting:* Outpatient clinic for cognitive disorders. *Participants:* Of 934 patients, 109 HYH patients met inclusion criteria to analyze effect of CFLN (N=86: median treatment duration and 90% confidence band = 17.3 [0-52.4] months) vs. no CFLN (N=23). *Intervention:* CFLN vs. no treatment. *Measurements:* Cognitive outcome measures included MCI Screen (memory), CERAD Drawings (constructional praxis), Ishihara Number Naming (object recognition), Trails A and B (executive function), and F-A-S test (verbal fluency). Dependent or predictor measures included demographics, functional severity, CFLN and no CFLN treatment duration, ADRD diagnosis, memantine and cholinesterase inhibitor treatment, apolipoprotein E (ApoE) genotype and quantitative MRI volumes. The data were analyzed using linear mixed effects models with covariate adjustment and random effects for functional severity. *Results:* Treatment duration on vs. off CFLN slowed decline in memory, constructional praxis, and executive function, even after controlling for ApoE genotype and regional brain volumes. CFLN treatment slowed cognitive decline significantly more for patients with milder baseline severity. *Conclusion:* This retrospective study showed that CFLN significantly slowed cognitive decline among HYH patients. Longer CFLN treatment duration and milder baseline severity were both significant factors. These findings warrant prospective validation.

Key words: Homocysteine, homocysteinemia, cognitive impairment, Alzheimer's disease, vascular dementia.

Introduction

Cognitive impairment (CI), in aging societies like the United States, is a major healthcare burden. While Alzheimer's disease (AD) is the leading cause of CI, other chronic conditions, such as atherosclerosis, diabetes, hypertension, heart disease, thyroid disease, pulmonary disease and sleep disorders, contribute to CI, and better management of these conditions can improve cognitive and functional outcomes while lowering CI-related healthcare costs, such as poor medication management.

Hyperhomocysteinemia (HYH) is another treatable condition that is associated with higher risk of mild cognitive impairment (MCI) (1), of which the prevalence in population studies ranges from 5.1% to 29% in persons aged 65 years and older (2-4). A study on cardio- and cerebro-vascular diseases in Switzerland found that the economic burden attributable to HYH (prevalence estimated to be 5-7%) was 10% (5), such that effective treatment of HYH could substantially reduce healthcare costs and improve quality of life if it is causally related to the development of vascular pathology.

One high potency formulation for treating HYH is a prescription medical food containing L-methylfolate, methylcobalamin and N-acetylcysteine (CFLN: Cerefolin®/Cerefolin-NAC®). Agents contained in CFLN have individually shown positive effects on amnestic MCI (6), particularly among individuals diagnosed with neurovascular oxidative stress or at increased risk for neurovascular oxidative stress (7-9) with or without HYH (10) or vitamin B12 deficiency (11-14). Previous studies have also shown positive effects of these individual agents in patients with vascular

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dementia (VD) (13, 15, 16) or AD (7 ,13, 15, 17). However, CFLN has not been well-studied in these clinical conditions or in various disease stages of these conditions.

The present study examined whether CFLN treatment of HYH delays decline in cognitive performance, and whether severity of cognitive impairment influences treatment response. To evaluate these issues, the effect of CFLN vs. no CFLN on cognitive change over time was analyzed among HYH patients with normal cognition, mild cognitive impairment (MCI) or dementia due to AD, VD, or another related disorder (ADRD).

Method

Sample

Data on 934 patients were extracted from a community memory clinic database, 109 of which met criteria for inclusion in the analysis. The criteria included: 1) laboratory-confirmed HYH, which is defined as a plasma homocysteine level ≥ 12 umol/liter because it is intermediate in the range of values considered to define HYH in the literature; and 2) no concomitant treatment with B12, folate, or B6 supplementation. 86 HYH patients received CFLN treatment for part or all of the study period (CFLN group). 23 HYH patients never received CFLN (No CFLN group). 39 and 59 of these 109 patients had quantitative MRI (qMRI) and apolipoprotein E (ApoE) genotype data, respectively.

