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N-Acetylcysteine for Hereditary Cystatin C Amyloid Angiopathy
A Nonrandomized Clinical Trial

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IMPORTANCE Hereditary cystatin C amyloid angiopathy (HCCAA) is a lethal, dominantly inherited disease primarily affecting Icelandic young adults that leads to severe cerebral amyloid angiopathy, with no effective therapy.

OBJECTIVE To investigate safety, tolerance, and therapeutic potential of *N*-acetylcysteine (NAC) in lowering disease-associated biomarkers in sequence variation carriers.

DESIGN, SETTING, AND PARTICIPANTS This phase 2a open-label clinical trial was conducted from March 2019 to December 2021 at a single study center at Landspitali University Hospital in Reykjavik, Iceland, and included 17 confirmed carriers of the L68Q-*CST3* sequence variation.

INTERVENTION High-dose NAC treatment was administered at 2400 mg daily for 9 months. Participants underwent regular monitoring for hemorrhages and disease progression, including blood and skin biopsy samples obtained every 3 months for biomarker testing.

MAIN OUTCOMES AND MEASURES The primary outcomes were drug tolerability and safety, cognitive status, and reduction in disease-associated biomarkers in skin biopsies. Secondary outcomes included changes in blood and plasma biomarker levels.

RESULTS Of 17 carriers treated, 6 were male and 11 were female, and mean (SD) participant age was 40.0 (4.2) years. Analysis of the primary outcomes showed that NAC was safe and well tolerated. Five cerebral bleeds occurred during the treatment period without permanent neurological sequela; no death occurred. There was significant reduction in median (IQR) disease-specific biomarker levels in skin after treatment, including collagen IV (baseline: 3.69% [2.48%-5.16%]; after treatment: 2.60% [1.99%-2.97%]; $P < .001$), fibronectin (baseline: 3.17% [2.09%-5.05%]; after treatment: 2.37% [1.87%-3.42%]; $P = .01$), vimentin (baseline: 1.60% [1.24%-2.37%]; after treatment: 1.31% [0.97%-1.68%]; $P < .001$), and SMAD (baseline: 2.25% [0.55%-4.36%]; after treatment: 1.56% [0.20%-2.54%]; $P < .001$) via Wilcoxon matched-pairs signed rank test. Secondary outcomes included a significant increase in reduced glutathione levels and decreased high-molecular-weight cystatin C aggregate levels in plasma after NAC treatment.

CONCLUSIONS AND RELEVANCE In this single-center nonrandomized clinical trial, NAC was safe and well tolerated and decreased disease-associated biomarker and amyloid deposition, suggesting NAC may offer a preventive strategy against HCCAA.

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Supplemental content

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Cerebral amyloid angiopathy (CAA) is among the world's leading cause of intracerebral hemorrhage, resulting in serious disability or death in patients. In CAA, amyloid is deposited in both the vessel walls and parenchyma of the central nervous system, as is also observed in Alzheimer disease.[1-3] Hereditary cystatin C amyloid angiopathy (HCCAA) is a subtype of CAA, an ultrarare Icelandic amyloid disease that causes cerebral hemorrhages in previously healthy young people.[4] HCCAA is a dominantly inherited disease caused by a sequence variation in the gene (*CST3*) coding for human cystatin C (hCC), which leads to leucine 68 to glutamine variant (L68Q-*CST3*) of hCC.[5] L68Q-hCC forms severe amyloid deposits in cerebral vessel walls in patients with HCCAA.[6-9] Most carriers of this sequence variation experience their first intracerebral hemorrhage in their 20s. The natural history of the disease shows that hemorrhages tend to increase in frequency and severity with associated dementia and paralysis, which ultimately leads to death, with a mean age at death of approximately 30 years, or 5 years on average after the first bleed. A small subset (2%-4%) of carriers have a longer life span.[4,7,10-12] HCCAA is rightly classified as familial CAA because of a strong cerebral presentation, although L68Q-hCC deposits can also be found in various internal organs and in skin.[13,14] In early stages, aggregation in the skin is only seen in the basement membrane (BM) between epidermis and dermis. As the disease progresses, the amount and distribution of L68Q-hCC deposits increase and clinical symptoms develop.[14] The L68Q-hCC deposit is always found in close connection with BM proteins in both the skin and in cerebral vessels, especially with collagen IV. In skin samples, increased cell density with activated fibroblasts occurs in the upper dermis.[9,14]

One of the most important drivers of the disease is the aggregation of L68Q-hCC into amyloid oligomers, as the monomer does not accumulate in the cerebral vasculature.[15-17] No treatment to avoid early death from brain hemorrhage is available; however, in a previous study of *N*-acetylcysteine (NAC), including NAC treatment in 6 L68Q-*CST3* sequence variation carriers, this class of drug showed promise as a potential treatment.[18] In the previous study, high-molecular-weight (HMW) L68Q-hCC aggregates were disrupted by NAC and related derivative compounds.[18] As the outcome of this previous study was encouraging, a formal phase 2a nonrandomized clinical trial of NAC in 17 patients with HCCAA was completed, the results of which are presented here and further support the benefits of NAC in reducing toxic disease-related biomarkers driving the pathogenesis of HCCAA.

