

ORIGINAL ARTICLE

Hyperhomocysteinemia exacerbates Alzheimer's disease pathology by way of the β -amyloid fibrinogen interaction

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Essentials

- Evidence suggests a comorbidity between hyperhomocysteinemia (HHC) and Alzheimer's disease (AD).
- Homocysteine (HC) could affect the β -amyloid ($A\beta$)–fibrinogen interaction in AD pathology.
- AD patients with concomitant HHC have increased fibrin and $A\beta$ deposits in their brains.
- HC contributes to AD pathology via the $A\beta$ –fibrinogen interaction.

Summary. *Background:* Accumulating clinical evidence suggests that hyperhomocysteinemia (HHC) is correlated with Alzheimer's disease (AD) and vascular dementia. *Objective:* This study was carried out to elucidate the specific role of elevated homocysteine (HC) levels in AD pathophysiology. *Methods:* Immunohistochemistry was used to examine β -amyloid ($A\beta$) deposition along blood vessels, also known as cerebral amyloid angiopathy (CAA), fibrin(ogen) deposition, and their correlation to each other in the brains of AD patients with and without HHC. To study AD-HHC co-morbidity in detail, an AD mouse model was administered a high methionine diet for several months. Parenchymal $A\beta$ plaques, CAA-positive vessels and fibrin deposits were then assessed by immunohistochemistry at different stages of AD progression. Memory deficits were evaluated with contextual fear conditioning and the Barnes maze. Additionally, the effect of HC and its metabolite, homocysteine thiolactone (HCTL), on the $A\beta$ –fibrinogen interaction was analyzed by pull-down, ELISA and fibrin clot formation and fibrinolysis assays *in vitro*. *Results:* We found increased fibrin (ogen) levels and $A\beta$ deposits in the blood vessels and

brain parenchyma of AD patients with HHC. We demonstrate that HC and HCTL enhance the interaction between fibrinogen and $A\beta$, promote the formation of tighter fibrin clots and delay clot fibrinolysis. Additionally, we show that diet-induced HHC in an AD mouse model leads to severe CAA and parenchymal $A\beta$ deposition, as well as significant impairments in learning and memory. *Conclusions:* These findings suggest that elevated levels of plasma HC/HCTL contribute to AD pathology via the $A\beta$ –fibrin(ogen) interaction.

Keywords: Alzheimer disease; amyloid beta-peptides; cerebral amyloid angiopathy; fibrinogen; homocysteine.

Introduction

There is increasing evidence that cerebrovascular dysfunction, such as altered cerebral blood flow, cerebral vasculature damage and abnormal hemostasis, plays a critical role in Alzheimer's disease (AD) pathogenesis [1–5]. Although the precise mechanism by which vascular dysfunction contributes to the development of AD remains unknown, it has been suggested that the β -amyloid peptide ($A\beta$) may act as a mediator for cerebrovascular impairment [2,6,7]. $A\beta$ accumulates in the brain parenchyma and cerebral blood vessels in AD. The deposition of $A\beta$ in cerebral blood vessels, known as cerebral amyloid angiopathy (CAA), has deleterious effects on the cerebrovasculature, promoting the degeneration of vessel wall components [1] while also reducing cerebral blood flow [7]. Several studies in AD mouse models demonstrate that a disrupted cerebrovasculature can induce cell damage and loss in CAA-positive vessels in early stages of AD [8,9].

We and others have shown that elevated plasma fibrinogen levels could be a potential risk factor for AD [10–18]. Fibrinogen, a large plasma glycoprotein, is converted by thrombin into fibrin, which polymerizes into blood clots. We have recently shown that $A\beta_{42}$ can directly interact with fibrinogen and induce its oligomerization *in vitro* [18]. Fibrin clots formed in the presence of $A\beta_{42}$ have an abnormal structure and are resistant to degradation by

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fibrinolytic enzymes *in vitro* and *in vivo* [19,20]. Fibrin(ogen) deposition leads to increased inflammation and disruption of the blood brain barrier (BBB) in AD patients and mouse models [13,19,21–23]. Furthermore, fibrin deposition parallels the severity of CAA in the brains of AD patients and mice [19,23]. Moreover, pharmacologic or genetic depletion of fibrinogen lessens CAA pathology [19], reduces cognitive impairment [19], suppresses microglial activation [13] and reduces BBB disruption [13] in AD mouse models. These studies support the possibility that A β can compromise cerebral blood flow through its interaction with fibrinogen, exacerbating AD pathology.

Elevated plasma levels of homocysteine (HC), a sulfur-containing amino acid derived from methionine [24], are known as hyperhomocysteinemia (HHC). HHC is associated with venous thromboembolism, atherosclerosis [25] and microvasculopathy [26]. Evidence suggests that HHC alters fibrin clot structure and stability *ex vivo* [27] by inducing fibrin accumulation, impairing perivascular fibrinolysis and causing prothrombotic defects *in vivo* [28]. Interestingly, high levels of HC are consistently observed and correlated with cognitive decline in AD and vascular dementia patients [29,30]. In addition, some studies have shown that HHC-inducing diets (high methionine or low folate) increase A β levels and/or deposition [31–33] and further impair cognitive decline [32,34] in various AD mouse models. Despite these critical insights, the exact impact of elevated HC levels on vascular dysfunction in AD remains undefined. We hypothesized that concomitant HHC could aggravate AD pathogenesis via the A β -fibrinogen interaction. We examined the correlation between plasma HC concentration and fibrin(ogen)-containing CAA-positive vessels in AD patients' brains. We also determined the effects of HC and its metabolite, homocysteine thiolactone (HCTL), on the A β -fibrinogen interaction, fibrin network structure, and fibrinolysis *in vitro*. Furthermore, we induced HHC in an AD transgenic mouse model to examine its effects on AD progression *in vivo*. Our studies suggest that HC and HCTL not only contribute to AD pathology, but they do so via the A β -fibrin(ogen) interaction.

Methods

A β ₄₂, homocysteinylated fibrinogen and FragD

A β ₄₂ or biotinylated A β ₄₂ peptides (Anaspec, Fremont, CA, USA) were reconstituted in 4% NH₄OH in 50 mM TBS, pH 7.4, and aliquots were stored at –80 °C. Before use, aliquots were centrifuged for 10 min at 12 000 × g to remove pre-aggregated material. A β ₄₂ concentration was determined by bicinchoninic acid assay.