Sample size, gender, age, years of education, follow-up duration, CFLN treatment duration, and baseline score of the MCI Screen's Memory Performance Index (MPI) are shown for the CFLN and No CFLN groups in Table 1. Table 2 summarizes patients' diagnoses and baseline severity. AD and VD diagnoses conformed to standardized criteria, which require confirmation of characteristic imaging features and regional atrophy patterns, exclusion of other diagnoses by laboratory assessment, cognitive testing results that are consistent with characteristic patterns seen in AD and VD, plus history and risk factors that are consistent with these diagnoses. Non-AD and non-VD diagnoses were based on exclusion of AD and VD diagnoses, plus inclusion of degenerative or non-degenerative etiologies, including Lewy body disease, frontal temporal lobe disease, normal pressure hydrocephalus and traumatic brain injury. About 20% of patients in each group did not have a defined ADRD etiology, but their baseline severity at the first cognitive assessment was established using the Functional Assessment Staging Test procedure (FAST) (18). Those whose FAST stage was not done at baseline were designated, "Not Assigned."

Table 1
Sample Characteristics

	CFLN	No CFLN
N	86	23
% Female	44%	39%
Age (years)	78.4 +/- 9.3	77.6 +/- 8.5
Education (years)	14.9 +/- 3.3	15.3 +/- 3.0
Months Follow-up*	19.7 (0.3-60.8)[a]	6.5 (0.0-62.2)
Months CFLN*	17.3 (0-52.4)	-
Baseline MPI*	33.7 (19.1-43.7)	31.4 (24.5-47.7)

*The median and 90% confidence bands (5-95%iles) are shown for these measures because their distribution is not Gaussian; a. Follow-up duration was significantly longer for CFLN vs. No CFLN groups (p = 0.0258).

Table 2
Sample Classification

	CFLN	No CFLN
By ADRD Diagnosis		
AD	25 (29.1%)[a]	5 (21.7%)
VD/Possible VD[1]	22 (25.6%)	7 (30.4%)
AD+VD	9 (10.5%)[b]	6 (26.1%)
LBD	2 (2.3%)	0
FTLD	1 (1.2%)	0
NPH	6 (7.0%)	0
TBI	0	0
Other ADRD NOS[2]	19 (22.1%)	5 (21.7%)
Normal Cognition	2 (2.3%)	0
Total	86 (100%)	23 (100%)
By Baseline FAST		
FAST 1,2 (NL/SCI)	9 (10.5%)	1 (4.3%)
FAST 3 (MCI)	20 (23.2%)	7 (30.4%)
FAST 4-7 (Dementia)	54 (62.7%)[c]	8 (34.8%)
Not Assigned	3 (3.5%)[d]	7 (30.4%)
Total	86 (100%)	23 (100%)

NL: normal; SCI: subjective cognitive impairment; MCI: mild cognitive impairment; 1. For CFLN and No CFLN groups, there were 4 and 2 Possible VD patients; 2. Other ADRD NOS consists of cognitively impaired and demented with no etiologic diagnosis; a. The proportion of AD patients was significantly higher in the CFLN vs. No CFLN group (p = 0.0534); b. The proportion of AD+VD patients was significantly lower in the CFLN vs. No CFLN group (p = 0.0534); c. The proportion of FAST Stages 1 or 2 patients was significantly greater in the CFLN vs. No LMF-B group (p = 0.0160); d. The proportion of unassigned FAST Stage patients was significantly lower in the CFLN vs. No CFLN group (p = 0.0001).

Assessments

Each patient was clinically followed every 3-6 months, and assessed cognitively and functionally using standardized tools. The cognitive assessment tasks included MCI Screen (MCIS) for memory (19), CERAD drawings for constructional praxis, Ishihara Number Naming for object recognition, Trails A and B for processing speed, working memory and set-shifting (executive function), and F-A-S for verbal fluency. FAST staging was used to assess functional severity. There are 7 major stages and 16 sub-stages, each with a published mean duration of the untreated course of AD.

