Targeting Coagulation Factor XII as a Novel Therapeutic Option in Brain Trauma

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Objective: Traumatic brain injury is a major global public health problem for which specific therapeutic interventions are lacking. There is, therefore, a pressing need to identify innovative pathomechanism-based effective therapies for this condition. Thrombus formation in the cerebral microcirculation has been proposed to contribute to secondary brain damage by causing pericontusional ischemia, but previous studies have failed to harness this finding for therapeutic use. The aim of this study was to obtain preclinical evidence supporting the hypothesis that targeting factor XII prevents thrombus formation and has a beneficial effect on outcome after traumatic brain injury.
Methods: We investigated the impact of genetic deficiency of factor XII and acute inhibition of activated factor XII with a single bolus injection of recombinant human albumin-fused infestin-4 (rHA-Infestin-4) on trauma-induced microvascular thrombus formation and the subsequent outcome in 2 mouse models of traumatic brain injury.
Results: Our study showed that both genetic deficiency of factor XII and an inhibition of activated factor XII in mice minimize trauma-induced microvascular thrombus formation and improve outcome, as reflected by better motor function, reduced brain lesion volume, and diminished neurodegeneration. Administration of human factor XII in factor XII–deficient mice fully restored injury-induced microvascular thrombus formation and brain damage.
Interpretation: The robust protective effect of rHA-Infestin-4 points to a novel treatment option that can decrease ischemic injury after traumatic brain injury without increasing bleeding tendencies.

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Traumatic brain injury (TBI) is life-threatening, and is a critical public health problem throughout the world. In Europe, the incidence rate of hospitalized TBI and TBI with fatal outcome is about 235 per 100,000 individuals.[1] Despite numerous clinical trials, attempts to find a safe and effective neuroprotective agent have all failed.[2,3] The primary brain damage that occurs due to an outside force causes irreversible mechanical disruption of the brain tissue. Subsequently, secondary injury processes contribute to the exacerbation of traumatic brain damage. Considering the clinical significance of these secondary injury processes, there is a pressing need to understand them in more detail.

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As well as well-characterized injury processes, such as excitotoxicity, inflammation, and blood–brain barrier damage, thrombus formation in the cerebral microcirculation probably contributes to secondary brain damage by causing pericontusional ischemia and reducing regional cerebral blood flow.[4–10] In clinical TBI, intracerebral vessel occlusion with subsequent ischemia worsens the outcome.[8,9,11,12] However, the potential use of conventional anticoagulants in patients with TBI is met with controversy, due to the increased risk of intracerebral hemorrhage.[13–18] Previous studies from our laboratory have shown that both genetic deficiency and pharmacological inhibition of the coagulation factor XII (FXII), a protease that initiates the activation of the intrinsic coagulation cascade, protect mice from ischemic stroke without increasing bleeding tendencies.[19,20] Therefore, we tested whether deficiency or selective inhibition of FXII has a beneficial effect on trauma-induced microvascular thrombus formation, lesion volume, and functional outcome in TBI. In view of the heterogeneity of TBI,[3,21] we used 2 complementary mouse models. A weight-drop closed-head injury model was used to induce concussion-like TBI with a prominent diffuse brain injury.[22,23] In addition, cortical cryolesion was used to produce a strictly focal cortical brain injury without a contrecoup but with a standardized cortical lesion that enables reliable quantification of well-established pathophysiological readouts after TBI, such as lesion volume and neuronal apoptosis.[24] Our results show that genetic deficiency of FXII in mice results in less thrombus formation and a better outcome in experimental TBI, without increasing the risk of abnormal intracerebral hemorrhage. A similar effect is achieved when mice are treated once after TBI with a selective inhibitor of activated FXII (FXIIa). In alignment with these results, we also found intravascular platelet aggregates in brain tissue from a series of well-characterized patients with TBI.

Materials and Methods

Animals

A total of 148 (134 male and 14 female) C57BL/6N (wild-type) mice, 60 (46 male and 14 female) FXII-deficient ($FXII^{-/-}$) mice,[25] and 8 male FXI-deficient ($FXI^{-/-}$) mice[26] were used in this study. Mice were housed in groups of 5 to 9, with free access to food and water and a 12-hour light/12-hour dark cycle. In this study, all experiments were approved by institutional and regulatory authorities and were conducted in accordance with the EU Directive 2010/63/EU, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and the ARRIVE criteria.[27]

Cortical Cryolesion Model

Cortical cryolesion was induced as described previously.[24] Briefly, 6-week-old mice were anesthetized with intraperitoneal injections of ketamine (0.1mg/g) and xylazine (0.005mg/g). After restraining the mouse head in a stereotactic frame (TSE Systems, Bad Homburg, Germany), surgery was performed on the right parietal cortex, the skull having been exposed through a scalp incision. A copper cylinder with a tip diameter of 2.5mm was filled with liquid nitrogen (−196°C) and placed on the right parietal cortex (coordinates from the bregma: 1.5mm caudal, 1.5mm lateral) for 90 seconds. Sham-operated animals underwent the same surgical procedure without cooling of the copper cylinder.

