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IN FOCUS

The Alzheimer's disease peptide β-amyloid promotes thrombin generation through activation of coagulation factor XII

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Essentials

- How the Alzheimer's disease (AD) peptide β-amyloid (Aβ) disrupts neuronal function in the disease is unclear.
- Factor (F) XII initiates blood clotting via FXI, and thrombosis has been implicated in AD.
- Aβ triggers FXII-dependent FXI and thrombin activation, evidence of which is seen in AD plasma.
- Aβ-triggered clotting could contribute to neuronal dysfunction in AD and be a novel therapeutic target.

Summary. *Background:* β-Amyloid (Aβ) is a key pathologic element in Alzheimer's disease (AD), but the mechanisms by which it disrupts neuronal function *in vivo* are not completely understood. AD is characterized by a prothrombotic state, which could contribute to neuronal dysfunction by affecting cerebral blood flow and inducing inflammation. The plasma protein factor XII triggers clot formation via the intrinsic coagulation cascade, and has been implicated in thrombosis. *Objectives:* To investigate the potential for Aβ to contribute to a prothrombotic state. *Methods and results:* We show that Aβ activates FXII, resulting in FXI activation and thrombin generation in human plasma, thereby establishing Aβ as a possible driver of prothrombotic states. We provide evidence for this process in AD by demonstrating decreased levels of FXI and its inhibitor C1 esterase inhibitor in AD patient plasma, suggesting chronic activation, inhibition and clearance of FXI in AD. Activation of the intrinsic coagulation pathway in AD is further supported by elevated fibrin levels in AD patient plasma. *Conclusions:* The ability of Aβ to promote coagulation via the FXII-driven contact system identifies new mechanisms by which it could contribute to neuronal dysfunction and suggests potential new therapeutic targets in AD.

Keywords: Alzheimer's disease; amyloid β-peptides; factor XI; factor XII; thrombin.

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Introduction

Alzheimer's disease (AD) is a fatal cognitive disorder affecting ~ 26 million people worldwide. There is evidence that the AD-related peptide β-amyloid (Aβ) is a primary driver of both early-onset and late-onset disease [1,2]. While the direct neuronal toxicity of Aβ *in vitro* is well documented, the mechanism by which Aβ disrupts neuronal function in AD patients is still unclear [1].

It is unlikely that direct toxicity of Aβ to neurons is the sole factor responsible for eliciting AD. Indeed, cerebrovascular pathology is present in the majority of AD patient brains upon autopsy [3,4], but whether it contributes to AD etiology or is simply a comorbidity is debated. A causative role for vascular pathology in AD is suggested by the increased risk of AD in vascular disease states such as atherosclerosis, diabetes, hypertension, and hypercholesterolemia [5]. In keeping with a link between AD and vascular dysfunction, accumulating evidence suggests that AD patients and mouse models exhibit a prothrombotic state: AD patients have numerous prothrombotic markers in the circulation [6], AD mouse models have a propensity to form thrombi

[7,8], and AD patients are at a higher risk for microinfarcts [9,10] and stroke [11]. Furthermore, prothrombotic conditions such as elevated levels of D-dimer, prothrombin fragment 1 + 2, coated platelets, plasma homocysteine, and fibrinogen, as well as the presence of factor V Leiden, silent brain infarcts, microinfarcts, and stroke, all contribute to AD onset and progression [6,10,12–15]. The idea that a prothrombotic state plays a role in AD is further supported by improvements in AD pathology and memory in patients [16,17] and mouse models [18,19] following treatment with anticoagulants.

Coagulation factor XII (FXII) is a circulating protein that, when converted to activated FXII (FXIIa), can initiate two pathways: the intrinsic coagulation pathway through activation of FXI, and the kallikrein–kinin system through activation of prekallikrein. Since Aβ has been shown to interact with and activate FXII [20–23], and FXII has been implicated in thrombosis in mice [24], one possibility is that Aβ may contribute to the prothrombotic environment in AD through FXII-dependent activation of FXI. Although Aβ has not been shown to trigger FXII-dependent FXI activation *in vitro* or *in vivo*, it has been implicated in FXII-dependent activation of the kallikrein–kinin system *in vitro* [20–22] and in mouse models [25]. Furthermore, increased activation of the kallikrein–kinin system is found in AD patient plasma [25], brain [26], and cerebrospinal fluid (CSF) [27], suggesting a role for FXII-driven processes in AD pathology. In the circulation, FXII can encounter increased concentrations of Aβ at sites of potential thrombosis, including the luminal side of cerebral capillary walls (where Aβ enters the blood through the blood–brain barrier [BBB]) [28], atherosclerotic lesions [29], sites of platelet activation [30], and areas of erythrocyte accumulation [31,32]. A role for circulating Aβ in AD is supported by increased levels of plasma Aβ in populations at high risk for AD (Down syndrome patients, familial AD patients, and family members of AD patients) [33,34], and in sporadic AD patients prior to the onset of symptoms [33–35]. Moreover, elevated plasma Aβ levels increase AD risk [36,37].

Here, we show that Aβ oligomers promote coagulation by inducing FXII-mediated thrombin generation through the intrinsic coagulation pathway. We demonstrate the relevance of these findings to AD by showing decreased levels of FXI and its inhibitor C1 esterase inhibitor (C1inh), and increased levels of fibrin, in AD patient plasma, suggesting activation of FXI and the intrinsic coagulation pathway. The promotion of coagulation by Aβ42 provides a possible new mechanism for the prothrombotic state observed in AD patients, and suggests new mechanisms by which it could contribute to neuronal dysfunction. Inhibition of the activation or activity of FXII in AD patients might therefore be an attractive novel therapeutic approach.

Materials and methods

Aβ preparation

Aβ42, Aβ40 and Aβ42 E22Q Dutch (Anaspec, Fremont, CA, USA) monomers and oligomers were prepared as in [38]. For fibrils, Aβ42 was dissolved in 60 μL of 1% NH_4OH, adjusted to 200 μM with 50 mM Tris (pH 7.4) and 150 mM NaCl, and incubated at 37 °C, with shaking, for 7 days. Aβ preparations were confirmed by transmission electron microscopy (TEM) at Rockefeller University's Electron Microscopy Resource Center.

Blood collection and plasma preparation

Experiments with human plasma were approved by Rockefeller's Institutional Review Board. Blood was drawn from healthy volunteers, who had given informed, written consent, with 21-gauge 0.75-inch butterfly needles with a multi-adapter for S-Monovette (Sarstedt, Newton, NC, USA) into S-Monovette tubes containing 1/10 volume 0.106 M trisodium citrate solution at Rockefeller University Hospital and Karolinska Institute Hospital. To obtain platelet-rich plasma (PRP), blood was centrifuged at 130 × *g* for 10 min, and the top half of the PRP was removed. To obtain platelet-poor plasma (PPP), blood was centrifuged twice at 2000 × *g* for 10 min. PPP was frozen immediately at − 80 °C. Microparticle-free plasma was prepared by ultracentrifugation at 100 000 × *g* for 30 min at 4 °C.