To homocysteinylate fibrinogen or fragment D (FragD), human plasminogen-free fibrinogen (29.4 μ M, Calbiochem) and purified FragD [18] (34 μ M) were incubated with various doses of HC (Santa Cruz Biotechnology, Dallas, TX,

USA) or HCTL (Sigma, St. Louis, MO, USA) at 37 °C for 18 h. To remove unreacted HC and HCTL, HC/HCTL-incubated fibrinogen samples were gel-filtered with a Sephadex G-25 column (GE Healthcare, Little Chalfont, UK). Control and homocysteinylated fibrinogen and FragD concentrations were adjusted to 2.94 μ M or 5.67 μ M and subjected to the assays described below.

Pull-down assay

Unmodified/control fibrinogen (0.5 nM) and FragD (25 nM) or HC/HCTL-modified fibrinogen (0.5 nM) and FragD (25 nM) were incubated with or without biotinylated A β ₄₂ (200 nM) for 1 h at room temperature (RT) in binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% NP-40, 0.1% BSA, and protease inhibitors). The samples were incubated with streptavidin-coupled magnetic beads (Invitrogen, Carlsbad, CA, USA) for 1 h and washed five times with binding buffer. A β -fibrinogen or A β -FragD complexes were eluted by heating to 80 °C for 5 min in non-reducing sample buffer and subjected to Western blot analysis using an anti-fibrin(ogen) antibody (Dako, Carpinteria, CA, USA). To compare the amounts of A β being pulled down, dot blots were performed (4G8, Covance, Princeton, NJ, USA).

ELISAs for A β -fibrinogen and A β -FragD interactions

Biotinylated A β ₄₂ was immobilized for 1 h at RT on a Reacti-Bind streptavidin-coated plate (ThermoScientific, Waltham, MA, USA). The plate was washed and incubated with either control fibrinogen (50 nM) or FragD (100 nM) or HC/HCTL-modified fibrinogen (50 nM) or FragD (100 nM) and then incubated with anti-fibrinogen antibody. The plate was washed and then incubated with HRP-conjugated secondary antibody and Ultra TMB solution (ThermoScientific). Absorbance was read at 405 nm.

Fibrin clot formation

Control fibrinogen or HC/HCTL-modified fibrinogen (2.7 μ M) was mixed with Alexa Fluor-488 fibrinogen (0.3 μ M; Invitrogen) in HEPES buffer, mounted on glass-bottom microwell dishes (MatTek, Ashland, MA, USA) and supplemented with 5 mM CaCl₂ and 0.5U mL^{–1} thrombin (Sigma) ± A β ₄₂ (3 μ M). Samples were then visualized by a Zeiss LSM510 (Oberkochen, Germany) confocal laser scanning microscope with a 40-Axiovert 1.2/water objective. To analyze A β -influenced fibrin clot structure and size, three to four images were obtained as Z-stack slices taken every 0.5 μ m (11 slices/image) at 50 μ m above the glass surface. Images containing fibrin(ogen) clumps were projected two-dimensionally to produce the final image, equally thresholded, and then analyzed using NIH ImageJ software (Bethesda, MD, USA).

Fibrin clot turbidity assay

Fibrin clot formation and lysis assays were performed in triplicate [20]. To measure fibrin polymerization, control fibrinogen (1.5 μ M) and HC/HCTL-modified fibrinogen were mixed with thrombin and CaCl₂. To measure clot formation and lysis, fibrinogen and HC/HCTL-incubated fibrinogen with or without A β ₄₂ (3 μ M) were mixed with plasminogen, thrombin, tPA (Genentech, South San Francisco, CA, USA) and CaCl₂ in HEPES buffer. Clot half-lysis time was calculated as the time between maximal and half-maximal turbidity. Plasminogen was purified from human plasma provided by the NY Blood Center [19,20].

Human brain immunohistochemistry

Human post-mortem frontal cortex and hippocampal samples from non-demented individuals and AD patients with or without HHC were obtained from The Thomas Willis Oxford Brain Collection at Oxford University Hospital. Paraffinized sections were prepared for immunohistochemical analysis [19]. Brain sections were incubated with rabbit anti-fibrinogen antibody (Dako), developed with deaminobenzidine, and counterstained with 1% Thioflavin S (ThioS; Sigma) in 70% ethanol or triple-immunostained using primary antibodies against fibrinogen, collagen IV (Fitzgerald, Acton, MA, USA) and A β (6E10, Covance), incubated with fluorescent secondary antibodies, and imaged using an inverted Zeiss Axiovert 200 microscope. To determine the total area of vascular fibrin(ogen) or CAA, 25–30 images from the frontal cortex or hippocampus were collected, thresholded using ImageJ, quantified, and normalized by area of fibrin(ogen)- or CAA-positive vessels in non-demented individuals for fibrin(ogen) staining or in AD control patients for CAA staining.

Animals and diet treatment

TgCRND8 AD mice [35] and wild-type (WT) littermates (7–8 weeks of age) were fed a standard control (CON), high methionine (MET) or high glycine (GLY) diet (Harlan) for 2 or 5 months. All animals had unrestricted access to food and water. Body weights and food consumption were monitored weekly. Mice were maintained and treated in accordance with Rockefeller's Institutional Animal Care and Use Committee.

Mouse brain immunohistochemistry

Following treatment, AD and WT mice were transcardially perfused with saline/heparin. Brains were removed and post-fixed overnight in 4% paraformaldehyde at 4 °C, stored in 30% sucrose at 4 °C until sinking, and sectioned with a sliding microtome into 30- μ m-thick

sagittal sections. Brain sections were stained and imaged as described above. The olfactory bulb, hippocampus and cortex of each mouse were analyzed for parenchymal A β deposition and CAA. Images were then thresholded using ImageJ and quantified.

Behavior

The Barnes maze was used to assess spatial learning and memory [36,37]. Animals were subjected to two trials/day for 5 days with a 30-min inter-trial interval. Trials were recorded and analyzed with Ethovision (Noldus, Leesburg, VA, USA). Fear conditioning was used to examine contextual memory [36].