Weight-Drop Model

Experimental closed-head injury was performed as previously described.[22,23] Briefly, after the induction of isoflurane anesthesia, spontaneously breathing 10- to 16-week-old mice were placed in a stereotactic frame and the skulls were exposed by a midline longitudinal scalp incision. After identification of the impact area, a weight with a silicone-covered blunt tip was dropped with a final impact of 0.01J. Sham operation included anesthesia and exposure of the skull, but without weight-drop injury. The neurobehavioral status of the mice was assessed using the Neurological Severity Score (NSS),[22,23] a composite score including tasks on motor function, alertness, and physiological behavior, with lower scores indicating less deficit. Mice were evaluated 1 hours, 1 day, 3 days, and 7 days after weight-drop injury. The personnel who performed the functional assays were blinded to the experimental groups.

Pharmacological Treatment

One hour after induction of focal cryolesion or diffuse weight-drop injury, wild-type mice received a single intravenous injection of the specific FXII inhibitor recombinant human albumin-fused infestin-4 (rHA-Infestin-4; CSL Behring, Marburg, Germany) at a dose of 200mg/kg body weight.[20] Control animals received equal volumes of 0.9% sodium chloride (vehicle). Intravenous injection of 2μg/g body weight human FXII (hFXII; ADG412H; Sekisui Diagnostics, Lexington, MA), or an hFXII variant that cannot be activated due to modifications in the activation domain (hFXII-3xAla; CSL Limited, Parkville, Victoria, Australia), administered 1 hour after injury induction and continually every 72 hours, resulted in reconstitution of $FXII^{-/-}$ mice.

Determination of Lesion Size after Cortical Cryolesion

Twenty-four hours or 3 days after cryolesion, mice were sacrificed; the brains were quickly removed and cut into five 1-mm-thick coronal sections using a mouse brain slice matrix (Harvard Apparatus, Holliston, MA). The slices were stained for 20 minutes at 37°C with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline to visualize the lesion. Lesion volumes were calculated by

volumetry (ImageJ; National Institutes of Health, Bethesda, MD) in a blinded fashion.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed repeatedly 1 and 7 days after cryolesion on a 3T unit (Vision; Siemens Healthcare, Erlangen, Germany) under anesthesia with ketamine (0.1mg/g) and xylazine (0.005mg/g). The protocol included a coronal T2-weighted sequence (slice thickness = 2mm) and a blood-sensitive coronal T2-weighted gradient-echo constructed interference in steady state (CISS) sequence (slice thickness = 1mm). Lesion volumes were calculated by planimetry of the hyperintense area on high-resolution CISS images. CISS images were additionally examined for possible intracerebral hemorrhage. To specifically analyze bleeding events after weight drop, MRI was performed repeatedly 1 and 7 days after weight drop, using a blood-sensitive coronal T2-weighted gradient-echo CISS sequence (slice thickness = 1mm) and a susceptibility-weighted imaging sequence, which is particularly suitable for the detection of blood.

Laser Doppler Flowmetry

Laser Doppler flowmetry (Moor Instruments, Axminster, UK) was used to monitor regional cerebral blood flow over the right parietal cortex (impact area). Cerebral blood flow was measured serially at baseline (before injury induction) and 1 hour, 3 days, and 7 days after injury induction.

Histology and Immunohistochemistry

Cryoembedded mouse brains were cut into 10-μm-thick or 15-μm-thick slices (cortical cryolesion model and weight-drop model, respectively) using a cryostat (Leica Biosystems, Wetzlar, Germany). For assessment of the thrombosis index, hematoxylin and eosin staining was performed according to standard procedures. Stainings were examined in a blinded fashion, and the numbers of occluded (N_{occ}) and nonoccluded (N_{open}) blood vessels within the lesioned hemispheres were counted in every 10th brain slice under a 20-fold magnification. The thrombosis index was calculated using the following equation: $(N_{occ}/[N_{open} + N_{occ}]) \times 100$. For immunofluorescence staining, the following primary antibodies were applied: anti–glycoprotein Ib (GPIb; 1:100; EMFRET Analytics, Eibelstadt, Germany), anti-CD31 (MCA2388GA, 1:100; Bio-Rad, Hercules, CA), and anti-NeuN (MAB377, 1:1,000; Millipore, Billerica, MA). As secondary antibodies, Cy2 anit-rat (122-225-167, 1:100; Dianova, Hamburg, Germany), Cy3 anti-rat (712-165-150, 1:100, Dianova), and DyLight 488 anti-mouse (ab96871, 1:100; Abcam, Cambridge, MA) were used. Neuronal apoptosis was assessed using a TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) in situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The number of apoptotic neurons was determined from 3 perilesional fields at a 40-fold magnification from the lesioned hemisphere of 2 brain slices under a Nikon Eclipse 50i microscope equipped with a DS-U3 DS camera control unit and NIS-Elements software (Nikon, Düsseldorf, Germany). To assess platelet aggregates, 2 brain slices per animal were quantified. For quantitative analysis, we used sections from near-identical brain regions for better comparison between groups.