Although the ADAS-Cog is most commonly used in clinical trials to assess treatment effect, a within-subject

comparison found that the MCIS discriminated treatment effect 39% better than the ADAS-Cog (20). The MCI Screen was therefore selected over the ADAS-Cog because of its higher signal-to-noise ratio, and because of its utility in detecting more subtle memory changes seen in MCI prior to the dementia phase.

Apolipoprotein E Genotype and Quantitative MRI

Apolipoprotein E (ApoE) genotype was obtained, when possible, as part of routine assessment of individuals with cognitive impairment. Quantitative MRI (qMRI) volumetrics were also measured, when possible, as part of routine evaluation of patients with CI. Reasons for not obtaining these data included clinical necessity, cost, contraindication for having an MRI, and availability of the quantitative volumetric software at time of initial patient assessment.

Primary Outcomes

The primary cognitive outcome measure was the Memory Performance Index (MPI) of the MCIS – a measure of working and episodic memory. Secondary cognitive outcome measures included: 1) Trails A and B time plus total correct and error scores; 2) F-A-S verbal fluency total correct plus error scores; 3) Ishihara object recognition time plus total correct; and 4) CERAD drawing time plus total correct and error scores.

Analyses

Multiple non-parametric comparisons (Stata's ranksum procedure for interval variables; Stata's prtest procedure for ordinal or nominal variables) were performed to determine if there were any statistically significant between-group differences for demographics, MPI score, and diagnosis (Tables 1 and 2).

To examine between-subject effects, we used a mixed effects linear model with random and fixed effects (Stata 12.0, xtmixed). The effect of functional severity category (FAST staging) on each patient's outcome was modeled as a random effect. Durations of CFLN and no CFLN treatment since the baseline cognitive assessment were included as fixed effects. By comparing the model's slope coefficients and p-values for CFLN vs. no CFLN treatment duration, one can determine if CFLN delayed cognitive decline in the outcome measure being examined.

Potential covariates were modeled as fixed effects on the outcome measures, and included age at baseline cognitive assessment, education, AD, VD, and non-AD non-VD diagnoses, functional severity (FAST staging), memantine treatment, and cholinesterase inhibitor treatment (Table 3, Base Model, N=109).

Table 3
P-values of mixed effects analyses assessing CFLN vs. no CFLN treatment effect for each model and cognitive outcome measure

	Base Model	CFLN by Severity Interaction	ApoE + Base Model	qMRI + Base Model
Sample Size	109	109	59	39
MPI	0.028	0.014[b]	0.062	0.009
Drawings correct	<0.001	0.333	<0.001	<0.001
Cube secs	0.230	0.287	0.029	0.009
Rectangle secs	0.045	0.264	0.047	0.523
Diamond secs	0.070	0.597	0.090	0.126
Circle secs	0.764	0.069[b]	0.071	0.015
INN secs	0.431	0.120	0.359	0.046
INN correct	0.106	0.068[b]	0.457	0.047
Trails A secs	0.283	0.192	0.063	0.636
Trails A errors	0.348	0.741	0.220	0.008
Trails B secs	0.098	0.989	0.013	0.016
Trails B errors	0.540	0.020[a]	0.556	0.324
FAS correct	0.070	0.154	0.166	0.274
FAS reps	0.085	0.047[a]	0.040	0.232
FAS set losses	0.746	0.075[a]	0.045	0.398

a. CFLN treatment effect was associated with significantly ($p < 0.05$) slower decline in hyperhomocysteinemic patients with normal cognition or subjective CI (FAST stages 1,2) compared to the corresponding MCI group (FAST stage 3). A trend for a significant association was considered as a p-value between 0.05 and 0.10; b. CFLNtreatment effect was associated with significantly ($p < 0.05$) slower decline in hyperhomocysteinemic patients with MCI (FAST stage 3) compared to the corresponding dementia group (FAST stages 4-7). A trend for a significant association was considered as a p-value between 0.05 and 0.10.