Statistical analysis

All values are expressed as mean \pm SEM. Statistical significance was assessed as described in the text. $P < 0.05$ was considered statistically significant.

Results

Fibrin deposits and CAA are increased in AD patients with high plasma HC

To examine the effect of elevated HC on fibrin homeostasis and CAA pathology, we analyzed vascular fibrin and A β deposition in post-mortem brain tissue from AD patients and non-demented individuals with normal or elevated levels of plasma HC (Table S1). Consistent with our previous reports [19,23], fibrin and A β co-deposition along large (≥ 20 μ m) vessels was elevated in AD patients compared with controls (Fig. 1A,B; Fig. S1). Brain tissue from AD patients with HHC had more fibrin deposits (Fig. 1B,E) and CAA (Fig. 1C,F) in the frontal cortex and hippocampus compared with samples from AD patients with normal HC levels. Furthermore, a highly significant correlation between fibrin deposition and CAA was found in both the frontal cortex and hippocampus of AD patients (Fig. S2A,B). Plasma HC levels did not influence fibrin or CAA deposition in non-demented individuals (not shown), but did so in the frontal cortex (Fig. S2C,E) and hippocampus (Fig. S2D,F) of AD patients. These data strongly suggest that high plasma HC levels contribute to fibrin accumulation and CAA in AD.

HC and HCTL enhance the A β -fibrinogen interaction via homocysteinylation of the FragD region of fibrinogen

Given that there are significantly greater levels of fibrin deposits in AD patients with concomitant HHC, we examined the effects of HC/HCTL on fibrinogen *in vitro*. Consistent with previous reports [27,38], HC/HCTL affected fibrin polymerization and degradation. We

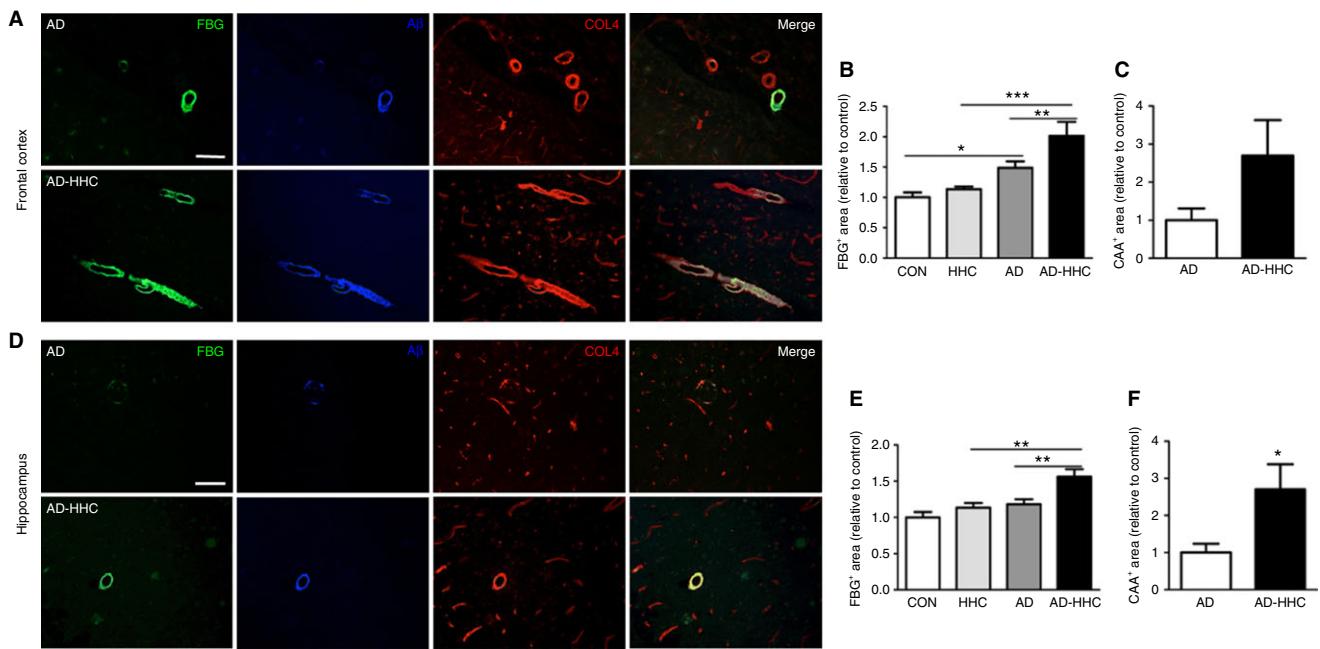


Fig. 1. Increased fibrinogen deposition and cerebral amyloid angiopathy (CAA) in the brains of Alzheimer's disease (AD) patients with hyperhomocysteinemia (HHC). We performed immunohistochemical analysis for fibrin(ogen) (FBG, green), A β (blue) and collagen IV (COL4, red) in frontal cortex (A) and hippocampus (D) samples from non-demented control individuals (Fig S1) and AD patients with normal (total homocysteine [HC] concentration < 15 $\mu\text{mol L}^{-1}$) or high (HHC; total HC concentration $\geq 15 \mu\text{mol L}^{-1}$) plasma HC levels. Scale bars = 100 μm . Fibrin(ogen) in the frontal cortex (B) and hippocampus (E) was quantified and normalized to control individual samples. Results represent mean \pm SEM (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; One-way ANOVA with Neuman–Keuls post-hoc test). CAA-positive vessels were defined by A β - and COL4-positive signal larger than 20 μm in diameter. CAA in the frontal cortex (C) and hippocampus (F) was normalized to AD control patient samples. Student's *t*-test revealed a significant difference in the hippocampus between AD patients with HHC and those with normal plasma HC levels (AD) (* $P < 0.05$). One-way ANOVA with the Tukey post-hoc test also revealed statistical significance. CON, non-demented control patients with normal plasma HC levels; HHC, non-demented patients with HHC; AD, AD patients with normal plasma HC levels; AD-HHC, AD patients with HHC.

observed significant dose-dependent effects of HC/HCTL on the lag time to clot (Fig. S3A,C) and maximal clot turbidity (Fig. S3B,D); the higher the levels of HC/HCTL, the slower the fibrin clots formed and the lower their turbidity (Fig. S3A–D). To visualize the effects of increased HC/HCTL on clotting, fibrinogen incubated with increasing amounts of HC or HCTL was analyzed by confocal microscopy (Fig. S3E). These results suggest that high HC or HCTL levels may modify fibrinogen structurally, thereby altering fibrin clot formation.