Mouse lines

Animal care and experimental procedures complied with the principles of laboratory and animal care established by the National Society for Medical Research, and were approved by the Stockholm Norra Djurförsöksetiska Nämnd. $FXII^{-/-}$ [39] and $FXI^{-/-}$ [40] mice backcrossed to C57BL/6 mice for > 10 generations and age-matched C57BL/6 control mice (Charles River, Wilmington, MA, USA) were used. Blood was collected with repel-gel (Sigma, St. Louis, MO, USA) coated glass capillary tubes into citrated Eppendorf tubes. PPP was prepared by centrifugation at 1500 × *g* for 15 min.

Thrombin generation in plasma

Thrombin generation in normal or FXII-deficient human plasma (George King Biomedical, Overland Park, KS, USA) was measured by calibrated automated thrombogram (CAT), as described in [41]. In some cases, plasma was preincubated for 30 min with an antibody against FXIIa [42] or active site-inhibited FVII (ASIS; Novo Nordisk, Bagsværd, Denmark). Some reactions also contained FXIa (Haematologic Technologies, Essex Junction, VT, USA; 3 pM) or phospholipids (Thrombinoscope BV,

Maastricht, Netherlands; 4 μM). Thrombin generation in FXII$^{-/-}$, FXI$^{-/-}$ and C57BL/6 mouse plasma was measured as described above, with modifications as in [43].

Aβ42–FXII binding

Human plasma diluted 1 : 5 in phosphate-buffered saline (PBS) containing 0.01% NP-40 and protease inhibitor cocktail (Roche, Indianapolis, IN, USA) was incubated with 500 nM biotinylated Aβ42 or amylin (Anaspec) for 2 h at room temperature, and this was followed by pulldown with streptavidin Dynabeads M-280 (Life Technologies, Carlsbad, CA, USA), and analysis by western blotting with mAb against FXII (Haematologic Technologies).

FXII and FXI activation in vitro *and in plasma*

Chromogenic substrate For FXII activation, 0.8 mM Pefachrome FXIIa (Centerchem, Norwalk, CT, USA) was added to 100 nM FXII (Haematologic Technologies) and 3 μM Aβ or vehicle. For FXII-dependent FXI activation, 0.8 mM Pefachrome FXIa (Centerchem) was added to 5 nM FXII, 15 nM FXI (Haematologic Technologies), and 3 μM Aβ or vehicle. Activity was monitored at 405 nm with a Molecular Devices (Sunnyvale, CA, USA) Spectramax Plus 384 reader at 37 °C in 96-well polystyrene plates (Fisher Scientific, Waltham, MA, USA) precoated with 1% poly(ethylene glycol) 20 000 in 20 mM HEPES containing 140 mM NaCl (HEPES-buffered saline [HBS]).

Western blotting For FXII activation, FXII (200 nM) and prekallikrein (150 nM) were incubated with Aβ42 (3 μM), dextran sulfate 500 kDa (DS500; Sigma; 10 μg mL^{-1}) or vehicle for 30 min at 37 °C. For FXI activation, FXII (200 nM), prekallikrein (150 nM), high molecular weight kininogen (HK) (Molecular Innovations, Novi, MI, USA; 300 nM) and FXI (150 nM) were incubated with Aβ (3 μM), kaolin (Fisher Scientific; 100 μg mL^{-1}) or vehicle for 30 min at 37 °C. Plasma from healthy volunteers was diluted 1 : 10 in HBS, and incubated with Aβ42 (20 μM) for 1 h at 37 °C. Reactions were stopped by addition of reducing sample buffer and heating for 5 min at 85 °C. Blots were probed with antibodies against FXII and FXI (Hematologic Technologies). FXI activation was quantified by densitometric analysis.

ELISA measuring FXIa–inhibitor complex formation Normal or FXII-deficient human plasma was diluted 1 : 10 in HBS, and incubated with Aβ42, Aβ42 Dutch (20 μM), kaolin (10 μg mL^{-1}) or vehicle at 37 °C for 1 h. Reaction mixtures were transferred to a plate precoated with a mAb against FXI (3 μg mL^{-1}; Hematologic Technologies), and blocked with PBS containing 2% milk (blocking buffer) for 1 h. Wells were then washed three times for 5 min each with PBS containing 0.05% Tween-20. A polyclonal C1 inhibitor (3 μg mL^{-1}; Cedarlane, Burlington, NC, USA) or α_1-antitrypsin (α_1AT) (3 μg mL^{-1}; Thermo Scientific, Waltham, MA, USA) antibody in blocking buffer was applied for 1 h. After washing, a horseradish peroxidase-conjugated anti-goat antibody (Jackson, Farmington, CT, USA; 1 : 2000) in blocking buffer was applied for 1 h. The ELISA was developed with 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Thermo Scientific).

FXI, C1inh and fibrin levels in human plasma

Plasma from AD patients and non-demented (ND) controls was obtained from the University of Kentucky Sanders-Brown Center on Aging (group 1) and Washington University Knight Alzheimer's Disease Research Center (group 2). Group 1 AD cases were defined by clinical diagnosis of AD and by a postmortem Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuritic plaque score [44] of B or C, corresponding to probable or definite AD, respectively. ND cases had a CERAD score of 0 and no clinical diagnosis of AD (Table S1). Group 2 AD cases had a Clinical Dementia Rating (CDR) score (measuring cognitive function) [45] of ≥ 0.5 and CSF Aβ42 levels of < 500 pg mL^{-1}, and ND cases had a CDR score of 0 and CSF Aβ42 levels of > 500 pg mL^{-1} (Table S2). For group 1, blood was drawn into heparinized plastic Vacutainer tubes. For group 2, blood was drawn with EDTA-coated syringes into polypropylene tubes containing a final concentration of 5 mM EDTA.

Equal amounts of total protein from each sample (as determined with the bicinchoninic acid assay) were analyzed by western blotting with antibodies against FXI (Haematologic Technologies), C1inh (Proteintech, Chicago, IL, USA), fibrin β-chain (59D8 [46]), D-dimer (AbD Serotec, Raleigh, NC, USA), and transferrin (Abcam, Cambridge, MA, USA). Purified FXI, C1inh (Athens Research and Technology, Athens, GA, USA) and FXI-deficient plasma (George King Biomedical) served as controls.