Human Brain Tissue

To assess platelet aggregates within vessels in the human brain, paraffin-embedded sections of brain samples from 10 male patients who underwent decompressive surgery and surgical removal of focal brain contusions following TBI and from 1 postmortem case of a female patient after a fatal TBI were stained against GPIb (bs-2347R; Bioss Antibodies, Woburn, MA) according to the manufacturer's protocol and then counterstained with hematoxylin to visualize nuclei. Control brain tissue was analyzed postmortem, obtained from 9 individuals (7 males, 2 females) without known central nervous system disorders and who were dying from unrelated causes. We obtained human brain samples from the Uppsala Biobank/archived sample collection at the Department of Pathology, University of Uppsala, and from the collection at the Department of Neuropathology, Institute of Pathology, University of Würzburg. Informed consent was obtained from each subject or their legal representative, and all clinical research procedures were approved by the regional ethical review board. Stainings were examined in a blinded fashion and the number of blood vessels occluded by platelet accumulations (N_{pa}) and nonoccluded blood vessels (N_{open}) within 1 slice was counted under 40-fold magnification. The percentage of vessels occluded by platelet aggregates was calculated using the following equation: $(N_{pa}/[N_{open} + N_{pa}]) \times 100$.

Western Blot Analysis

Immunoreactivity for GPIb (anti-GPIb, 1:500, EMFRET Analytics) in lesioned cortices was detected by Western blot analysis as previously described.[24] Densitometric analysis of GPIb was performed in a blinded way using ImageJ software with β-actin (A5441, 1:500,000; Dianova) as loading control to normalize the levels of GPIb detected.

Spectrophotometric Hemoglobin Assay

The hemoglobin concentration in the brain parenchyma, which correlates with the extent of hemorrhage, was determined spectrophotometrically.[28] Twenty-four hours after trauma, animals were sacrificed and the brains were removed. The lesioned hemispheres were sonified for 60 seconds in 1.5ml ice-cooled water and afterward centrifuged at 4°C for 30 minutes. Drabkin solution (1ml) was added to 250μl of the supernatant and incubated at room temperature for 15 minutes. The absorbance was measured at 540nm (MultiskanEX; Thermo Scientific, Waltham, MA).

Experimental Design

The numbers of animals necessary to detect a standardized effect size on lesion volumes ≥ 0.2 on day 1 after cortical cryolesion or NSS ≥ 0.2 on day 1 after weight-drop injury, respectively, were determined via a priori sample size calculation with the following assumptions: $\alpha = 0.05$, $\beta = 0.2$, mean, and standard deviation (G*Power 3.0.10). Mice were randomly assigned to treatment groups (block randomization after cryolesion and, to achieve balanced groups, stratified randomization

TABLE 1. Patient Characteristics

Patient	Age, yr	Sex	Severe Diseases/Injuries	Cause of Death (in case of autopsy)	Origin of Brain Tissue
#1	65	M	TBI, facial fracture	—	Surgery
#2	50	M	TBI	—	Surgery
#3	58	M	TBI, radial fracture	—	Surgery
#4	52	M	TBI	—	Surgery
#5	25	M	TBI	—	Surgery
#6	51	M	TBI	—	Surgery
#7	48	M	TBI	—	Surgery
#8	68	M	TBI	—	Surgery
#9	52	M	TBI	—	Surgery
#10	25	M	TBI	—	Surgery
#11	43	F	TBI	TBI	Autopsy
#12	63	M	Acute myeloid leukemia	Sepsis	Autopsy
#13	62	F	Cardiac failure	Multiorgan failure	Autopsy
#14	50	M	—	Pulmonary dysfunction	Autopsy
#15	43	M	—	Pancreatitis	Autopsy
#16	69	M	—	N/A	Autopsy
#17	52	M	—	Anterior myocardial infarction	Autopsy
#18	56	M	—	Epilepsy?	Autopsy
#19	22	M	—	N/A	Autopsy
#20	45	F	—	N/A	Autopsy

Human brain samples were analyzed for the presence of intracerebral glycoprotein Ib–positive platelet accumulations.
F = female; M = male; N/A = not available; TBI = traumatic brain injury.

after weight-drop injury). To avoid bias, experiments were performed and analyzed in a blinded fashion.

Statistical Analysis

All results were expressed as mean ± standard error of the mean except for the NSS scales, which are depicted as scatter plots, including median with the 25% percentile and the 75% percentile given in brackets in the text. For statistical analysis, the PrismGraph 5.0 software package (GraphPad, La Jolla, CA) was used. Data were tested for Gaussian distribution with the Kolmogorov–Smirnov test and, in the case of measuring the effects of 2 factors simultaneously, analyzed by 2-way analysis of variance (ANOVA) with post hoc Bonferroni correction for multivariate analyses or, in the case of nonparametric data (NSS), the Kruskal–Wallis test with post hoc Dunn correction. In the case of measuring the effect of 1 factor, 1-way ANOVA with post hoc Bonferroni correction was applied. If only 2 groups were compared, an unpaired, 2-tailed Student t test was performed. Probability values < 0.05 were considered to be statistically significant.