Key Points

Question What is the safety, tolerability, and efficacy of *N*-acetylcysteine (NAC) for patients with hereditary cystatin C amyloid angiopathy (HCCAA)?

Findings In this open-label, nonrandomized clinical trial of 17 participants carrying the L68Q-*CST3* sequence variation with HCCAA, high-dose NAC therapy reduced levels of biomarkers of disease progression in skin and plasma. NAC was safe and well tolerated; a total of 5 cerebrovascular bleeds occurred during the study without permanent neurological sequela.

Meaning Data from this open-label phase 2a nonrandomized clinical trial support the safety and potential efficacy of NAC as a therapeutic treatment of neurological consequences in patients with HCCAA.

Methods

Participants and Study Design

A total of 22 individuals who were aged 18 years or older and determined to be eligible for this open-label, nonrandomized study were sequenced for the L68Q-*CST3* sequence variation in the screening phase. Of these, 17 individuals (11 female and 6 male) were confirmed to be L68Q-*CST3* carriers and were offered participation in the study (**Figure 1**). We note that HCCAA is an ultrarare disease, and no other living L68Q-*CST3* carrier was known to exist outside of those who enrolled who was eligible for the study. For study end points and for other inclusion and exclusion criteria, see the eMethods in Supplement 2. The study's design and conduct complied with all relevant regulations regarding the use of human biological specimens, and this study was conducted in accordance with the criteria set by the Declaration of Helsinki. All participants provided written informed consent and all necessary permits were obtained from the Icelandic National Bioethics Committee, the Data Protection Authority in Iceland, and the Icelandic Medicines Agency (EudraCT 2017-004776-56). See the full trial protocol in Supplement 1 for details. The study was monitored by Vistor, an independent monitoring agency in Iceland.

Primary End Points

Clinical Monitoring and Safety End Points

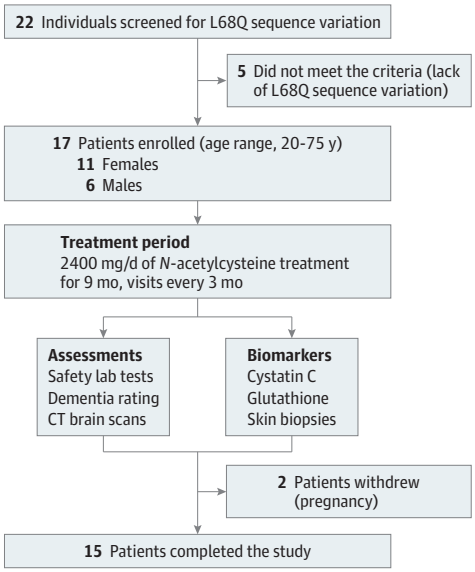
The study took place at a single study site: Landspítali University Hospital in Reykjavík, Iceland. All visits included meetings with a neurologist who completed comprehensive neurological and physical examinations. Safety and tolerability of NAC were assessed by monitoring treatment-emergent adverse events, which were coded by system organ class and preferred term using the Medical Dictionary for Regulatory Activities (version 24.1), clinical laboratory tests, and physical examination findings. All patients were closely monitored for adverse events (AEs) and provided blood, urine, and skin samples for laboratory testing at each visit. A computed tomography (CT) scan of the brain was acquired if new onset of neurological symptoms occurred. Furthermore, the Dementia Rating Scale-2 (DRS-2) was used to assess the cognitive status of the patients and to monitor global cognition.[19,20] See detailed description in the eMethods in Supplement 2.

Punch Skin Biopsies

Biopsies were obtained from all patients and 7 healthy family members (6 female and 1 male; age range, 30-68 years), the latter of whom were used as controls. The detailed method and quantification are described in the eMethods in Supplement 2.

Figure 1. Study Flow Diagram and Patient Characteristics

A Study flow diagram



B Characteristics of patients with HCCAA at baseline

Characteristics	No. (%)
Patients, No.	17
Age, y	
Median (range)	35 (20-75)
Mean (SD)	40 (4.2)
Sex	
Female	11 (64.7)
Male	6 (35.3)
NAC before trial, 1200-1800 mg	6 (35.3)
NAC before trial, 2400 mg	3 (17.6)
NAC naive before trial	8 (47.1)
Finished trial	15 (88.2)
Withdrew due to pregnancy	2 (11.8)

A, Study flow diagram showing enrollment of patients with hereditary cystatin C amyloid angiopathy (HCCAA) and analyses. B, Characteristics of patients with HCCAA at baseline. CT indicates computed tomography; NAC, *N*-acetylcysteine.

Secondary End Points

Detailed methods for glutathione assay and Western blot (WB) are described in the eMethods in Supplement 2.

Statistical Analysis

The statistical analysis plan of the data was generated using GraphPad Prism version 10.3.1 (GraphPad Software) after data were collected and prior to data analysis.

Primary Analysis

DRS-2

The DRS-2 includes 24 subtests that are used to generate 5 subscales: attention, initiation/perseveration, construction, conceptualization, and memory. In total, 15 patients finished all tests; however, 1 patient's score was an outlier that affected the normal distribution. Therefore the data were not normally distributed (confirmed using Kolmogorov-Shapiro test). However, as the patient cohort was very small, the Friedman test (nonparametric, used for analyzing repeated matched or measured data) was used for the analysis to test the null hypothesis if there were no differences between the 4 time points (visit 1 [V1]-V4).