Statistical analysis

Data are presented as vertical scatter plots with medians, and are reported as medians with 25th and 75th percentile ranges (median [25th–75th percentile range]), or presented as bar graphs (mean ± standard deviation). Comparisons between groups were performed with the unpaired, two-tailed Mann–Whitney test or one-way ANOVA followed by Tukey's multiple comparison test. Correlations were analyzed by the use of Spearman's correlation coefficient (r). P-values of ≤ 0.05 were considered to be significant. Statistical analyses were performed with GRAPHPAD PRISM 5 (GraphPad Software, La Jolla, CA, USA).

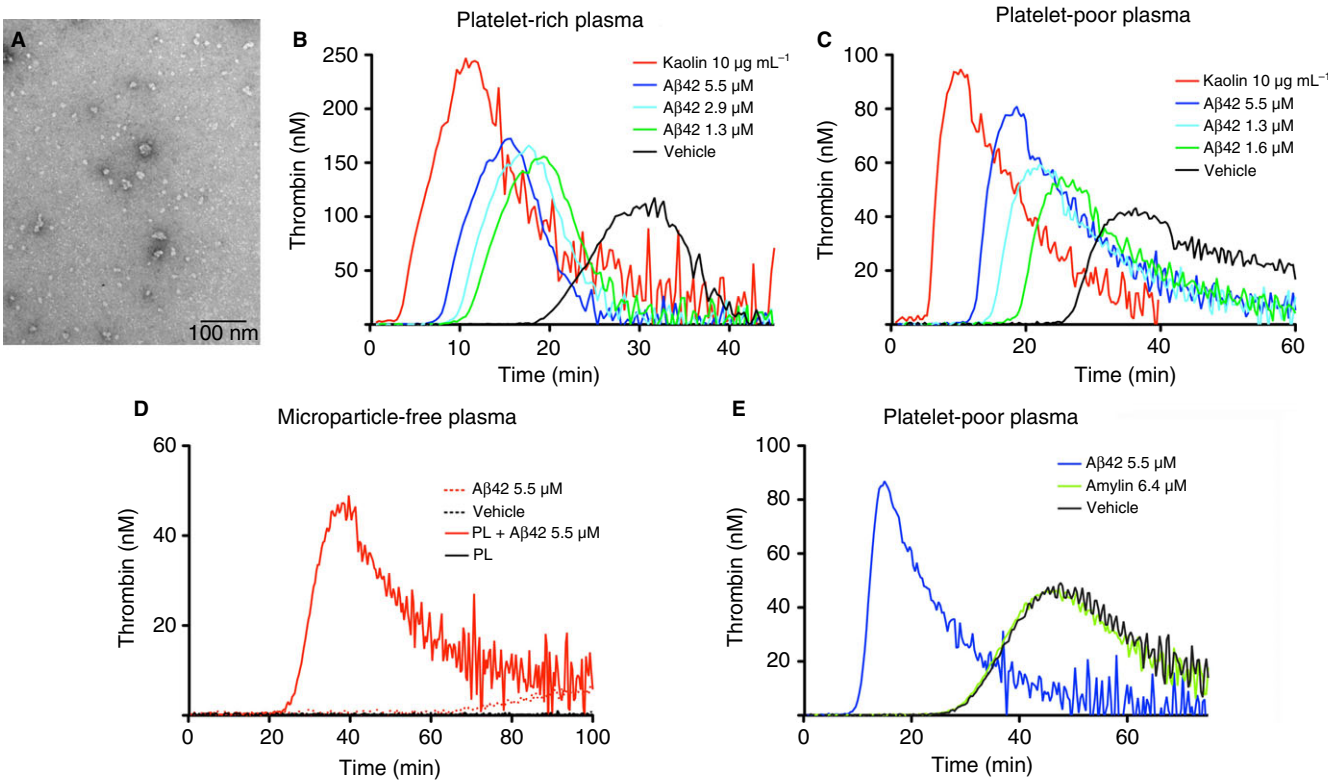


Fig. 1. Aβ42 triggers thrombin generation in human plasma. (A) Representative transmission electron microscopy image of Aβ42 oligomers used. (B–E) Real-time thrombin generation was measured by calibrated automated thrombogram. (B) Platelet-rich plasma was incubated with Aβ42 at the concentrations indicated or with kaolin (a known activator of thrombin generation). The lag time to thrombin generation was decreased and the thrombin peak height was increased in the presence of Aβ42 in a dose-dependent manner. (C) As in (B), except that platelet poor-plasma (PPP) was used. (D) Aβ42 had no effect in platelet and microparticle-free plasma. Addition of phospholipids (PL; 4 μM) restored Aβ42's ability to trigger thrombin generation. (E) The lag time to thrombin generation was decreased and the maximum peak height was increased in PPP with Aβ42 but not with amylin. All experiments were performed in duplicate, and averaged curves are presented. Aβ, β-amyloid.

Results

Aβ42 promotes thrombin generation in plasma

To determine whether Aβ42 is prothrombotic, we quantified thrombin generation in human plasma by CAT [41] in the presence of oligomeric Aβ42, a toxic assembly that correlates with disease severity [47]. The oligomeric composition of Aβ42, which is stable for 24 h at room temperature and 37 °C (Fig. S1), was confirmed by electron microscopy (Fig. 1A). In the absence of exogenous activators, a small thrombin burst was detectable after a long lag period (vehicle; Fig. 1B). Addition of Aβ42 to PRP promoted thrombin generation in a dose-dependent manner, as indicated by a shortening of the lag time to the thrombin burst and an increase in peak height (maximum thrombin formed) (Fig. 1B). A similar prothrombotic effect was observed in PPP (Fig. 1C), indicating that platelets are not required for the effect. However, Aβ42 had no effect in microparticle-free plasma (Fig. 1D). Supplementing microparticle-free plasma with phospholipids restored Aβ42's ability to trigger thrombin generation (Fig. 1D), indicating that the presence of phospholipid surfaces (found on platelets and microparticles) is required for Aβ42-mediated thrombin generation. The prothrombotic effect is specific to Aβ42, since amylin, another amyloid-forming peptide, failed to induce thrombin generation (Fig. 1E).