Because fibrinogen directly binds to A β_{42} and this interaction increases fibrinogen oligomerization *in vitro* [18], we investigated whether HC or HCTL affected fibrinogen oligomerization and its interaction with A β . We incubated biotinylated-A β_{42} with control, HC-modified or HCTL-modified fibrinogen and performed pull-down assays. There was a stronger interaction between HC/HCTL-modified fibrinogen and A β_{42} than between HC/HCTL-modified fibrinogen and control fibrinogen (Fig. 2A). At higher concentrations of HC or HCTL, fibrinogen oligomerized in the absence of A β_{42} (Fig. S4). Consistent with these data, ELISAs showed increased binding absorbance of HC- or HCTL-modified fibrinogen

with A β_{42} , suggesting that modification of fibrinogen by HC/HCTL increases the interaction between fibrinogen and A β_{42} (Fig. 2B).

We have shown that fibrinogen binds to A β at its fragment D (FragD) region [18]. To examine whether the enhanced interaction between HC/HCTL-modified fibrinogen and A β_{42} is a result of direct homocysteinylation of FragD, a pull-down assay (Fig. 2C) and ELISA (Fig. 2D) were performed. HC and HCTL dramatically increased the FragD–A β_{42} interaction, suggesting that HC/HCTL positively influences the fibrinogen–A β_{42} interaction through homocysteinylation of FragD.

HC and HCTL affect fibrin clot formation and fibrinolysis

To further investigate how HC and HCTL affect fibrin clot structure, we induced clot formation using Alexa Fluor488-conjugated fibrinogen in the absence or presence of A β_{42} and HC/HCTL. Consistent with previous results [19,20], A β_{42} induced the formation of non-homogeneous fibrin fibers while also significantly increasing fibrin aggregate size ($P < 0.001$; Fig. S5A–C). HC- or HCTL-modification of fibrinogen not only enhanced the formation of

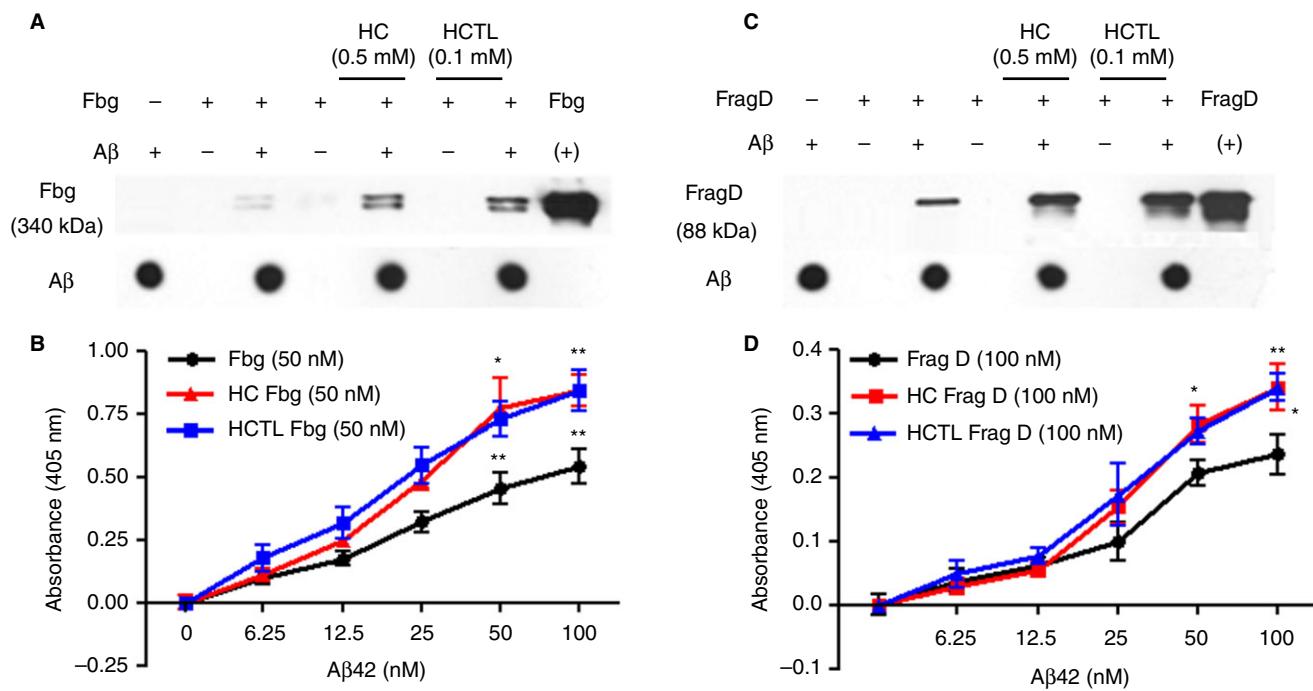


Fig. 2. Enhanced A β 42-fibrinogen interaction via homocysteinylation of FragD/fibrinogen. Control, homocysteine (HC)-modified (0.5 mM) or homocysteine thiolactone (HCTL)-modified (0.1 mM) human fibrinogen (Fbg; 29.4 μ M) and FragD (34 μ M) were incubated in the presence or absence of biotinylated A β 42. Unreacted HC/HCTL was removed as described in Methods. Pull-down assays were performed using streptavidin-conjugated magnetic beads. More fibrinogen (A) or FragD (C) was pulled down when the incubation reactions contained HC or HCTL. Dot blots against A β 42 showed that comparable amounts of A β 42 were pulled down in all experiments. Pull-down assays were performed three to four independent times and representative blots are shown. (B, D) Binding curves for the interactions of fibrinogen (B; 50 nM) and FragD (D; 100 nM) with A β 42 in the absence or presence of HC or HCTL were determined by ELISA. The results are shown as mean \pm SEM from four different experiments. The A β 42-fibrinogen interaction is significantly enhanced in the presence of HC or HCTL (* P < 0.05, ** P < 0.01).

irregular clusters in the presence of A β (Fig. S5A), it also significantly increased their size (Fig. S5B,C). These data suggest that modification of fibrinogen by HC/HCTL promotes and enhances the formation of A β -induced irregular clusters within fibrin clots.