Skin Biopsies

The Wilcoxon matched-pairs signed rank test was used to test if there was difference in immunoreactivity values (percentage per region of interest [ROI]) for hCC, collagen IV, fibronectin, vimentin, and SMAD in carriers before (V1) and after treatment (V4, 15 patients). The data were not normal distributed (confirmed using the Kolmogorov-Shapiro test). Due to the nature of the comparison between healthy controls (V1 baseline values only available from 7 healthy family members) and patients (V1 and V4 values), conventional analysis of variance (ANOVA) comparison could not be performed.

Secondary Analysis

Glutathione Analysis

Statistical analysis from the glutathione assay was conducted from the 15 patients for whom results from V1 and V4 were compared in regard to treatment efficiency. Participants were grouped into all patients with HCCAA (n = 15), NAC-naive (NAC-) patients (n = 6), and controls (n = 5). The Wilcoxon matched-pairs signed rank test was used for comparing values for carriers, as the data were not normally distributed (confirmed using Kolmogorov-Shapiro test), and the Mann-Whitney test was used for comparison to control samples.

HMW hCC Levels

HMW aggregates of hCC were determined by WB. Values were quantified by optical density and normalized to the V1 time point of each patient for accurate comparison (15 patients for time points V1, V2, and V3 and 11 for time point V4). Normal distribution was tested using the Kolmogorov-Smirnov test, and comparisons between the V1 normalized values and V4 sample population were made using a 2-tailed *t* test. In addition, unbalanced ANOVA was used on proteomic analysis to identify differences among populations. A *P* value less than .05 was considered statistically significant for all tests.

Results

Participant Characteristics

In total, 17 patients participated in the trial; age ranged from 20 to 75 years, with a mean (SD) age at enrollment of 40.0 (4.2) years and a median age of 35 years (Figure 1). Included in this study were the original 6 participants from the previously published case study.[18] All but 2 of the 17 participants completed visits at 4 time points—at baseline (V1) and then at 3 months

(V2), 6 months (V3), and 9 months (V4)—with the other 2 participants completing 3 time points, with early termination due to pregnancy. Several participants had been taking NAC as a dietary supplement prior to enrolling in this trial, including 6 participants who were taking low to medium doses of NAC (1200-1800 mg/day) and 3 participants who were taking high-dose NAC (2400 mg/day) ranging from 6 to 24 months prior to the trial. These 9 participants were self-medicating with NAC prior to enrollment without physician involvement; upon enrollment, they continued taking high-dose NAC therapy, 2400 mg per day, for an additional 9 months. Eight participants were drug naive and started high-dose NAC treatment after the first biological samples were collected. The 2 female participants who withdraw due to pregnancy were from the drug-naive group. Study participants were monitored for 9 months while receiving therapy of NAC, 1200 mg, twice per day. Twelve of the 17 participants had history of clinical symptoms consistent with 1 or more hemorrhagic strokes prior to study enrollment, and 3 participants had clinical evidence of cognitive impairment when entering the study based on neurological examination—2 with mild and 1 with moderate impairment.

Primary End Points

Drug Tolerability and Safety

During the trial, there were no abnormalities observed that were attributed to drug effects on blood or urine parameters (eFigure 1 in Supplement 2). Two female patients became pregnant during the study. This was reported as a serious AE (SAE), and both participants withdrew from the study. They delivered successfully and are doing well. No SAEs were reported related to drug effects, and no significant drug-related AEs were reported. No major strokes occurred for the duration of the study in any of the patients; however, in 5 participants, a CT scan was done in response to onset of clinical symptoms that resulted from intracranial hemorrhage during the study. All clinical symptoms were mild and resolved within 2 weeks and without permanent neurological sequela.

DRS-2

Fifteen participants completed all 4 DRS-2 tests (mean education level, 15.1 years) and fell into the following clinical categories relative to the age-corrected and education-corrected scaled scores in the DRS-2 Professional Manual: average (intact) (n = 6), below average (intact) (n = 6), mildly impaired (n = 2), or moderately impaired (n = 1). Little to no clinically significant changes in cognition were uncovered during the treatment period (Friedman test, with significance level set at $P < .05$). See additional results in the eResults and eFigure 2 in Supplement 2.