Aβ42-mediated thrombin generation is FXII-dependent

Thrombin is generated through activation of the intrinsic (FXII-driven) or extrinsic (tissue factor [TF]-driven) coagulation pathways. To determine which pathway is activated by Aβ42, CAT experiments were performed in the presence of a FXIIa function-blocking antibody [42] (to block the intrinsic pathway), or with active site-inhibited FVII (ASIS; to block the extrinsic pathway). The FXIIa antibody abolished Aβ42-induced thrombin generation (Fig. 2A), whereas ASIS had no inhibitory effect (Fig. 2B), indicating that Aβ42 is prothrombotic via the FXIIa-driven intrinsic coagulation pathway. The FXIIa antibody specifically blocks FXIIa-mediated thrombin generation, since it abolished thrombin generation

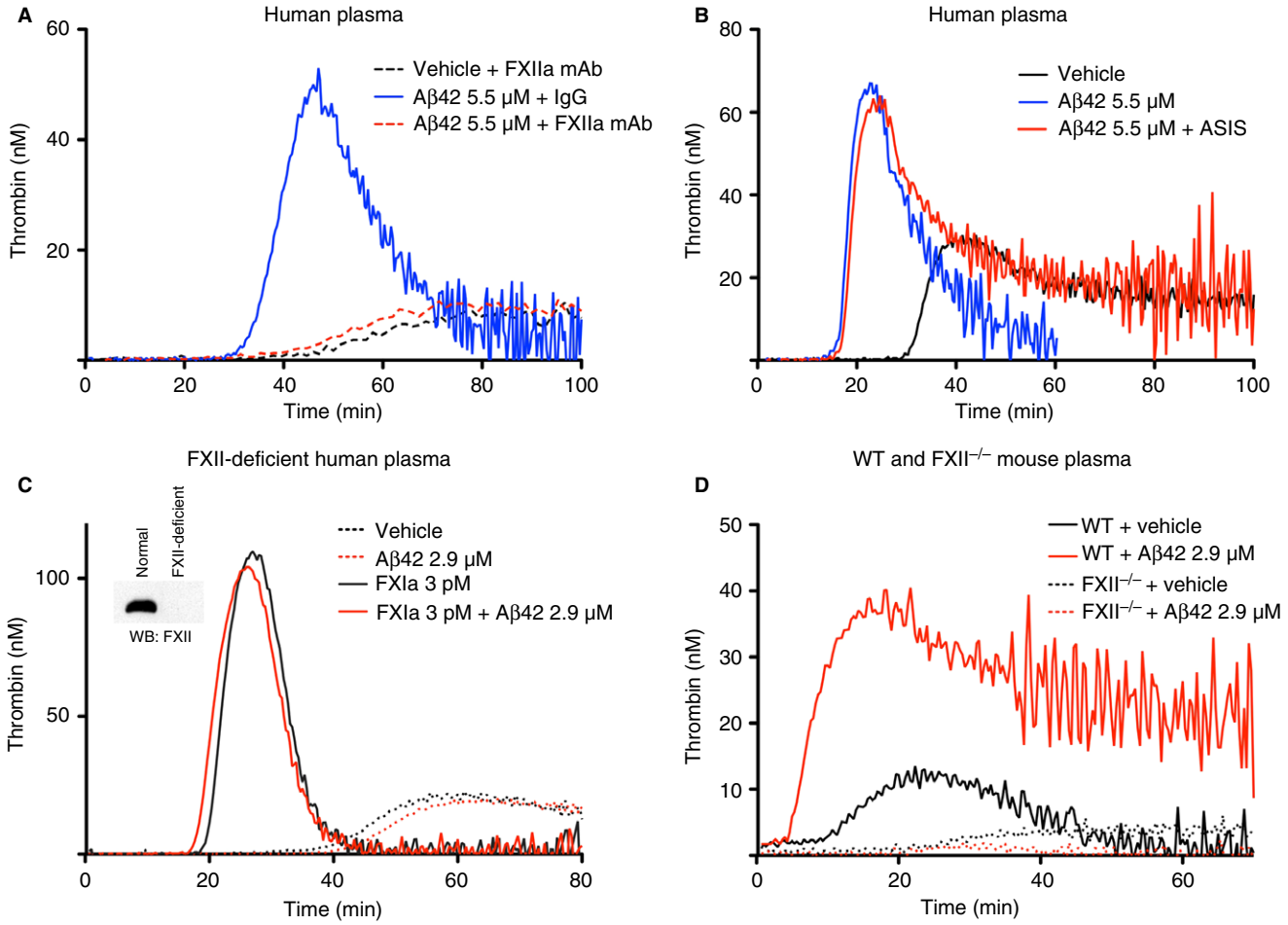


Fig. 2. Aβ42 promotes thrombin generation in an FXII-dependent manner. Thrombin generation was measured by calibrated automated thrombogram. (A) Aβ42-induced thrombin generation was blocked by a mAb against activated FXII (FXIIa) [69] (4 μM), but not by IgG. (B) Aβ42's enhancement of thrombin generation was not inhibited by the extrinsic coagulation pathway inhibitor ASIS (60 nM). (C) Thrombin generation was not enhanced in human plasma from an FXII-deficient individual in the presence of Aβ42. Deficiency of FXII in this plasma was confirmed by western blot (WB; inset). Aβ42 had no effect when thrombin generation was triggered by 3 pM activated FXI (FXIa). (D) Thrombin generation was enhanced in WT mouse plasma but not in $FXII^{-/-}$ mouse plasma in the presence of Aβ42. Mouse plasma contained 240 nM ASIS to block tissue factor (TF)-mediated thrombin generation stemming from TF contamination during blood draw. ASIS did not affect Aβ42-mediated enhancement of thrombin generation (Fig. 2B). All experiments were performed in duplicate, and averaged curves are presented. Aβ, β-amyloid.

initiated by kaolin (an FXII activator) but did not interfere with TF-initiated thrombin generation. As expected, ASIS inhibited TF-initiated thrombin generation (Fig. S2).

To further examine the role of FXII in Aβ42-mediated thrombin generation, we analyzed the effect of Aβ42 in FXII-deficient human plasma (with no detectable plasma FXII antigen; Fig. 2C inset). Aβ42 failed to trigger thrombin generation in FXII-deficient plasma (Fig. 2C, dashed curves). To examine the role of Aβ42 in a system in which FXII is completely absent, we tested plasma from mice that do not express any FXII ($FXII^{-/-}$). Whereas Aβ42 promoted thrombin generation in wild-type (WT) mouse plasma, no effect was seen in $FXII^{-/-}$ mouse plasma (Fig. 2D).

Since FXII-deficient or FXII-neutralized plasmas have normal levels of downstream coagulation factors, the results also indicate that thrombin generation is not driven through direct activation of these factors by Aβ42. However, Aβ42 may potentiate downstream factors when they are in the activated state, which may be produced by low-level, well surface-mediated FXII activation (e.g. the background thrombin signal in Fig. 1B). To address this possibility, thrombin generation in FXII-deficient human plasma was measured following activation with FXIa, which activates downstream members of the coagulation cascade. Aβ42 had no effect on thrombin generation in plasma activated with low levels of FXIa (Fig. 2C, solid curves), indicating that it does not enhance the activity of FXIa or any downstream factors. Furthermore, Aβ42 had no effect on thrombin generation in plasma from mice that have normal levels of FXII but do not express FXI (Fig. S3), confirming that the pathway enhanced by Aβ42 involves FXIIa-mediated activation of FXI and not FXIIa-mediated activation of another substrate.