To investigate whether HC or HCTL affects fibrinolysis, *in vitro* degradation assays were performed with fibrinogen (control or HC/HCTL-modified) with or without A β 42. Consistent with our previous data [19,20], the dissolution of A β 42-altered fibrin clots was significantly delayed (blue vs. black, Fig. S5D–G). However, modification of fibrinogen by HC/HCTL further increased the half-lysis time in the presence of A β 42 (red vs. blue, Fig. S5D–G), but did not affect fibrinolysis in the absence of A β (gray vs. black, Fig. S5D–G). Collectively, our data suggest that HC/HCTL affects the structure of A β -altered fibrin clots, resulting in their delayed dissolution.

Concomitant HHC and AD enhances A β deposition in the mouse brain

We examined the effects of HHC on vascular and parenchymal A β deposition in TgCRND8 AD mice [35]. HHC was induced by feeding 7–8-week-old AD and WT mice a high methionine (MET) diet for 2 or 5 months.

Standard (CON) and high glycine (GLY) diets were used as controls; the GLY diet was used as a control for the increase in a single amino acid in the experimental diet (Table S2). No gross changes in appearance or morbidity were observed, although MET-fed mice gained significantly less weight than control groups (Table S3). Consistent with previous reports [35], we observed more A β plaques in the cortex, olfactory bulb and hippocampus of MET-fed AD mice compared with CON-fed and GLY-fed mice (at 4 and 7 months of age) (Figs 3,S6; not shown). Compared with controls, the MET diet caused a notable increase in the total A β -positive area in all regions examined at 4 months of age (Fig. 3B) and in the cortex at 7 months of age (Fig. 3A,C). Large vessel ($\geq 20 \mu$ m) CAA was detected at a relatively low abundance in the brains of AD mice at 4 months of age but was dramatically increased by 7 months of age (Fig. 3A, D,E,S6), suggesting that large vessel CAA develops after A β plaque formation in these mice. Although the MET diet only led to an increase in large vessel CAA in the AD cortex at 4 months of age (Fig. 3D), it markedly enhanced the CAA-positive area in all brain regions examined by 7 months of age (Fig. 3A,E,S6). Furthermore, CAA in capillaries ($< 20 \mu$ m) was dramatically increased in MET-fed AD mice compared with control