Skin Biopsies

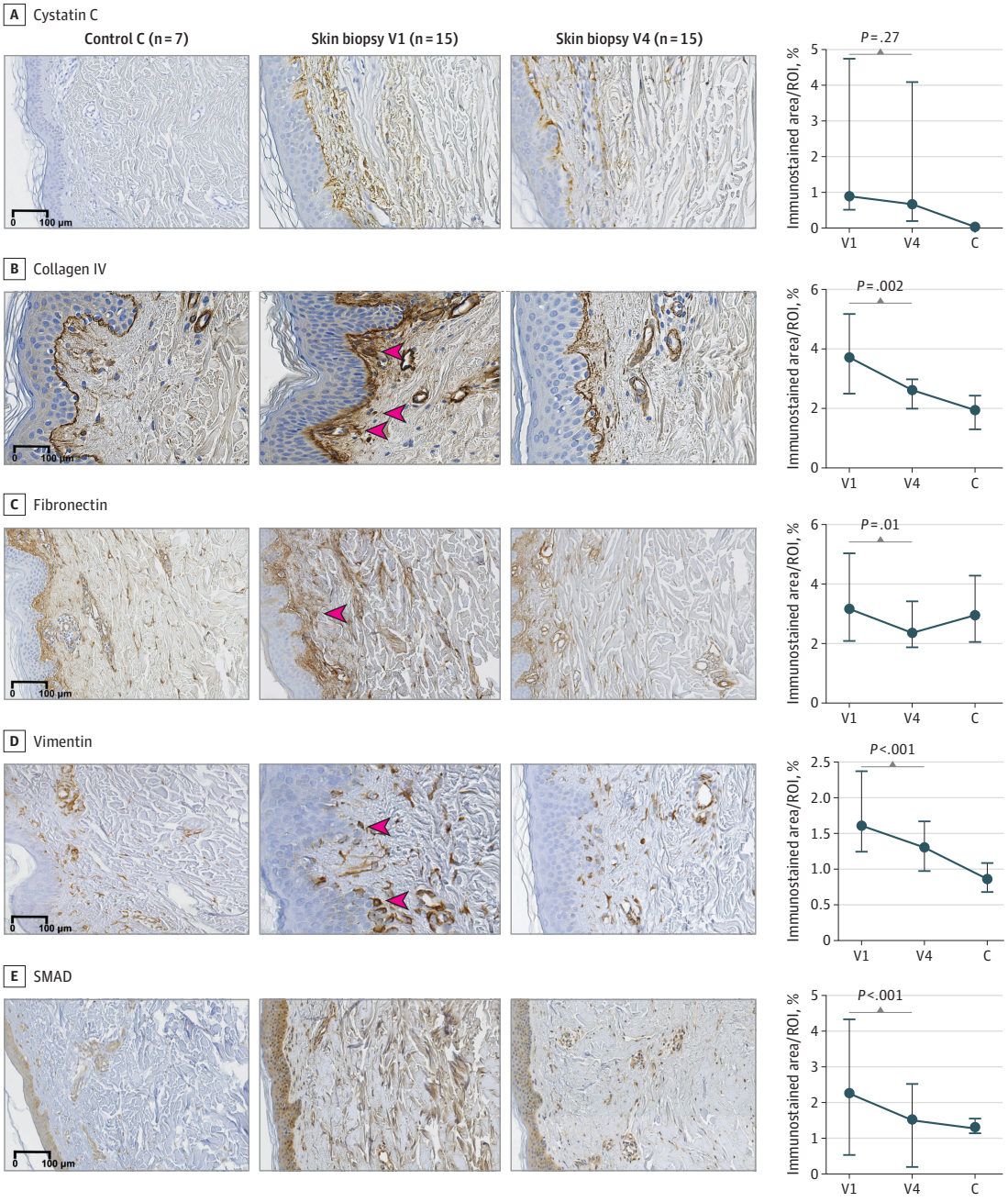
All skin biopsies from 15 patients were stained for cystatin C/hCC amyloid complexes, collagen IV, fibronectin, vimentin, and SMAD 2/3. The distribution and intensity of the staining was visually scored (eFigure 3 in Supplement 2) and percentages of immunoreactivity per ROI were compared between biopsies from V1 and V4 in a masked way with the Wilcoxon matched-pairs signed rank test, with significance level set at $P < .05$. **Figure 2** shows the results of the immunostaining and quantification for each antibody (percentage per ROI) for patients with HCCAA and controls. L68Q-hCC immunoreactive deposits were observed in skin biopsies from V1 in 14 of 15 patients, with 1 young patient only staining positive in a few fibroblasts—5 patients had weak staining in BM between epidermis/dermis and in upper dermis and in fibroblasts right beneath epidermis, 5 patients had moderate staining, and 4 patients had intense staining in BM between epidermis/dermis and dermis (Figure 2A, V1). After treatment, the deposition was less prominent in BM and in fibroblasts in the upper dermis (Figure 2A, V4). The quantification of the staining intensity and distribution (percentage per ROI) was lower in biopsy from V4 in 12 of 15 patients—however, the difference between V1 and V4 median (IQR) values was not significant (V1: 0.89% [0.51%-4.75%]; V4: 0.65% [0.19%-4.10%]; $P = .27$) (Figure 2A). For collagen IV, the median (IQR) change in percentage immunoreactivity between V1 and V4 was statistically significant (V1: 3.69% [2.48%-5.16%]; V4: 2.60% [1.99%-2.97%]; $P < .001$). The intensity was especially lower in the BM between epidermis/dermis (Figure 2B). The change in median (IQR) percentage immunoreactivity for fibronectin, a biomarker not previously assessed in patients, between V1 and V4 was significant (V1: 3.17% [2.09%-5.05%]; V4: 2.37% [1.87%-3.42%]; $P = .01$) (Figure 2C). Reduced staining intensity for collagen IV and fibronectin was especially evident in fibroblasts in the upper dermis (see arrowheads in Figure 2B and C). Vimentin staining for fibroblasts in the upper dermis was similarly lower in biopsies from V4 (median [IQR] values, V1: 1.60% [1.24%-2.37%]; V4: 1.31% [0.97%-1.68%]; $P < .001$) (Figure 2D). Fibroblasts in biopsy V1 showed an increase in cell density and activated appearance, while fibroblasts in controls and V4 from patients had more quiescent appearance (Figure 2D). Extensive immunoreactivity was seen for SMAD 2/3 in fibroblasts in biopsies at V1, whereas the immunoreactivity in biopsies at V4 was notably lower (median [IQR] values, V1: 2.25% [0.55%-4.36%]; V4: 1.56% [0.20%-2.54%]; $P < .001$) (Figure 2F). Comparison with healthy control biopsy has been previously reported.[14]