Results

Microvascular Thrombosis Is a Common Pathological Feature in TBI in Humans and Mice

First, we analyzed 20 human brain samples (Table 1) obtained from patients with TBI or control patients, and found significantly more microvascular platelet accumulations in the brain tissue of TBI cases than in control cases (Fig 1, $p < 0.01$). Consequently, we tested whether similar pathophysiological changes can be detected in experimental TBI in mice using a weight-drop model resulting in a predominantly diffuse brain trauma. Intravascular accumulations of GPIb-positive platelets were found significantly more often in the brain tissue of wild-type mice subjected to weight-drop compared with sham-treated mice. In the purely focal brain cortical cryolesion, numerous occluded vessels and intravascular accumulations of GPIb-positive platelets were also found in the

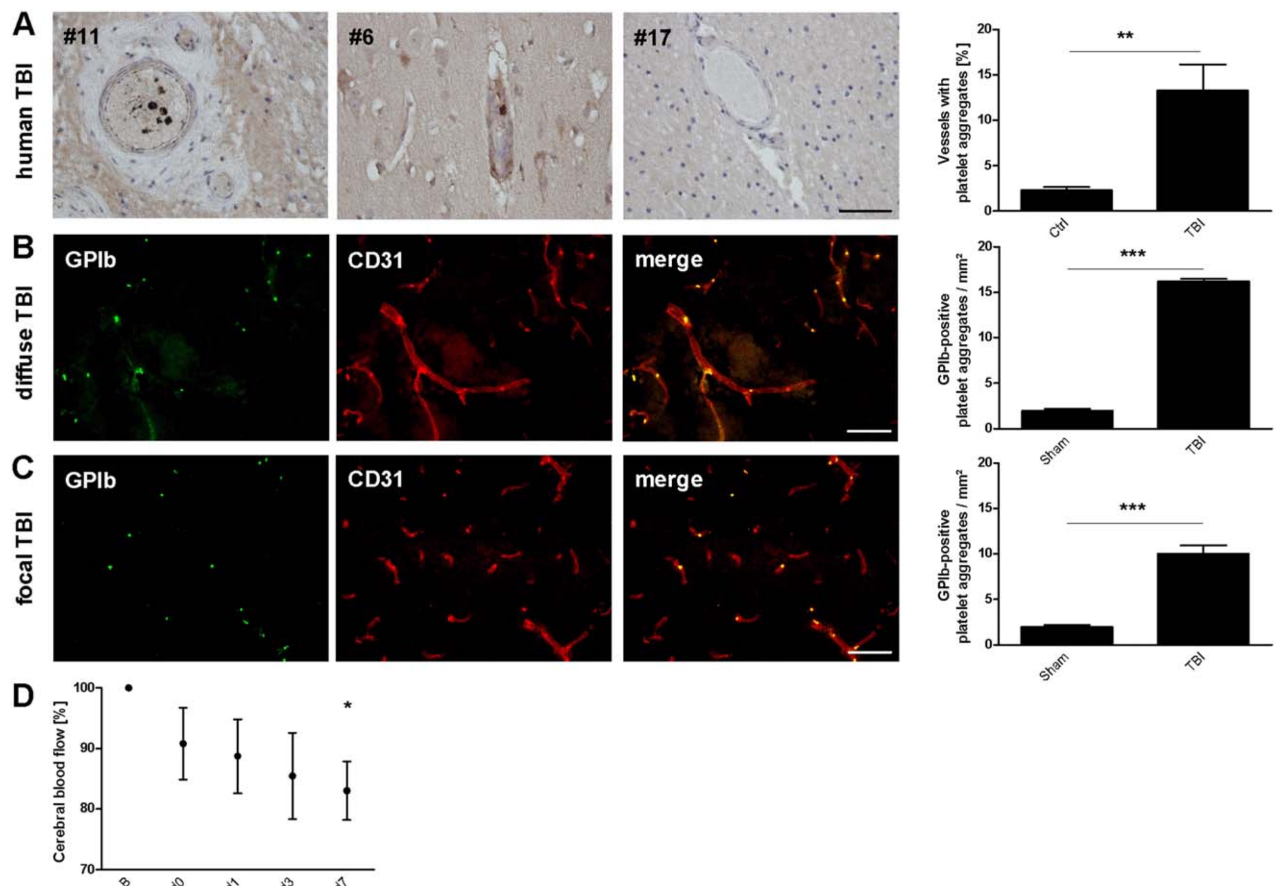

FIGURE 1: Intracerebral platelet accumulation and thrombosis are pathological features of traumatic brain injury (TBI). (A) The left panel shows representative immunohistological stainings for the platelet marker glycoprotein Ib (GPIb) of human brain tissue (patients with TBI: #6 and #11; control patient: #17; scale bar represents 100μm). Quantitative analysis of immunohistological stainings using GPIb (right panel) reveals that significantly more intravascular platelet depositions are found in human brain tissue of patients with TBI (n = 11) compared with control (Ctrl) patients (n = 9; **$p < 0.01$). (B) The left panel shows representative immunofluorescence staining for the platelet marker GPIb and CD31, which is found on the surface of platelets and endothelial cells, from a mouse brain section on day 7 after weight-drop injury (scale bar represents 100μm). Quantitative analysis of immunohistological stainings using GPIb (right panel) reveals that significantly more intravascular platelet depositions are found in mouse brain sections of mice on day 7 after weight-drop injury (n = 4) compared with sham-treated mice (Sham; n = 3; ***$p < 0.001$). (C) The left panel shows representative immunofluorescence staining for the platelet marker GPIb and CD31 from a mouse brain section on day 1 after cryolesion (scale bar represents 100μm). Quantitative analysis of immunohistological stainings using GPIb (right panel) reveals that significantly more intravascular platelet depositions are found in mouse brain sections of mice on day 1 after cryolesion (n = 5) compared with sham-treated mice (Sham; n = 3; ***$p < 0.001$). (D) The cerebral blood flow over the right parietal cortex (impact area) decreases significantly over 7 days after weight-drop injury (n = 5; *$p < 0.05$). B = baseline; d = day.

perilesional brain tissue on days 1 and 3 after injury induction. Interestingly, after weight-drop injury the cerebral blood flow at the impact area slightly decreased over time with significantly reduced cerebral blood flow on day 7. These results strongly support the hypothesis that microvascular thrombosis is a common pathological feature in TBI.

FXII Contributes to Microvascular Thrombosis in TBI

To assess the impact of FXII on intracerebral thrombus formation after TBI, we first analyzed contusioned brain tissue of FXII$^{-/-}$ mice in comparison with wild-type mice or FXII$^{-/-}$ mice that were reconstituted with intravenous injections of hFXII (FXII$^{-/-}$/hFXII) or with intravenous injections of hFXII-3xAla (FXII$^{-/-}$/hFXII-3xAla), a hFXII variant that possesses no anticoagulant activity due to a mutation in its activation domain. On day 7 after weight-drop injury, histological analysis of brain sections stained with hematoxylin and eosin demonstrated fewer occluded cerebral microvessels in FXII$^{-/-}$ and FXII$^{-/-}$/hFXII-3xAla mice compared with wild-type or FXII$^{-/-}$/hFXII mice (Fig 2B). We consistently detected less intravascular GPIb-positive platelet accumulation in the brains of FXII$^{-/-}$ and FXII$^{-/-}$/hFXII-3xAla mice (see Fig 2A).