ApoE genotype (number of E2, E3 and E4 alleles) and qMRI volumetric data (hippocampus, inferior lateral ventricles, lateral ventricles, cortex, hippocampus/(hippocampus + inferior lateral ventricle) ratio, and cortex/(cortex+lateral ventricles) ratio) were also available for a subset of the 109 subjects. These sub-samples were also analyzed with the mixed effects linear model with covariate adjustment after including the ApoE (Table 3, ApoE + Base Model, N=59) and qMRI data (Table 3, qMRI + Base Model, N=39) respectively.

Of particular interest was whether severity of impairment influenced treatment response to CFLN. To test for such an interaction, three severity groups were constructed – normal subjects with no functional impairment (FAST stage 1 or 2), MCI subjects (FAST stage 3) and dementia subjects (FAST stages 4-7). Tests for interaction between CFLN treatment and severity pairs – Normal vs. MCI (N=37, repeated measures = 175) and MCI vs. Dementia (N=72, repeated measures = 434) – were then constructed to examine for CFLN by severity differential treatment effects. The aforementioned mixed effects linear model with covariate adjustment was then run after excluding FAST staging (Table 3, CFLN by Severity Interaction, N=109).

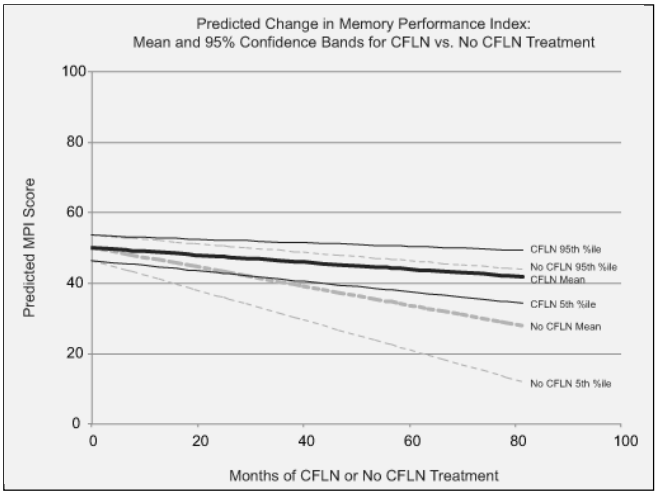
Results

The baseline mean and median homocysteine levels were 14.8 and 14.9 (95% confidence limits = 7.4-22.3, and 7.9-24.4 respectively), indicating an approximately Gaussian distribution. Tables 1 and 2 also show that there were demographic, diagnostic and severity (FAST Stage) differences between the CFLN and No CFLN treatment groups, warranting inclusion of these variables as covariates that could influence cognitive test performance over time.

Effect of Duration of CFLN vs. No CFLN Treatment

Figure 1 shows the change in mean MPI score and its associated 95% confidence band for the estimated parameters of the duration on vs. off CFLN treatment. The slopes on vs. off CFLN treatment significantly differed ($p = 0.046$, Chi-square test). It also showed that longer CFLN treatment duration increased the difference in MPI score compared to no CFLN treatment.

Figure 1
Effect of duration of treatment with vs. without CFLN on the primary outcome measure



A mixed effects regression was used to predict Memory Performance Index (MPI) score changes over time. A random effects intercept per patient, plus fixed effects for duration of CFLN and no CFLN treatment was used for this model. The slopes of the change in MPI score per month of CFLN or no CFLN treatment significantly differed ($p = 0.046$) and indicated slower decline on CFLN treatment. To illustrate the impact of CFLN vs. no CFLN treatment over time, the mean and 95% confidence bands are plotted for two hypothetical patients whose initial MPI scores were both 50 out of 100, and were treated for 7 years with or without CFLN.