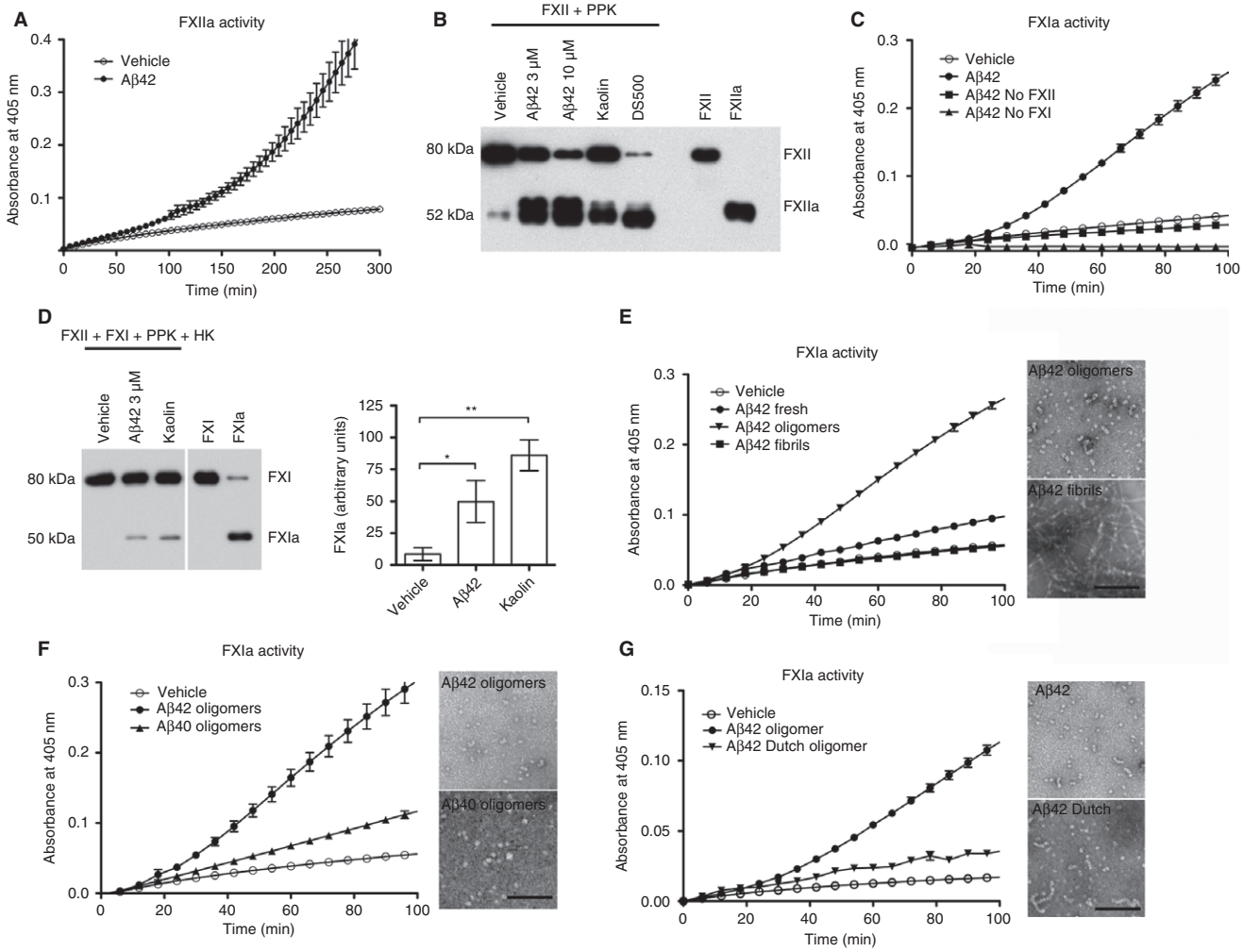


Fig. 3. Aβ42 promotes FXII-dependent FXI activation *in vitro*. (A) Aβ42 (3 μM) triggered autoactivation of FXII as determined by chromogenic substrate assay. (B) In the presence of both FXII and prekallikrein (PPK), Aβ42 dose-dependently promoted activation of FXII, as shown by the reduction in FXII zymogen levels at 80 kDa and the appearance of the activated FXII (FXIIa) heavy chain at 52 kDa. Dextran sulfate 500 kDa (DS500) and kaolin were used as positive controls. (C) Aβ42 (3 μM) triggered FXII-dependent activated FXI (FXIa) generation in a chromogenic substrate assay. The signal was not attributable to non-specific cleavage of chromogenic substrate by FXIIa, or by autoactivation of FXI, as seen in controls where FXII or FXI was omitted. (D) FXI activation can be seen through the appearance of the 50-kDa FXIa heavy chain band following incubation of FXII, FXI, PPK, and high molecular weight kininogen (HK) with Aβ42 or kaolin. Levels of FXIa heavy chain were increased in Aβ42-treated ($P < 0.05$) and kaolin-treated ($P < 0.01$) samples as compared with vehicle. All lanes presented are from the same blot. (E) Aβ42 oligomers were more potent in promoting FXII-dependent FXI activation than freshly dissolved Aβ42. Aβ42 fibrils had no effect. All Aβ42 concentrations were 3 μM. (F) Aβ42 oligomers were more potent than Aβ40 oligomers in promoting FXII-dependent FXI activation. (G) Aβ42 oligomers (transmission electron microscopy [TEM] image, top inset) promoted FXII-dependent FXI activation much more strongly than Aβ42 Dutch oligomers (TEM image, bottom inset). Chromogenic substrate assays were performed in triplicate. Representative immunoblots are from three experiments. *$P \leq 0.05$; **$P \leq 0.01$.

Aβ triggers FXII-dependent FXI activation in vitro

FXII undergoes autoactivation on negatively charged surfaces. Since autoactivation of FXII has only been shown with fibrillar Aβ40 and in the presence of $ZnCl_2$ [20], we first determined that Aβ42 oligomers can directly induce FXII autoactivation (Fig. 3A). Physiologically, contact system activation takes place in the presence of prekallikrein, which is activated by FXIIa to kallikrein, which, in turn, activates additional FXII, amplifying the reaction. Aβ42 dose-dependently promoted FXII activation in the presence of prekallikrein (Fig. 3B), as seen through the reduction of FXII zymogen levels (80 kDa) and the appearance of the FXIIa heavy chain (52 kDa).