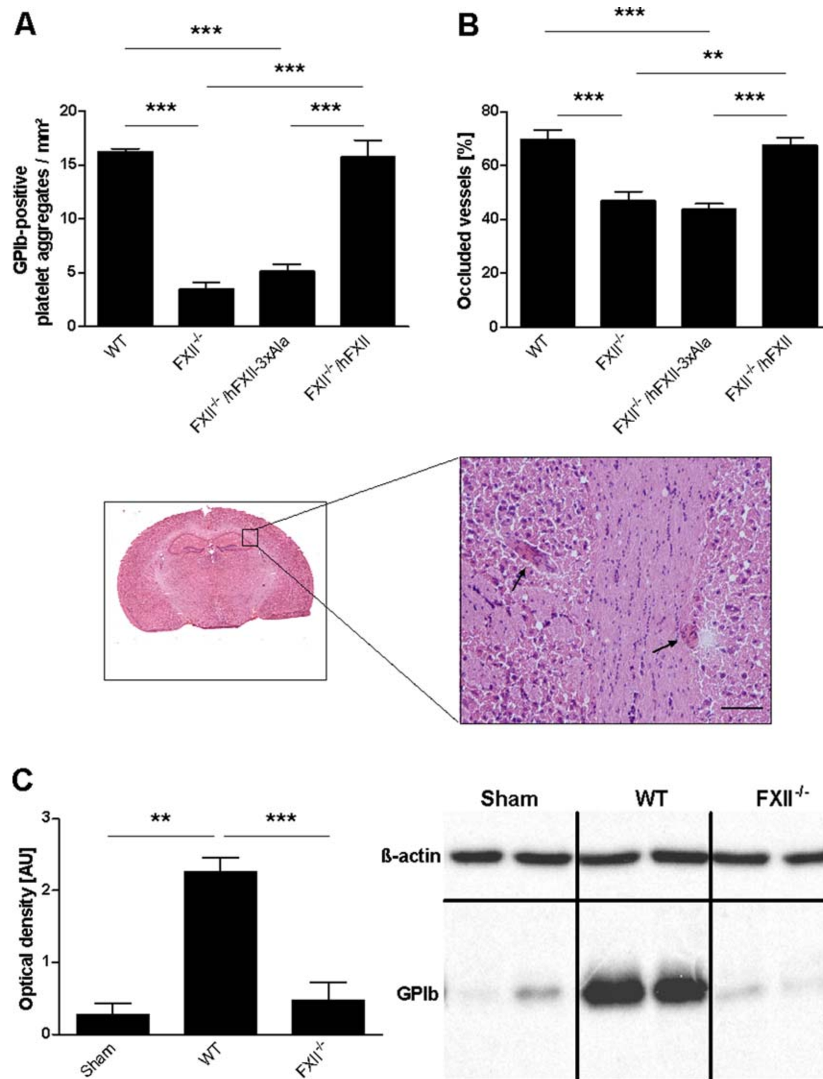

FIGURE 2: Intracerebral thrombosis after weight-drop injury is diminished in factor XII (FXII)$^{-/-}$ mice. (A) Analysis of immunofluorescence stainings using glycoprotein Ib (GPIb) and CD31 antibodies reveals a significant reduction in intravascular platelet depositions on day 7 after injury induction in FXII$^{-/-}$ mice and FXII$^{-/-}$ mice reconstituted with human FXII–3xAla (FXII$^{-/-}$/hFXII-3xAla) compared with wild-type (WT) and FXII$^{-/-}$ mice reconstituted with hFXII (FXII$^{-/-}$/hFXII; n = 4 per group; ***$p < 0.001$). (B) Calculation of the thrombosis index from brain sections stained with hematoxylin and eosin shows that the number of thrombotic vessels is decreased in FXII$^{-/-}$ and FXII$^{-/-}$/hFXII-3xAla mice compared with WT and FXII$^{-/-}$/hFXII mice (n = 5 per group; **$p < 0.01$, ***$p < 0.001$). The lower panel shows a representative hematoxylin and eosin staining from a mouse brain section of the lesioned hemisphere on day 7 after weight-drop injury with numerous small vessels that are occluded by a thrombus (*arrows;* scale bar represents 100μm). (C) Western blot analysis using a GPIb antibody confirms that platelets accumulate to a lesser extent in FXII$^{-/-}$ mice compared with WT controls. Bands were quantified by densitometry in relation to β-actin control. The right panel shows 2 representative blots of each group (n = 5 per group; **$p < 0.01$, ***$p < 0.001$). AU = arbitrary units.

Furthermore, Western blot analyses confirmed that the number of platelets was significantly diminished in the brain tissue of FXII$^{-/-}$ mice (see Fig 2C). Similar to weight-drop injury, fewer thrombus-occluded brain vessels (Fig 3A), a decreased number of platelets in the brain tissue (see Fig 3B), and less platelet accumulation in the brain vasculature (see Fig 3C) were detected 1 and 3 days after focal cryolesion in FXII$^{-/-}$ mice compared with wild-type mice. Consequently, we conclude that FXII contributes to microvascular thrombosis independently of the nature of TBI.

FXII Deficiency Results in a Better Outcome after TBI

To evaluate the pathological significance of reduced intracerebral thrombosis in FXII$^{-/-}$ mice, we next determined the impact of FXII deficiency on functional outcome after weight-drop injury. Intravascular thrombosis after experimental TBI in rodents increases within the first 3 days.[29] Accordingly, trauma severity at the early stages (1 hour and 1 day after injury induction) was comparable in all groups, whereas FXII$^{-/-}$ mice and FXII$^{-/-}$/hFXII-3xAla mice had recovered significantly