Adjustment for Potential Confounding Factors

Primary Outcome

Figure 1 shows that longer CFLN treatment duration was associated with significantly slower decline of the memory performance index (MPI) ($p = 0.046$). Table 3 shows that the beneficial effect of CFLN treatment duration on the rate of decline in MPI score persisted after covariate adjustment ($p = 0.028$, Base Model).

Secondary Outcomes

Table 3 shows, for each model and cognitive task measure examined, the p-values of the effects of CFLN treatment duration compared to duration of no CFLN treatment. At a p-value of 0.05 or less, 3 of the 60 comparisons (4 models x 15 significance tests) would be expected due to chance alone, whereas 20 of the 60 comparisons had a p-value less than 0.05.

For the base model (ApoE genotype and qMRI data excluded), CFLN treatment duration was associated with significantly slower cognitive decline on tasks of memory and constructional praxis ($p < 0.05$). A trend for slower cognitive decline with CFLN treatment ($0.05 < p < 0.10$) was observed for measures of executive function and verbal fluency.

For the model that tested for interaction between disease severity and CFLN treatment, there was significantly greater slowing of decline in milder subjects for measures of memory, executive function and verbal fluency ($p < 0.05$). A trend for slower cognitive decline in milder subjects treated with CFLN ($0.05 < p < 0.10$) was observed for measures of constructional praxis and object recognition.

For the model that included ApoE genotype data (N=59), CFLN treatment duration was associated with significantly slower decline on measures of constructional praxis, executive function and verbal fluency ($p < 0.05$). A trend for slower decline with CFLN treatment ($0.05 < p < 0.10$) was observed for memory performance.

For the model that included qMRI data (N=39), CFLN treatment duration was associated with slower cognitive decline on measures of memory, constructional praxis, object recognition, and executive function ($p < 0.05$).

Discussion

In this study, across all three data models (base, ApoE genotype, and qMRI), CFLN treatment compared to no CFLN treatment duration consistently associated with slower cognitive decline for measures of memory, constructional praxis and executive function. Significantly slower cognitive decline with CFLN treatment was also found for object recognition and verbal fluency on some but not all of these data models. Also of potential importance is the association of a greater benefit of CFLN treatment for patients started earlier in the course of their cognitive decline. The findings of a cumulative benefit of CFLN treatment duration plus benefit when starting it in milder subjects have not been previously reported to our knowledge. These findings

imply that cognitive decline in ADRD patients with HYH could be delayed with early and prolonged CFLN treatment, and warrant validation in a prospective study.

Comparison with Previous Studies

There is compelling experimental and epidemiological data suggesting that B-vitamin deficiency and/or HYH can cause a variety of neurological deficits, which has provided motivation for large randomized, double-blind clinical trials aimed at determining the safety and efficacy of B-vitamin supplementation for preserving cognitive function in older adults.

Among non-randomized studies of subjects with HYH versus normal homocysteine levels, HYH has been found to be associated with increased risk for cognitive impairment or decline in global cognition, executive function, constructional praxis, processing speed, verbal fluency, and episodic memory, as well as white matter atrophy (21-28).

Among studies assessing effect of B6, B12 and folate treatment, a meta-analysis of randomized, double-blind, placebo-controlled trials found no effect on cognitive decline (29). However, other similarly designed trials (not meta-analyses) have found slower rates of decline in global cognition, executive function, semantic and episodic memory, and whole brain atrophy (30, 31). While the majority of these studies have not demonstrated that B-vitamin supplementation has protective or therapeutic cognitive benefit, there are a number of possible explanations for this perceived lack of efficacy, which are discussed in the context of the AD Cooperative Study (ADCS) trial.