Aβ42 led to FXIIa-dependent FXIa generation in the absence (Fig. 3C) and presence (Fig. 3D) of prekallikrein, indicating that FXII activated by Aβ42 is capable of cleaving its substrate FXI. Previously, FXIIa-dependent FXI activation and procoagulant effects were not detected in the presence of Aβ [22]. The main difference between our experiments is that the previous study used 'amorphous aggregates' of Aβ42 with the Dutch mutation (E22Q) instead of the WT oligomeric Aβ42 used here. This discrepancy prompted us to analyze the ability of

Aβ42 in different states of aggregation, and of other Aβ variants, to trigger FXII-dependent FXI activation. We found that Aβ42 oligomers had a much greater ability to trigger FXII-dependent FXI activation than freshly dissolved Aβ42 (Fig. 3E) or Aβ40 oligomers (Fig. 3F), while Aβ42 fibrils produced no FXI activity at all (Fig. 3E). Furthermore, even the most active (oligomeric) form of Aβ42 Dutch was substantially less potent than oligomeric Aβ42 in stimulating FXII-dependent FXI activation (Fig. 3G), indicating that the discrepancy between our results is attributable to the use of Aβ42 Dutch and the different state of Aβ aggregation in the previous study.

Aβ42 oligomers trigger FXII-dependent FXI activation in plasma

We next examined FXII-mediated FXI activation by Aβ42 in human plasma. Biotinylated Aβ42 (TEM of oligomeric preparation in Fig. S4), but not biotinylated amylin, was able to bind FXII in plasma, as shown by pulldown assay (Fig. 4A), demonstrating that the Aβ42–FXII interaction is specific and occurs in the presence of plasma proteins. This interaction leads to FXII activation, since plasma incubated with Aβ42 had decreased FXII zymogen levels and increased FXIIa heavy chain levels compared with plasma incubated with vehicle (Fig. 4B). Activation of FXI in plasma can be sensitively measured by quantifying FXIa–inhibitor complex levels, since FXIa generated in plasma is rapidly bound by inhibitors [48]. Incubation of plasma with Aβ42 but not with Aβ42 Dutch oligomers resulted in increased levels of FXIa–C1inh complex (Fig. 4C; $P < 0.001$). The activation of FXI by Aβ42 was FXII-dependent, since Aβ42 did not promote FXIa–C1inh complex formation in FXII-deficient plasma. The levels of FXIa in complex with α_1AT, another FXIa inhibitor, were also increased in normal plasma following activation with Aβ42 (Fig. 4D; $P < 0.0001$).

Levels of FXI zymogen and C1inh are decreased and levels of fibrin are increased in AD patient plasma

We next investigated whether the FXII-driven intrinsic coagulation pathway is activated in AD patient plasma.

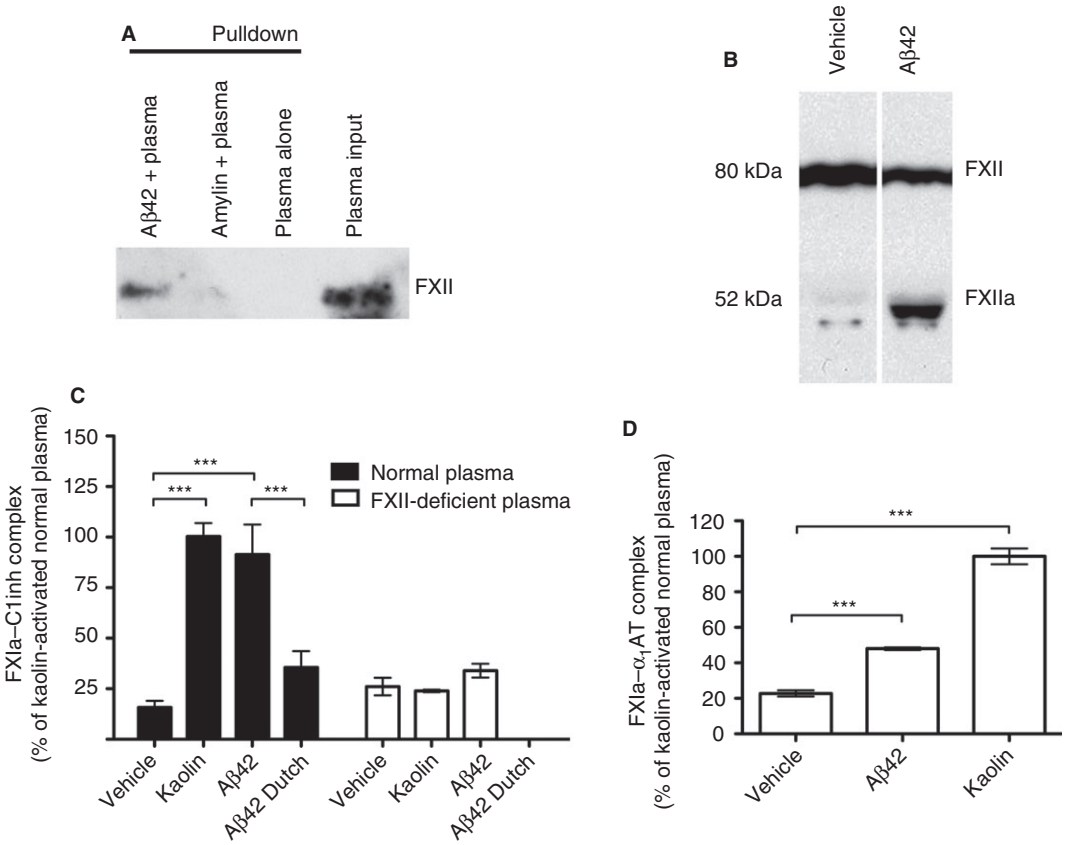


Fig. 4. Aβ42 promotes FXII-dependent FXI activation in plasma. (A) Western blot demonstrating that biotinylated Aβ42 oligomers pull down FXII from human plasma. (B) Incubation of Aβ42 oligomers with human plasma led to FXII cleavage. Lanes presented are from the same blot. (C) ELISA measuring activated FXI (FXIa)–C1 esterase inhibitor (C1inh) complex formation in normal and FXII-deficient human plasma. Oligomers of Aβ42 but not of Aβ42 Dutch promoted the formation of FXIa–C1inh complex ($P < 0.001$; Aβ42 versus vehicle). (D) ELISA measuring FXIa–α_1-antitrypsin (α1AT) complex formation in normal human plasma. Aβ42 oligomers promoted increased FXIa–α_1AT complex formation ($P < 0.0001$; Aβ42 versus vehicle). Results in (C) and (D) are expressed as percentage of kaolin-activated normal plasma and presented as mean ± standard deviation of experiments performed in triplicate. ***$P \leq 0.001$. Aβ, β-amyloid.