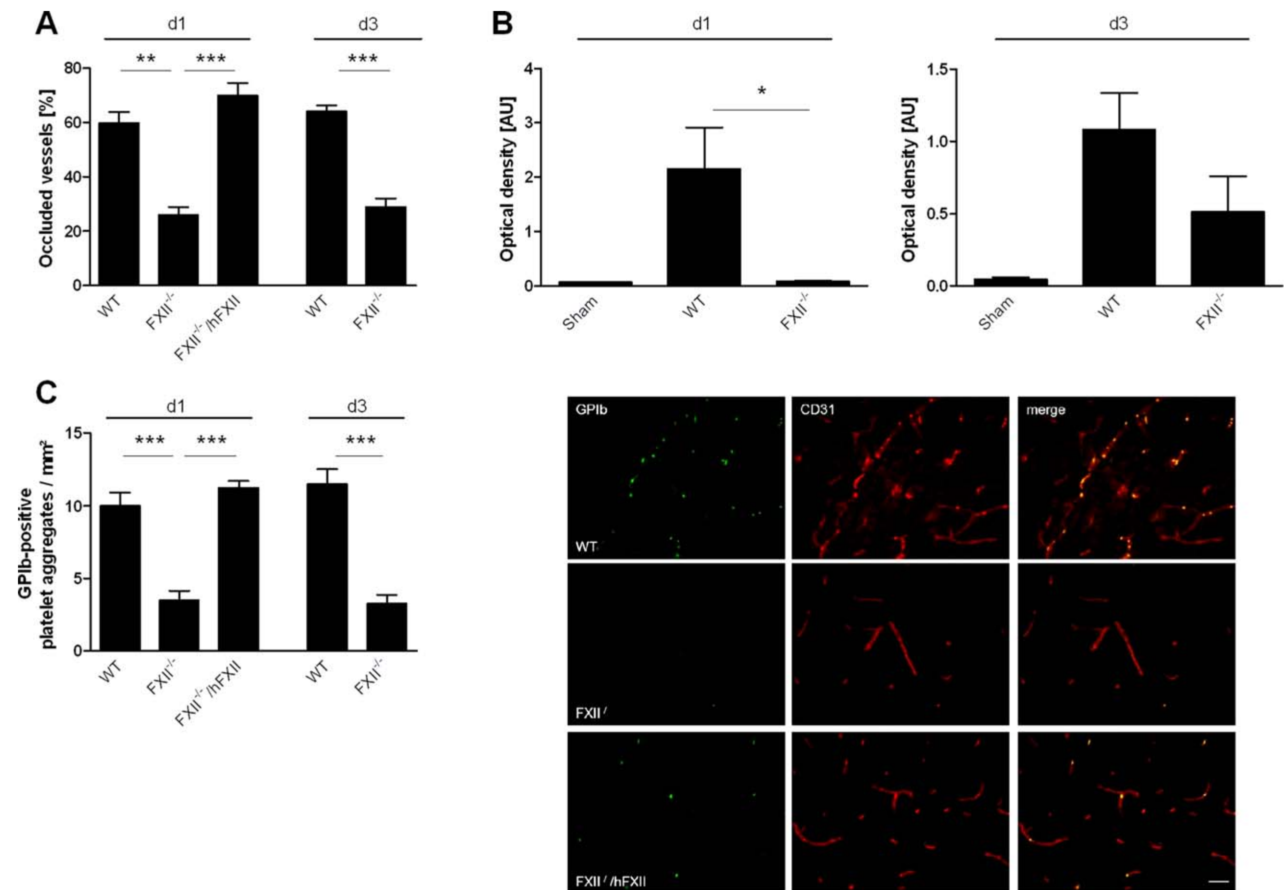

FIGURE 3: Intracerebral platelet accumulation on day 1 (d1) and d3 after cryolesion is diminished in factor XII (FXII)$^{-/-}$ mice. (A) Calculation of the thrombosis index from brain sections stained with hematoxylin and eosin shows that the number of thrombotic vessels is decreased in FXII$^{-/-}$ mice compared with wild-type (WT) and FXII$^{-/-}$ mice reconstituted with human FXII (FXII$^{-/-}$/hFXII; n = 5 per group; ** $p < 0.01$, *** $p < 0.001$). (B) Western blot analysis using a glycoprotein Ib (GPIb) antibody confirms that platelets accumulate to a lesser extent in FXII$^{-/-}$ mice compared with WT controls. Bands were quantified by densitometry in relation to β-actin control (n = 5 per group; * $p < 0.05$). (C) Analysis of immunofluorescence staining using GPIb and CD31 antibodies reveals a marked reduction in intravascular platelet depositions in FXII$^{-/-}$ mice compared with WT and FXII$^{-/-}$/hFXII mice. The right panel shows representative images (n = 4 per group; *** $p < 0.001$; scale bar represents 50μm).

better than wild-type or FXII$^{-/-}$/hFXII mice 3 days after weight-drop injury (median NSS [25th percentile, 75th percentile]: 4.0 [3.0, 4.0] in wild-type mice, 3.5 [3.0, 4.0] in FXII$^{-/-}$/hFXII mice, 2.0 [1.0, 3.0] in FXII$^{-/-}$ mice, 2.0 [1.0, 1.0] in FXII$^{-/-}$/hFXII-3xAla mice; Fig 4). Importantly, the better neurological outcome in FXII$^{-/-}$ mice and FXII$^{-/-}$/hFXII-3xAla mice was persistent until day 7 (median NSS [25th percentile, 75th percentile]: 3.0 [2.0, 3.0] in wild-type mice, 2.5 [2.0, 3.0] in FXII$^{-/-}$/hFXII mice, 1.0 [1.0, 2.5.0] in FXII$^{-/-}$ mice, 2.0 [1.0, 1.0] in FXII$^{-/-}$/hFXII-3xAla mice; see Fig 4).

We next evaluated the impact of FXII deficiency on cortical lesion volume and neurodegeneration on days 1 and 3 after cryolesion. In male mice, FXII deficiency resulted in significantly reduced lesion volumes on days 1 and 3, as assessed by TTC staining of brain sections (Fig 5). As gender might have a significant impact on the clinical outcome following TBI,[30] we subjected female mice to cryolesion. Similar to male mice, FXII deficiency in female mice resulted in significantly smaller brain lesions on days 1 and 3 compared with wild-type mice. These observations were corroborated by studies using brain MRI, showing that FXII deficiency resulted in sustained reduction in lesion size after focal brain injury. Reduction in lesion volume was accompanied by significantly diminished neuronal apoptosis in FXII$^{-/-}$ mice on day 1 after cryolesion compared with control mice. An even more pronounced difference in the number of apoptotic cells was observed on day 3 after cryolesion.