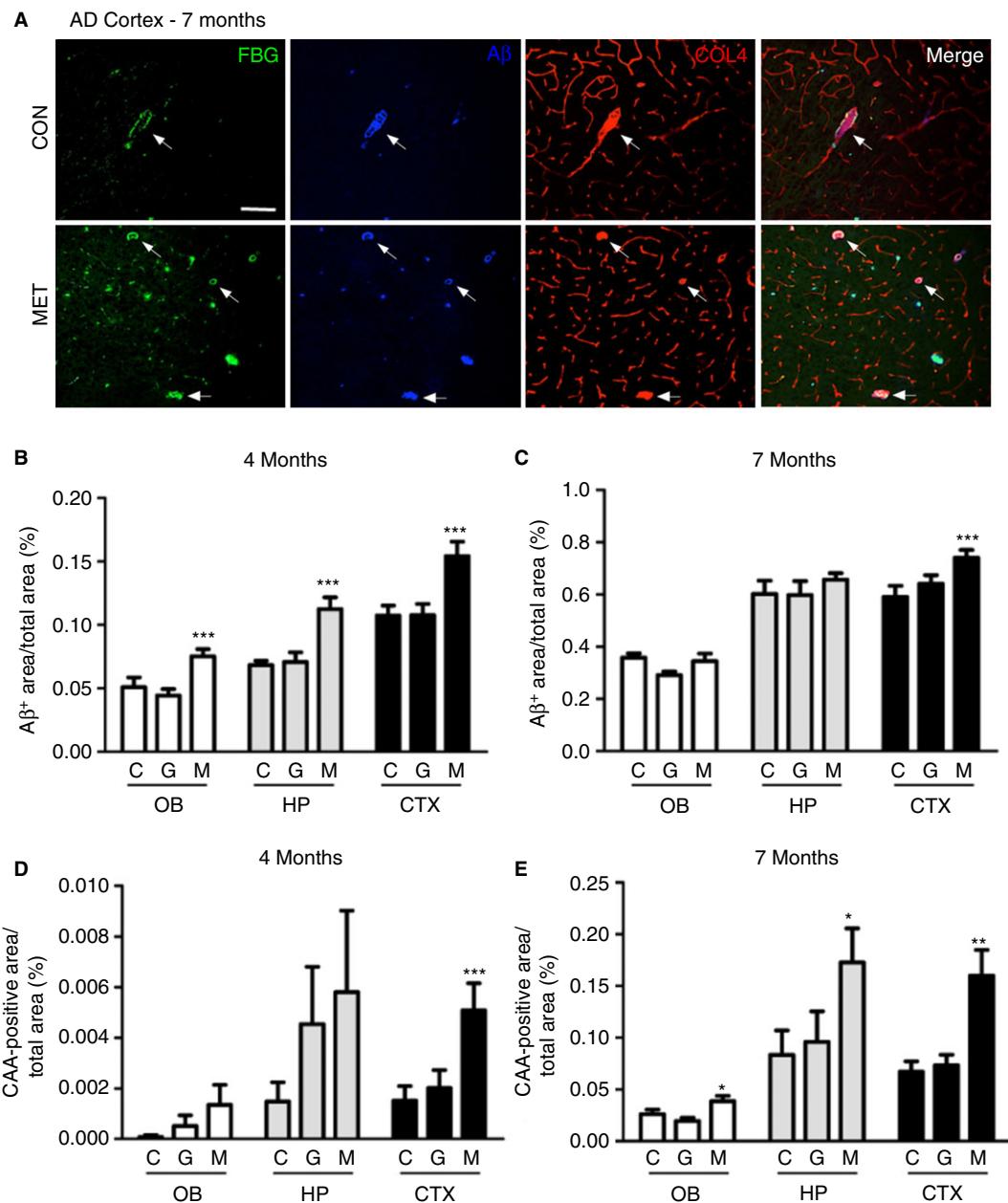


Fig. 3. Hyperhomocysteinemia (HHC) increases parenchymal and vascular A β deposition in the brains of transgenic Alzheimer's disease (AD) mice. TgCRND8 AD mice and their wild-type (WT) littermates (7–8 weeks-of-age) were treated with control (CON), high glycine (GLY) or high methionine (MET) diet for 2 or 5 months. (A) Immunohistochemical analysis was performed for fibrin(ogen) (FBG, green), A β (blue) and collagen IV (COL4, red) in the cortex of AD and WT mice. A β staining represents both parenchymal plaques and cerebral amyloid angiopathy (CAA). Arrows indicate representative large ($\geq 20 \mu\text{m}$) COL4-positive vessels that were also fibrinogen and A β positive. As in humans, the A β signal often localized with FBG. (B,C) Bar graphs depict the total A β -positive area (parenchymal and vascular A β deposits) throughout the olfactory bulb (OB), hippocampus (HP) and cortex (CTX) in AD mice treated for 2 months (B) or 5 months (C). There was significantly more A β deposition in brain regions of MET-fed AD mice compared with CON and GLY diet-fed AD mice ($***P < 0.001$; one-way ANOVA with the Newman–Keuls *post hoc* test). There was no A β -positive staining in any of the WT mouse groups (not shown). (D,E) Quantification of large CAA-positive vessels (via A β -positive staining aligned with COL4 signal) in each brain region. The number of CAA-positive vessels was significantly increased in the CTX of AD mice fed a MET diet for 2 months compared with that of mice fed CON and GLY diets. Mice treated with a MET diet for 5 months showed a significant increase in the number of CAA-positive vessels in all brain regions examined compared with CON and GLY diets ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$; one-way ANOVA with the Newman–Keuls *post hoc* test). Statistical significance indicates a difference when compared with CON diet-treated AD mice. The parenchymal and vascular A β signal was quantified in four to five sections from six to nine mice per experimental group. C, control diet; G, high glycine diet; M, high methionine diet.

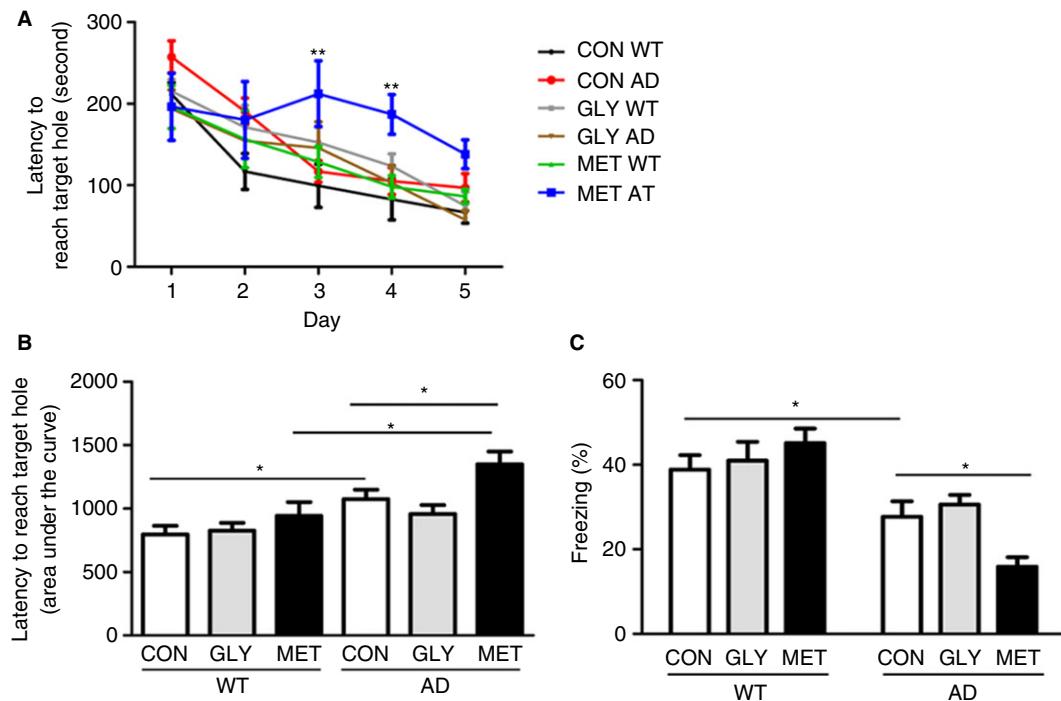


Fig. 4. Hyperhomocysteinemia (HHC) exacerbates memory impairment in Alzheimer's disease (AD) mice. (A,B) Spatial learning and memory were measured using the Barnes maze, which consisted of 10 training trials over 5 days (two trials/day) at 7 months of age (after 5 months of diet treatment, $n = 6\text{--}9/\text{group}$). (A) Latency to reach the escape hole. There was a significant difference between high methionine (MET)-diet-fed AD mice (blue) and control (CON)-diet-fed mice (black and red) in the latency to reach the target hole on the third and fourth days of training ($**P < 0.01$; two-way ANOVA with Bonferroni post hoc test [$F(15,115) = 2.152, P < 0.05$]). (B) Bar graph represents area under the curve derived from (A) ($*P < 0.05$; mean \pm SEM by Student's *t*-test). (C) Contextual fear conditioning was used to measure learning and memory in AD and wild-type (WT) mice fed CON, high glycine (GLY) or MET diets ($n = 7\text{--}10/\text{group}$). Bar graph depicts per cent freezing (immobility) during contextual testing (mean \pm SEM) and shows a significant difference between CON-diet-fed AD and WT mice and between CON-diet-fed and MET-diet-fed AD mice ($*P < 0.05$; two-way ANOVA with Bonferroni post hoc test [$F(2,41) = 4.982, P < 0.05$]).

groups at both 4 and 7 months of age (not shown). Neither control nor MET diets affected the formation of A β deposits in the parenchyma or vessels of the WT mouse brains (not shown).