A frequently cited, negative result is the ADCS randomized, double-blind, placebo controlled trial of homocysteine lowering with B12, folate and B6 supplementation (32). The generalizability of the ADCS study's findings may be limited because: 1) it included patients with normal homocysteine levels; 2) the sample was restricted to mild to moderate dementia AD patients, who, according to the present study's findings, may be less likely to benefit from homocysteine lowering than milder patients; 3) the primary cognitive outcome measure, the ADAS-Cog, has been shown to be less sensitive in subjects with milder forms of memory impairment than those with dementia; and 4) the treatment using vitamin B supplementation differs from CFLN (methylcobalamin, L-methyl-folate and N-acetyl-cysteine), whose effect may not solely be due to homocysteine lowering. Further studies are needed because there are a number of possible explanations of the negative results of some trials that need to be excluded to more clearly understand the benefits or lack thereof of CFLN treatment or vitamin B supplementation.

Physiologically, homocysteine regulation may not only be critical for proper neuronal functioning, and cerebrovascular health, but may also be critical to proper circadian gene expression and protein transcription in neurons and other cells. Homocysteine and adenosine triphosphate produce S-adenyl-methionine – the main methyl group donor in cells. These methyl groups attach to exposed histone regulatory sites in the nucleus to control the circadian rhythm of cellular protein synthesis (via gene transcription) (33). Elevated or reduced homocysteine levels alter the circadian pattern of proteins transcribed and may significantly interfere with neuronal function. This potential mechanism of deleterious effects of abnormal homocysteine levels needs to be investigated in humans and animal models.

Study Limitations

This study used statistical covariate adjustment to account for differences between CFLN and no CFLNtreatment groups that may influence the outcome measures. Given the relatively small sample sizes, it is possible that these adjustments were inadequate to account for the effects of these covariates. While other approaches can be used, such as a matched sample design, or exclusion of cases based on some criterion, they each have their strengths and weaknesses. Whether or not the covariate adjustments were adequate in the present study is being addressed by an ongoing prospective study.

This study also included some cases with shorter CFLN treatment durations than would be considered acceptable for analysis of disease modification. Both symptomatic benefit and disease modification are potential explanations for the observed findings of this retrospective study. The median treatment duration of 17.3 months is comparable to the durations of treatment used in FDA clinical trials to evaluate disease modification. The present data allow one to initially evaluate the hypothesis of disease modification, which necessarily must be further evaluated by a prospective study.

This study is retrospective, which introduces bias from potential covariates not analyzed or measured. Another weakness of this study is its non-random sampling, which could lead to differences in the outcome measures that arise from potential differences in the CFLN treated vs. untreated samples. These relative weaknesses will be more accurately addressed in a prospective study with larger sample sizes of ApoE and qMRI. Given these limitations, the present study extends the findings of HYH treatment to a broader range of cognitive processes that may be ameliorated, and to potentially greater benefit when treatment is started earlier.

Conclusion

This retrospective analysis of a clinical sample of HYH patients treated with CFLN for up to 7 years yielded two important hypotheses that merit further validation: 1) a broader range of cognitive abilities may benefit (delayed cognitive decline) from CFLN treatment than from vitamin supplementation with folate, B12 and/or B6; and 2) initiating treatment in less impaired patients may produce greater benefit. This benefit may be observed in the context of co-existing vascular cognitive impairment and AD. Prospective validation of these findings is warranted.

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Conflict of Interest Statement: Drs. Shankle and Hara are employees and shareholders of Medical Care Corporation. Also they are on the board of directors. Dr. Rafii is a medical advisor for Medical Care Corporation and consultant to Pamlab.

Author Contributions: The authors had full access to all study data and had uniformly agreed to submit this paper for publication. Drs. Shankle and Hara have equally contributed to the study design/conceptualization, and the development of the manuscript for intellectual content. Dr. Shankle conducted statistical analyses for the study, and Dr. Hara confirmed the results. Dr. Rafii provided manuscript review and made significant contribution to the interpretation and writing of the results. Ms. Russell has contributed to the assembly of the data and was responsible for the quality of the data for this study.

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