Secondary End Points

Glutathione

Results for calculated concentrations of free glutathione (GSH) and the GSH:glutathione disulfide (GSSG) ratio for all patients with HCCAA and NAC- patients are shown in **Figure 3**, calculated using the Wilcoxon matched-pairs signed rank test, with significance level set at $P < .05$. The change in median (IQR) free GSH (V1: 1.68% [0.90%-10.85%]; V4: 1.56% [0.76%-18.72%]; $P = .93$) and the GSH:GSSG ratio (V1: 1.64% [0.96%-13.52%]; V4: 2.01% [0.06%-80.53%]; $P = .12$) was not significant in patients with HCCAA between V1 and V4 (Figure 3A-B). The change in median (IQR) free GSH was significantly higher for NAC- participants between V1 and V4 (V1: 6.64% [1.18%-18.02%]; V4: 18.64% [16.57%-18.78%]; $P = .03$) (Figure 3C), but not for GSH:GSSG ratio (V1: 18.26% [1.67%-22.15%]; V4: 25.31% [12.62%-35.40%]; $P = .12$) (Figure 3D). See the eResults and eFigure 4A-B in Supplement 2 for comparison of baseline measurement in patients with HCCAA and controls.

Figure 2. Skin Biopsies at Baseline (V1) and Following 9 Months of Treatment (V4)



Statistical analysis was done with the Wilcoxon matched-pairs signed rank test ($P < .05$) for comparing percentage immunoreactivity per region of interest (ROI) in biopsy V1 to biopsy V4 in 15 patients. All plots show change in median (IQR) percentage biomarker staining per ROI from V1 to V4 in carrier biopsies and controls. A, Skin biopsy from control (healthy family member) showing no L68Q–human cystatin C (hCC) amyloid complex deposition. In carriers, moderate hCC amyloid complex deposition is seen in the basement membrane (BM), between epidermis and dermis, and in upper dermis in V1 and less intensive deposition in V4. For hCC, V1: 0.89% (0.51%-4.75%); V4: 0.65% (0.19%-4.10%); $P = .27$; controls: 0.003% (0.0015%-0.01%). B, Skin biopsy from control with collagen IV staining in BM between epidermis and dermis and BMs in dermis. In carriers, more intense staining is seen in V1, especially in the BM between epidermis/dermis and in fibroblasts in upper dermis (arrowheads), and less extensive staining in seen in V4, mainly in fibroblasts. For collagen IV, V1: 3.69% (2.48%-5.16%); V4: 2.60% (1.99%-2.97%); $P < .001$; control: 1.97% (1.29%-2.44%). C, Skin biopsy from control with fibronectin staining in BMs, more intense staining in V1 from carrier, especially in fibroblasts in upper dermis (arrowhead) and less intense staining in fibroblasts in V4. For fibronectin, V1: 3.17% (2.09%-5.05%); V4: 2.37% (1.87%-3.42%); $P = .01$; control: 2.95% (2.06%-4.29%). One control biopsy had intense fibronectin immunoreactivity. D, Skin biopsy from control with vimentin staining, showing normal fibroblasts in upper dermis, biopsy V1 from carriers showing an elevated number of fibroblasts in the upper dermis with activated appearance (arrowheads) and less staining and more normal appearance in fibroblasts in V4. For vimentin, V1: 1.60% (1.24%-2.37%); V4: 1.31% (0.97%-1.68%); $P < .001$; control: 0.88% (0.68%-1.08%). E, Skin biopsy from control participant showing normal fibroblasts with SMAD 2/3 immunoreactivity in upper dermis, biopsy V1 from carrier showing an increased number of fibroblasts with SMAD 2/3 immunoreactivity in the upper dermis between V1 and V4. For SMAD, V1: 2.25% (0.55%-4.36%); V4: 1.56% (0.20%-2.54%); $P < .001$; control: 1.35% (1.16%-1.56%). All figures were taken with 20 × objective. Scale bar represents 100 µm on all figures. C indicates controls.

Reduction in HMW hCC Levels Following NAC Therapy

The oligomerization status of hCC was determined by WB in plasma samples from patients under NAC treatment at 4 time points (0, 3, 6, and 9 months). Of 17 L68Q-hCC carriers, 8 participants started high-dose NAC treatment during the trial (group 1), while the other participants were taking a moderate dose of NAC (group 2, including 6 participants taking 1200-1800 mg NAC/day), and 3 participants (group 3) were taking a high dose of NAC (2400 mg/day) for 6 to 24 months before enrolling in the trial. As shown by the 2 representative case samples in **Figure 4**A, long-term treatment with NAC notably reduced the presence of HMW species of hCC in L68Q-hCC carriers at 3 (V2), 6 (V3), and 9 (V4) months compared to baseline.