Concomitant HHC exacerbates cognitive deficits in AD mice

To examine whether the HHC-induced increase in CAA exacerbates cognitive deficits in AD mice, we performed the Barnes maze using 7-month-old mice that had been administered a CON, GLY or MET diet for 5 months. During acquisition training, two-way ANOVA revealed significant effects of genotype and diet on the time to reach the escape hole, as well as a significant effect of the interaction between genotype and diet on time to enter the escape hole (Fig. 4A). Furthermore, MET-fed AD mice took significantly longer to find and enter the escape hole compared with CON-treated AD mice on days 3 and 4 (Fig. 4A). When the latency to the target hole was quantified as area under the curve, CON-treated AD mice took significantly longer to reach the target hole compared with CON-treated WT mice (Fig. 4B). Notably, MET-treated AD mice exhibited significantly longer entry latencies compared with control AD mouse groups (Fig. 4B).

We also performed contextual fear conditioning to assess memory impairment. Consistent with previous reports [39], control AD groups demonstrated a robust deficit in contextual fear conditioning at 7 months of age compared with the control WT groups (Fig. 4C). Analysis by two-way ANOVA showed significant effects of genotype and diet as well as a significant interaction between genotype and diet. Compared with AD mice fed a CON or GLY diet, a MET diet induced an even more significant deterioration in their contextual memory (Fig. 4C). There was no difference between genotype and diet in basal freezing activity (data not shown). These data suggest that HHC-induced cerebrovascular changes could exacerbate cognitive decline in AD.

Discussion

This study is the first to demonstrate significant increases in fibrin(ogen) deposition and CAA severity in the brains of AD patients with concomitant HHC compared with those with normal plasma HC levels. HHC, induced by a high methionine diet, also enhanced parenchymal and vascular A β deposition and aggravated cognitive decline in transgenic AD mice compared with AD mice

administered control diets. Furthermore, our *in vitro* and *in vivo* data both suggest that high plasma HC has deleterious effects on vascular AD pathophysiology via the A β -fibrinogen interaction. These conclusions are based on two findings: (i) HC/HCTL affected fibrin clot formation and amplified the A β -fibrinogen interaction via modification of FragD, and (ii) in the presence of A β , HC/HCTL-modified fibrinogen augmented clot size and delayed fibrin clot dissolution.

Recent clinical studies have shown that AD pathogenesis is associated with cerebrovascular dysfunction, such as reduced cerebral blood flow and altered hemostasis [3,5], implying an association between vascular and neurological pathologies [2]. Several studies suggest that fibrinogen also plays a crucial role in these abnormalities [13,14,19]. Not only does fibrin(ogen) localize with CAA in the cerebral vessels and brain parenchyma of AD patients and mice [14,19,23], but fibrin(ogen) deposition increases with age and correlates with A β plaque levels in the brains of AD mice and patients [10]. Furthermore, pharmacological or genetic depletion of fibrinogen attenuates CAA pathology and synaptic dysfunction in AD mice [10,13,19]. Consistently, we found that fibrin(ogen) deposition was enriched along CAA-containing cerebral vessels in AD patients and was associated with CAA severity in the frontal cortex and hippocampus of AD patients (Fig. S2), indicating that the degree of fibrin(ogen) deposition may affect the development of CAA in the AD neurovasculature.

We also show that HHC results in increased fibrin (ogen) deposition and CAA in AD patients (Fig. 1). Correlation analyses showed that plasma HC level was significantly associated with both fibrin accumulation and CAA severity in the frontal cortex and hippocampus of AD patients (Fig. S2). Other studies have suggested that elevated plasma HC may contribute to vascular fibrin accumulation and reduced fibrinolysis in vascular diseases [40]. HHC is known to disturb hemostasis and shift hemostatic mechanisms, such as altered thrombosis and fibrinolysis [41]. Several studies demonstrated that HC-modified fibrinogen was more resistant to fibrinolysis [42] and formed fibrin clots with thinner fibers than normal fibrinogen *in vitro* [38]. We considered the possibility that HC/HCTL directly alters fibrin clot formation by modifying fibrinogen. We show by turbidity assay and confocal microscopy that abnormally high levels of HC or HCTL can modify fibrinogen, inducing its oligomerization and abnormal fibrin clot formation *in vitro* (Fig. S3). These results indicate that homocysteinylation leads to atypical fibrin(ogen) properties.

The concentrations of HC/HCTL used in *in vitro* experiments were higher than physiological levels. We initially performed experiments with physiological concentrations of HC (5–100 μ m) and HCTL (0.05–0.5 μ m) (Table S1), but these concentrations did not show a difference in lag time and maximal turbidity during clot formation. The higher concentrations used accelerated the reaction and

allowed us to model this long-term disease *in vitro* in a reasonable time frame.

We also examined the possibility that HC/HCTL could increase A β ₄₂ fibrillization. Incubation of A β ₄₂ with various doses of HC (5–50 μ m) or HCTL (0.05–5 μ m) for up to 96 h did not affect total β -sheet content via the Thioflavin T assay. Therefore, HC/HCTL-modified fibrinogen is crucial to the changes we observed in A β deposition.

It is known that A β can specifically interact with fibrinogen and subsequently alter fibrin clot structure [13,18–20]. A β -influenced abnormal fibrin clots are resistant to fibrinolysis because of their tighter network of thinner fibers [18,19]. The present studies confirm that HC/HCTL-modified fibrinogen more strongly interacts with A β ₄₂ (Fig 2A,B) and forms enlarged, irregular clots in the presence of A β ₄₂ (Fig. S5A–C). Moreover, A β -influenced HC/HCTL-modified fibrin clots showed a delayed half-lysis time during fibrinolysis (Fig. S5D–G), suggesting that HC/HCTL alters A β -influenced fibrin clot structure and causes delayed fibrin clot dissolution. Therefore, abnormally high levels of plasma HC/HCTL could be critically important in enhancing the deposition of fibrin and A β in the cerebral vasculature, via the A β -fibrinogen interaction.

HC/HCTL-modified FragD interacted more strongly with A β than control FragD in pull-down assays and ELISAs (Fig. 2C,D). Importantly, Sauls and colleagues demonstrated that fibrinogen has 12 lysine residues that can be modified by HCTL, four of which are within FragD [38]. Because HC/HCTL-modified fibrinogen produced significantly larger aggregates during clot formation with A β but we previously showed that A β does not induce FragD oligomerization [18], our data suggest that HCTL-modified residues within FragD account for the increased interaction with A β , supporting an important functional mechanism in AD/HHC.