Two sets of AD patient and ND control plasmas were obtained from two plasma banks. Group 1 consisted of 10 AD and 10 ND samples matched with respect to age, gender, and apolipoprotein E genotype (Table S1), and group 2 consisted of 10 AD and 10 ND samples matched with respect to age (Table S2). Plasma was analyzed by western blotting, with results normalized to a transferrin loading control, the levels of which are unchanged in AD patients [49]. Increased activation of FXI in plasma can be detected as decreased plasma FXI zymogen levels, which are often observed in disease states accompanied by FXI activation [48,50,51], with decreased plasma FXI zymogen levels possibly reflecting continuous consumption of FXI due to its activation and clearance. AD plasma had decreased levels of FXI zymogen as compared with ND plasma in both group 1 (0.46 [0.36–0.50] versus 0.69 [0.54–0.87], $P = 0.008$; Fig. 5A,B) and group 2 (0.84 [0.61–1.15] versus 1.43 [1.20–1.96], $P = 0.0003$; Fig. 5D,E).

If AD plasma FXI levels were decreased because of its activation and clearance, levels of its main inhibitor C1inh [48] would also be expected to decrease. Indeed, C1inh levels were decreased in AD versus ND plasma in both group 1 (0.52 [0.45–0.75] versus 1.39 [1.01–1.79], $P = 0.0008$; Fig. 5A,C) and group 2 (1.04 [0.91–1.11] versus 1.18 [1.11–1.43], $P = 0.012$; Fig. 5D,F), suggesting its consumption. Decreased levels of FXI and C1inh in AD versus ND control plasma were accompanied by increased levels of FXIIa (FXIIa levels were derived from previous results [25], and are designated by asterisks in Fig. 5B,C). This relationship suggests that depletion of FXI and C1inh in AD patient plasma is attributable to FXII activation.

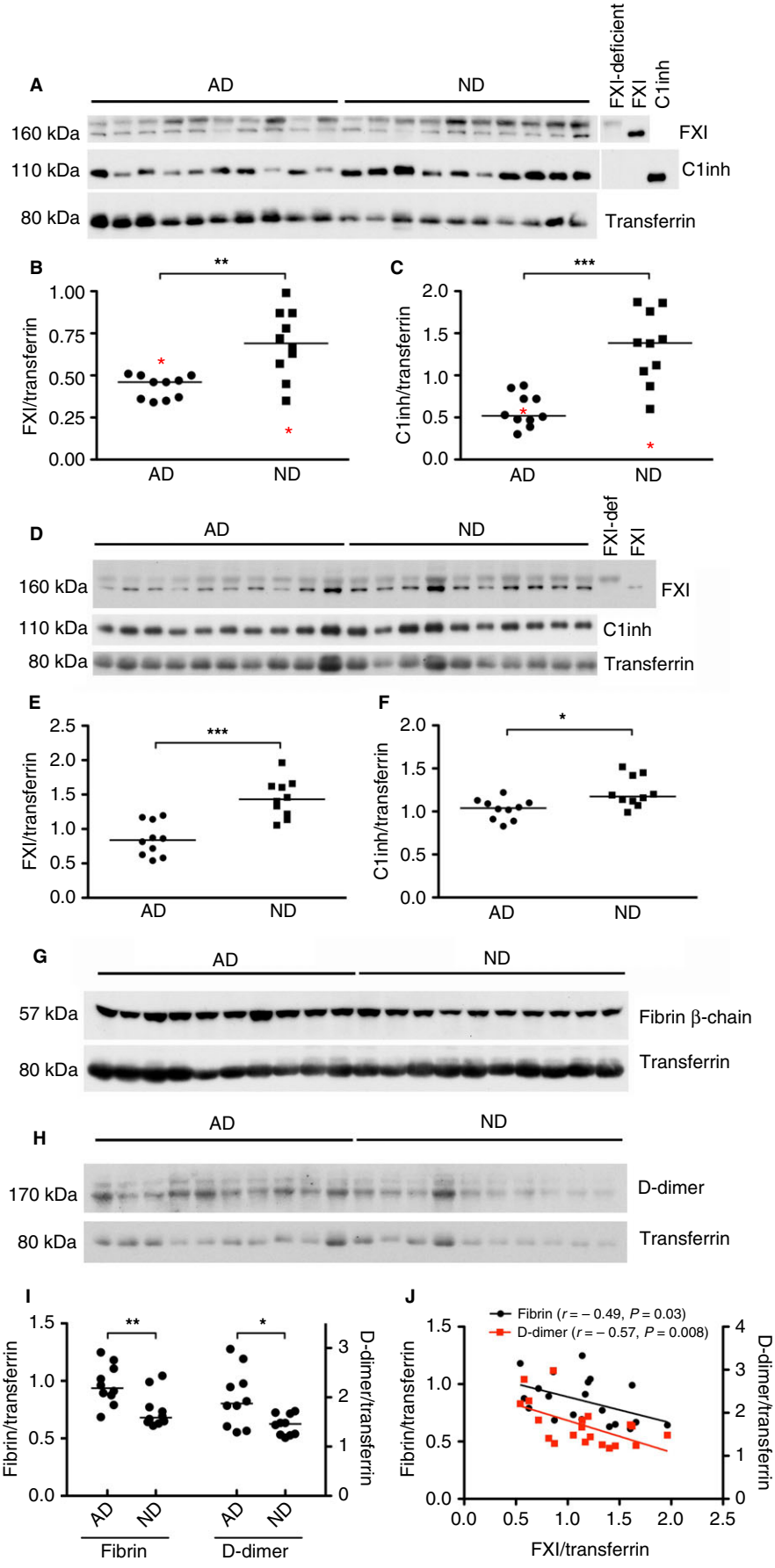
Activation of the intrinsic pathway of coagulation would be expected to result in increased thrombin generation and fibrin formation. Indeed, AD patients from group 2 had elevated plasma fibrin (0.94 [0.85–1.13] versus 0.68 [0.64–0.83], $P = 0.009$); and D-dimer (1.88 [1.39–2.41] versus 1.46 [1.24–1.68], $P = 0.018$) levels compared to controls (Fig. 5G–I). Levels of fibrin and D-dimer were inversely correlated with FXI levels ($r = -0.46$, $P = 0.04$ for fibrin; $r = -0.57$, $P = 0.008$ for D-dimer; Fig. 5J), suggesting that activation and subsequent clearance of FXI results in thrombin generation and fibrin formation. In group 1, there was a non-significant trend towards increased fibrin levels in AD plasma (data not shown), which could be attributable to differences in blood draw and anticoagulation methods between the groups (see Materials and methods). Another possible explanation is the more advanced disease stage of patients in group 1 than of patients in group 2 as determined by the CDR score measuring cognitive function, where 0 = no dementia and 3 = severe dementia [45] (2.0 ± 1.1 for group 1 versus 1.0 ± 0.6 for group 2, $P = 0.028$; Tables S1 and S2). Since group 1 patients are likely to have been exposed to FXI activation for a longer time due to more advanced disease the fibrin formed may have been progressively deposited, thereby depleting soluble fibrin from plasma.