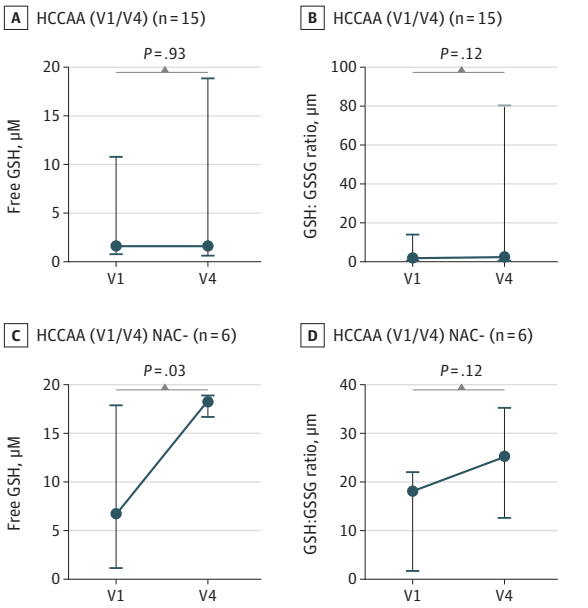
To gain further insight into the effects of NAC treatment on the HMW species of hCC in each of the 3 groups of L68Q-hCC carriers, we examined the ratio of the amount of HMW species detected at V4 to the amount detected at V1 (Figure 4B). As shown in Figure 4B, with all 3 groups combined for optimized study power, there was a time-dependent reduction trend observed in HMW cystatin C species during treatment with NAC, with 9 months of NAC therapy (V4) showing lower mean (SD) HMW values compared to study entrance (baseline/V1) values that were normalized to 100% (V4: 73.6% [27.9%] vs V1: 100% [0]; P = .001).

To better characterize the impact of NAC treatment on hCC, hCC levels in the V1 and V4 samples from 15 of the L68Q-hCC carriers were analyzed. We performed analysis of the quantity of hCC peptides detected by mass spectrometry in plasma samples and compared the ratio of V4:V1 hCC peptides. As shown in Figure 4C, 2 of 6 patients (33%) who started taking a high dose of NAC (2400 mg/day) in the trial displayed higher levels of hCC peptides in plasma after treatment compared to baseline (V4/V1). One of 6 patients (17%) who had been taking a low dose of NAC (1200-1800 mg/day) before the trial exhibited higher levels of hCC peptides after treatment compared to baseline (Figure 4C). All 3 carriers (100%) who were already taking a high dose of NAC (2400 mg/day) before the trial showed increased levels of hCC peptides after treatment compared to baseline (Figure 4C), suggesting higher levels of soluble hCC in plasma in patients who are taking a high dose of NAC. See the eResults and eFigures 5-7 in Supplement 2.

Discussion

CAA is one of the major causes of recurrent intracerebral hemorrhages. The clinical presentation of CAA is complex, with progressive cognitive decline and shortened life span.[3,21-23] Severe hemorrhages are the dominant clinical symptom of HCCAA associated with dementia and paralysis, with an average life expectancy of 30 years.[4,7,10,12] Encouraging therapeutic results were previously reported in 2021 in 6 patients who were taking NAC as a food supplement.[18] Otherwise, there has been no available treatment option for carriers. In our previous study, we showed that short-term incubation with NAC and/or reduced GSH breaks L68Q-hCC amyloid oligomers into monomers of both intracellular and secreted L68Q-hCC.[18] A

Figure 3. Free Glutathione (GSH) and GSH:Glutathione Disulfide (GSSG) Ratio Analysis in Patients With Hereditary Cystatin C Amyloid Angiopathy (HCCAA)