In neuropathological studies, CAA is commonly observed in AD patients [7,43,44] and can induce neurovascular damage and disrupt blood flow in the brain, resulting in cognitive impairment [7,45]. Studies have shown that amelioration of CAA in the brain improves memory deficits in AD mice [19,46]. Recently, Li and Pratico reported that HHC induced by a diet deficient in folate and vitamins B6 and B12 increased CAA in AD transgenic mice [47]. Here, we report that HHC induced by a methionine-rich diet increased CAA severity and exacerbated spatial and contextual memory loss in AD mice (Figs 3, 4). These data strongly suggest that plasma HC can influence CAA pathology and cognitive impairment in AD. In the brains of AD mice, parenchymal A β can be cleared by way of the vasculature, but severe CAA hinders this clearance route [7]. Our studies showed that a

MET diet significantly increased both parenchymal and vascular A β deposition in AD mice compared with CON-fed AD mice (Fig. 3). It is possible that the increased deposition of parenchymal A β could ultimately lead to impaired clearance through the vasculature, facilitating A β -induced abnormal fibrin clot formation. It is also possible that high plasma HC/HCTL levels could modify fibrinogen and induce its oligomerization, leading to accumulation of A β -fibrinogen deposits. Given that homocysteinylation of fibrinogen induced its oligomerization (Fig. S3) and strengthened its interaction with A β_{42} (Fig. 2 and S4) *in vitro*, increased HC/HCTL levels may prompt more A β -fibrinogen accumulation within the cerebral vasculature. This hypothesis is supported by a report that fibrinogen and A β deposits are elevated in the brains of transgenic HHC mice [48]. Our results are the first to suggest that HC/HCTL may aggravate cognitive dysfunction and pathology in AD by way of the A β -fibrinogen interaction.

Many clinical studies suggest co-morbidity of AD and HHC, yet homocysteinylated-fibrinogen in the blood or brain of AD patients has not yet been reported. Interestingly, HHC patients deficient in cystathione β -synthase (CBS), an enzyme responsible for converting homocysteine to cystathione, have significantly elevated plasma levels of N-Hcy-fibrinogen [49,50]. This fibrinogen N-homocysteinylation leads to resistance to fibrin clot lysis [27,38] and increases risk of thrombosis [27,51]. Moreover, experimental studies provide evidence that MET diet-induced HHC results in increased fibrin deposition in the vasculature and delayed fibrinolysis in the kidneys, lung and liver [28], which complements the symptoms of CBS-deficient patients. Although we did not show evidence of fibrinogen N-homocysteinylation in our HHC mouse model, our study provides the first evidence that HC/HCTL-modified fibrinogen may be responsible for aggravating AD pathology by way of the A β -fibrinogen interaction.

Several studies have shown that AD mice have abnormal clotting abilities compared with controls [10,19,52]. We have also shown that AD patients have increased cerebral fibrin deposition, corresponding to their CAA severity [10,23]. Moreover, our *in vitro* studies demonstrate in detail how A β affects fibrin clot structure and interferes with clot lysis by preventing the binding of plasminogen to fibrin [18–20]. Altogether, these results could suggest that vessel obstruction results from the formation of persistent A β -laden fibrin clots, implying the existence of a close relationship between AD pathophysiology and stroke/microinfarcts. Many clinical studies have shown that asymptomatic spontaneous cerebral emboli [53] and microinfarcts [54] are more common in AD patients. Furthermore, these ischemic events lead to weakening of blood vessels, increasing the likelihood of subsequent microhemorrhage and hemorrhagic stroke, a process referred to as hemorrhagic transformation [55].

Thus, our findings that HC/HCTL-modified fibrinogen induced bigger A β -fibrin aggregates in clots and delayed fibrinolysis suggest that HHC could aggravate CAA pathology, including hemorrhage, in both AD and stroke.

Emerging evidence strongly indicates diverse roles of HHC in AD pathophysiology. Although several studies have explored the effect of HHC on A β plaque formation and tau phosphorylation in AD mice [56], there are still conflicting results about the role of HHC in AD pathophysiology. Here, we show that heightened plasma HC levels enhance the A β -fibrinogen interaction and exacerbate vascular pathogenesis and cognitive decline in AD. This finding and further studies that show HHC deteriorates the cerebrovascular environment through the A β -fibrinogen interaction will provide novel insights and may help develop therapeutics for AD.

Addendum

Y. C. Chung performed the research, analyzed data and wrote the manuscript; A. Kruyer performed experiments, analyzed data and assisted in manuscript writing; Y. Yao assisted in manuscript writing; E. Feierman assisted in experiments; A. Richards assisted in manuscript editing; S. Strickland assisted in study design and data analysis; and E. H. Norris assisted in study design, data analysis, and writing and editing the manuscript.

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Disclosure of Conflicts of Interests

The authors state that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Representative images of fibrinogen (FBG, green), A β (blue) and vascular (COL4, red) staining in post-mortem samples of the frontal cortex and hippocampus of non-demented control individuals.

- Fig. S2.** Plasma homocysteine (HC) concentration correlates with fibrinogen deposition and cerebral amyloid angiopathy (CAA) in Alzheimer's disease (AD) patients.
- Fig. S3.** Effects of homocysteine (HC) and homocysteine thiolactone (HCTL) on fibrin polymerization *in vitro*.
- Fig. S4.** Homocysteinylation enhances the interaction between A β 42 and fibrinogen.
- Fig. S5.** Homocysteine (HC) and homocysteine thiolactone (HCTL) alter fibrin clot formation and fibrinolysis.
- Fig. S6.** Hyperhomocysteinemia (HHC), caused by administration of a high methionine diet, leads to increased parenchymal and vascular A β deposition in 4- and 7-month-old Alzheimer's disease (AD) mice.
- Table S1.** Demographic characteristics of individuals analyzed in this study.
- Table S2.** Nutrient composition of control (CON), high methionine (MET) and high glycine (GLY) diets administered to mice.
- Table S3.** Body weight changes in mice administered control (CON), high glycine (GLY) or high methionine (MET) diets.

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