Discussion

Our results identify Aβ as a prothrombotic factor that can trigger thrombin generation via FXII-dependent activation of FXI. Aβ42-mediated, FXII-dependent FXI activation was previously not found [22], likely because amorphous aggregates of Aβ42 with the Dutch mutation (E22Q) were used. While that study found no FXI activation with Aβ42 Dutch amorphous aggregates, our results with Aβ42 Dutch oligomers showed low levels of FXII-dependent FXI activation, highlighting the importance of the assembly state of Aβ42 Dutch in FXI activation. We also showed that WT oligomeric Aβ42 was a more potent FXI activator than monomeric and fibrillar preparations, further supporting the importance of Aβ aggregation state. Finally, WT Aβ42 oligomers were much more potent in FXI activation than Aβ42 Dutch oligomers, possibly because of differences in peptide charge (-2.7 for Aβ42 versus -1.7 for Aβ42 Dutch at pH 7). A more negative charge and/or the presence of glutamic acid at position 22 of Aβ42 therefore appears to be crucial for its activation of FXII and FXI, and it is possible that this region is more optimally exposed in oligomers.

Aβ's role as a prothrombotic factor is interesting in the context of the anticoagulant properties of its parent molecule amyloid precursor protein (APP). Some isoforms of APP contain a Kunitz protease inhibitor domain that

Fig. 5. Alzheimer's disease (AD) patient plasma has lower levels of FXI and C1 esterase inhibitor (C1inh) and increased levels of fibrin. (A) Non-reducing western blot analysis of FXI, C1inh and transferrin loading control in plasma of 10 AD patients and 10 non-demented (ND) controls from group 1. Lanes loaded with FXI purified protein (FXI) and FXI-deficient human plasma show that the band just above the FXI band is non-specific. (B) FXI levels normalized to transferrin were lower in AD plasma than in ND plasma ($P = 0.008$). Levels of FXIIa normalized to transferrin in these samples were determined previously [25], and mean values for each group are designated by asterisks. (C) C1inh levels were lower in AD plasma than in ND plasma ($P = 0.0008$). Levels of FXIIa normalized to transferrin in these samples were determined previously [25], and mean values for each group are designated by asterisks. (D) Levels of FXI, C1inh and transferrin were analyzed in 10 AD and 10 ND plasmas from group 2. (E) FXI levels were lower in AD plasma than in ND plasma ($P = 0.0003$). (F) C1inh levels were lower in AD plasma than in ND plasma ($P = 0.01$). (G) Levels of fibrin monomer were analyzed under reducing conditions in 10 AD and 10 ND plasma samples from group 2 with antibody 59D8 specific for fibrin β-chain [46]. (H) D-dimer levels were analyzed under non-reducing conditions in 10 AD and 10 ND plasma samples from group 2. (I) Fibrin ($P = 0.009$) and D-dimer ($P = 0.018$) levels were increased in group 2 AD plasma compared to control. (J) Fibrin ($r = -0.49$; $P = 0.03$) and D-dimer ($r = -0.57$, $P = 0.008$) levels were negatively correlated with FXI levels in samples from group 2. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.



inhibits several coagulation factors [52–54]. As protease activation is often accompanied by activation of corresponding inhibitory pathways, the release of soluble APP (sAPP) during Aβ production may attenuate the prothrombotic pathways initiated by Aβ. Dysregulation of the balance between Aβ and sAPP may lead to thrombosis or hemorrhage, depending on their abundance and/or localization. For example, an excess of Aβ over sAPP may occur in the cerebral circulation of AD patients because of transport of Aβ from the brain parenchyma [55] without corresponding transport of sAPP.

Our hypothesis that circulating Aβ triggers a prothrombotic state in AD is supported by increased levels of Aβ42 oligomers (in the nanomolar range, similar to the levels used in our thrombin generation studies) in AD patient plasma [56]. Interestingly, higher Aβ42 levels are found in sporadic and familial AD at early stages of disease, prior to and just at the onset of symptoms [33–35,57]. It is thus tempting to speculate that the Aβ/FXII-dependent prothrombotic state suggested by our results together with Aβ/FXII-mediated proinflammatory events [25] may trigger AD-related vascular pathology when combined with age-mediated or vascular risk factor-mediated changes.

In AD and pre-AD states, circulating Aβ may induce chronic, low-level FXII-dependent FXI activation. This is supported by increased FXIIa levels [25] and decreased levels of FXI zymogen and its inhibitor C1inh in AD patient plasma, which could reflect the clearance of the FXIa–C1inh complex following FXII-dependent FXI activation [58]. C1inh depletion in AD plasma may also stem from inhibition of FXIIa and kallikrein, which are activated in the intrinsic coagulation pathway. Our results are in agreement with the decreased levels of C1inh previously found in plasma from individuals with mild cognitive impairment and AD [59,60]. Although our data suggest that FXI consumption is attributable to its activation by FXII, it is possible that some FXI consumption results from feedback activation by thrombin following extrinsic pathway activation.

Chronic FXI activation could mediate the production of low 'idling' levels of thrombin, which may contribute to the chronic formation of fibrin; this is supported by increased fibrin monomer and D-dimer levels in the plasma of AD patients from group 2. Elevated plasma fibrin monomer levels are observed in procoagulant states such as coronary artery disease [61], venous thrombosis [62], and myocardial infarction [63], among others. In AD, chronic thrombin generation could lead to the formation of persistent clots with decreased susceptibility to fibrinolysis, due to Aβ's ability to induce the formation of a fibrinolysis-resistant fibrin network [64]. Indeed, formation of persistent clots is observed in an AD mouse model [7], and increased deposition of fibrin occurs in the brains of AD patients and mouse models [65].

Increased thrombosis in AD would have significant consequences for vascular and neuronal health. Thrombus formation in arterioles and venules decreases blood flow in surrounding capillaries [66,67], limiting oxygen and nutrient supply to neurons. Furthermore, thrombin mediates BBB breakdown [68], and the resulting plasma protein access to the vessel wall and brain parenchyma could contribute to vascular and neuronal damage. Targeting FXII, the intrinsic coagulation pathway, thrombin activity and thrombus formation in AD could therefore provide novel therapeutic opportunities.

Addendum

D. Zamolodchikov designed the study, performed experiments, analyzed data, and wrote the manuscript. T. Renné and S. Strickland designed the study, analyzed data, and participated in manuscript preparation.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

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

Fig. S1. Stability of Aβ42 oligomers.
Fig. S2. Efficacy of the anti-FXIIa function-blocking antibody and active site-inhibited FVII (ASIS).
Fig. S3. Aβ42 fails to trigger thrombin generation in plasma from $FXI^{-/-}$ mice.
Fig. S4. Biotinylated Aβ42 forms oligomers.
Table S1. Characteristics of AD and ND cases from group 1.
Table S2. Characteristics of AD and ND cases from group 2.

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