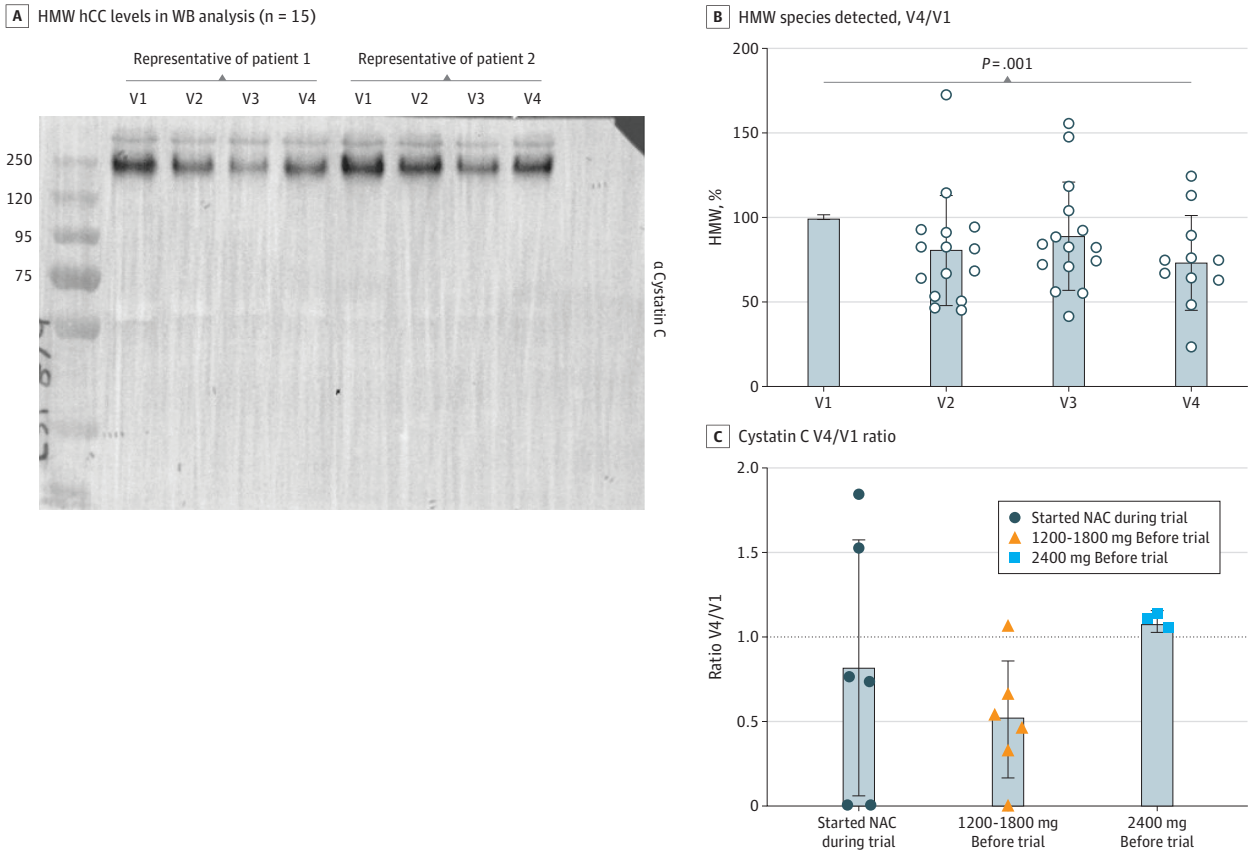
The plots show the results from analysis for free GSH and GSH:GSSG ratio at baseline (V1) and following 9 months of treatment (V4) for all 15 patients with HCCAA and for 6 *N*-acetylcysteine (NAC)–naive (NAC-) patients. Statistical analysis was done with the Wilcoxon test (P < .05) and plots were conducted in GraphPad and represent median with interquartile range. A, There was not a significant change in median (IQR) free GSH between V1 and V4 in patients with HCCAA (V1: 1.68% [0.90%-10.85%]; V4: 1.56% [0.76%-18.72%]; P = .93). B, There was not a significant change in median (IQR) GSH:GSSG ratio in patients with HCCAA (V1: 1.64% [0.96%-13.52%]; V4: 2.01% [0.06%-80.53%]; P = .12). C, There was a significant change in median (IQR) free GSH between V1 and V4 in NAC- patients (V1: 6.64% [1.18%-18.02%]; V4: 18.64% [16.57%-18.78%]; P = .03). D, There was a measurable change, albeit not significant, in median (IQR) GSH:GSSG ratio between V1 and V4 in NAC- patients (V1: 18.26% [1.67%-22.15%]; V4: 25.31% [12.62%-35.40%]; P = .12).

total of 17 individuals took part in this phase 2a trial to monitor safety, tolerability, and efficacy of NAC in patients with HCCAA. Blood and urine parameters were normal, and the drug was well tolerated.

Apart from 5 cerebral bleeds observed in 5 participants that resolved without sequela and 2 pregnancies, the study participants did not have any other SAEs during the study, and no drug-related SAE was recorded. Importantly, no major strokes or death occurred for the duration of the study in any of the patients. As the patients' arterial vessel walls are weakened due to amyloid deposition, the disease progression is usually rapid and the recurrence of bleeds becomes more frequent and more severe.[4] Indeed, the natural history of the disease shows that the magnitude or severity and frequency of cerebral hemorrhages tend to increase (average 3.2-3.9 bleeds per participant over 5 years based on different studies), until it ultimately leads to death at a young age.[4,10,12,24] During the study, no evidence of cognitive decline was observed.

Amyloid deposition in the brain cannot be directly monitored, but skin samples provide an excellent proxy for the po-

Figure 4. *N*-Acetylcysteine (NAC) Treatment Reduced High-Molecular-Weight (HMW) Human Cystatin C (hCC) Levels in Plasma Samples of Patients With Hereditary Cystatin C Amyloid Angiopathy (HCCAA)



High-molecular-weight (HMW) human cystatin C (hCC) was detected by Western blot (WB) in plasma samples of L68Q-hCC carriers. A, Representative figure of HMW hCC levels in WB analysis shows reduction in HMW hCC levels following NAC therapy. B, HMW hCC levels were determined by WB and quantitated by optical density. Bars represent means, error bars represent standard deviation, and individual data points are depicted as dots. Data were normalized to the V1 time point of each patient for accurate comparison (n = 15 patients for time points V1, V2, and V3; n = 11 for time point V4). Normal distribution was tested, and comparisons between the V1 and V4 sample population were made using 2-tailed *t* tests. There was significant reduction in mean (SD) HMW hCC levels between baseline sample (V1) (100% [0]) and treatment at 9 months (V4) (73.6% [27.9%]) with 2-tailed Student *t* test (P = .001). C, hCC ratio in L68Q-hCC carriers analyzed by mass spectrometry who were not previously treated with NAC prior to clinical trial enrollment (left group) and patients who were receiving a low- to medium-dose NAC (middle group) vs high-dose NAC (right group) upon study enrollment. Bars represent mean and error bars represent standard deviation of hCC ratio between V4 and V1 and show reduction in HMW levels at V4. Individual data points are depicted as dots.

tential impact of NAC treatment. The results in this study show that L68Q-hCC amyloid deposition stayed stable or went down during treatment. Few patients had very little hCC deposition in the skin at enrollment and had no clinical cerebral hemorrhage prior or during the trial. Ongoing monitoring of these patients with mild or pre-disease progression to assess drug benefits is of the highest relevance to determine if the disease could be prevented (ie, if NAC blocks amyloid oligomerization, there is no amyloid precipitation occurring in these patients).

Patients' skin and brain samples have shown that the hCC amyloid deposition is always connected to extracellular matrix (ECM) protein changes in the BM.[9,14] Activated fibroblasts are seen in the skin and are the main cell type responsible for the pathogenesis.[14] These BM changes are driven by activated fibroblasts, which are activated by TGF-β, which then produces collagen IV and fibronectin.[14,25,26] Collagen IV and fibronectin are therefore critically important biomarkers besides hCC to assess disease status, along with fibroblasts activation markers. There was a significant change in immunoreactivity of collagen IV and fibronectin after NAC treatment.

Significant changes were also observed in immunoreactivity of vimentin and SMAD 2/3 in fibroblasts, which are biomarkers reflective of activated fibroblasts. Studies have shown that SMAD 2/3 is more active among carriers, which is a part of the TGF-β signaling pathway, and that TGF-β interacts with gene expression of ECM proteins through SMAD 2/3.[14] This study showed reduced SMAD 2/3 immunoreactivity in skin after NAC intake. NAC has been studied in connection to idiopathic pulmonary fibrosis and has been shown to downregulate TGF-β and reduce ECM material.[27] Decreased GSH concentration activates TGF-β[28] and leads to dramatic increase of fibrotic markers (ie, vimentin, collagen IV, and fibronectin).[29] NAC increases GSH[30] and therefore could inhibit TGF-β signaling; this is very important for the pathogen-

esis of the disease, and these markers in the BM and in fibroblasts in skin samples are good indicators to assess drug efficacy.

Secondary end points were to assess NAC influence on plasma biomarkers. In this study, treatment increased the amount of GSH—the body's major antioxidant—in plasma from NAC- patients.[31-33] Thus, NAC appears to affect protein aggregation by restoring GSH levels through its antioxidant properties and its ability to break disulfide bonds.[18,32,33]

In terms of plasma biomarkers, a significant reduction in the HMW fraction of hCC vs monomeric hCC was observed in plasma samples between baseline and posttreatment groups. These results suggest that the effects of the drug observed in vitro may be translated into therapeutic effects in patients. With increase in the monomer protein, which is nontoxic, the opportunity exists to prevent the disease from being expressed if the drug is administered early and before protein precipitation in various tissues begins. Taken together, these results suggest that NAC may have beneficial effects on biomarkers that are directly involved in the pathogenesis of HCCAA.

While these results suggest that NAC may be beneficial in reducing levels of toxic biomarkers in patients with HCCAA, 5 cerebral bleeds occurred during the study, suggesting that any potential clinical benefit of NAC is suboptimal despite very high doses of the drug, which ideally requires dosing 3 times per day, creating compliance challenges. In this regard, our recent in vitro data demonstrate that the NAC-amide analogue, NACA, has more than 10-fold higher potency in preventing or dispersing hCC amyloid complex aggregates than NAC.[18] Moreover, its higher bioavailability and ability to penetrate the blood-brain barrier compared with NAC support the assumption that NACA will be a more effective treatment. NACA can be given in a lower dose that may be better tolerated, with fewer adverse effects in the long term.

Limitations

Given the rarity of this disease, it is impossible to execute a placebo-controlled study or a sufficiently powered analysis due to the small sample size. This also limits assessment of the sustainability of the results or long-term compliance with drug adherence. It is also unclear how nonhereditary forms of CAA would respond to NAC. While the analysis of the data was masked to the best of our ability, the potential for biased scoring is high, particularly when using a subjective scale, such as the skin pathology scoring, and is acknowledged as a potential limitation.

Conclusions

Results from this open-label phase 2a nonrandomized clinical trial support the safety and tolerability of NAC in the treatment of patients with HCCAA. However, due to a relatively low bioavailability of NAC, a derivative of the drug that is lipophilic may have better ability to reach higher concentration in the brain and in cells, conferring greater benefits. Consequently, following recent European Medicines Agency approval, an open-label clinical registration study has been initiated to monitor safety, tolerability, and efficacy using NACA in patients with HCCAA.

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