Pharmacological Inhibition of FXIIa Results in Reduced Microvascular Thrombosis and a Better Outcome after TBI

To test the efficacy of pharmacological FXIIa inhibition for the treatment of pathological thrombosis in TBI, we administered rHA-Infestin-4, a selective inhibitor of

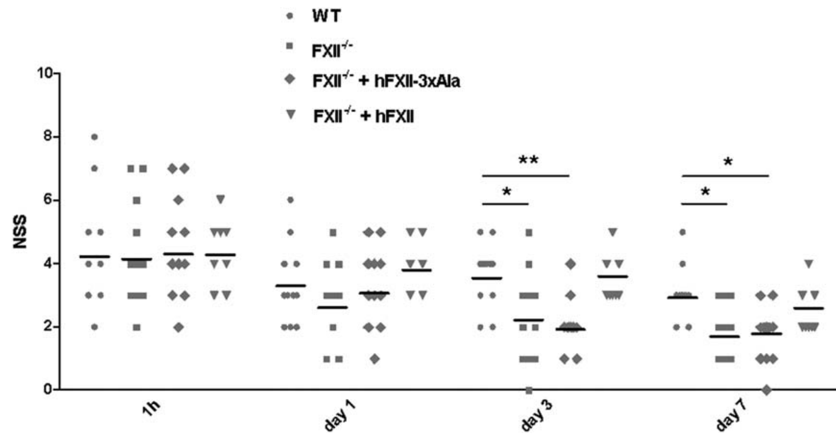

FIGURE 4: Factor XII (FXII) deficiency improves functional outcome after weight-drop injury. FXII$^{-/-}$ and FXII$^{-/-}$ mice reconstituted with human FXII–3xAla (FXII$^{-/-}$ + hFXII-3xAla) had a significantly lower Neurological Severity Score (NSS) than wild-type (WT) and FXII$^{-/-}$ mice reconstituted with hFXII (FXII$^{-/-}$ + hFXII) on days 3 and 7 after diffuse brain trauma. At 1 hour (day 0) and 1 day after trauma, animals had similar neurological deficits (n = 10–13 per group; *$p < 0.05$, **$p < 0.01$).

FXIIa,[20] at a dosage of 200mg/kg body weight intravenously and monitored microvascular thrombosis, functional outcome, and lesion volumes in mice subjected to weight-drop injury or cryolesion. Detailed analysis of brain sections stained with hematoxylin and eosin and immunohistochemistry visualizing platelets and endothelium

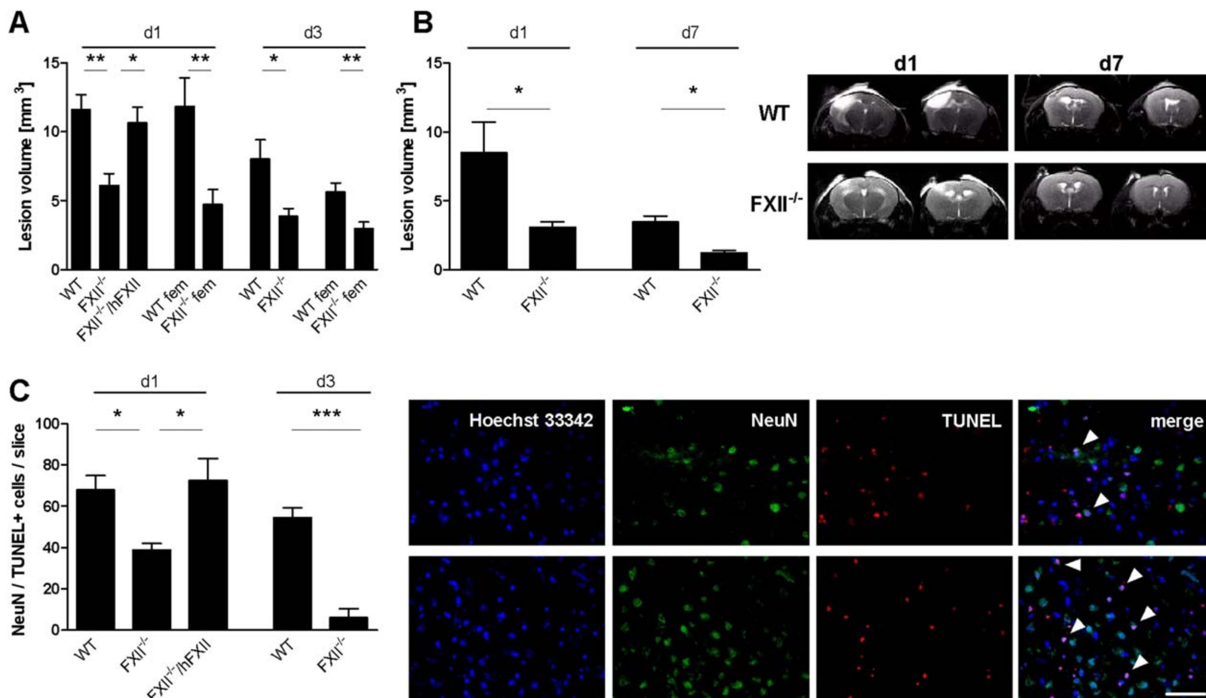

FIGURE 5: Factor XII (FXII) deficiency improves outcome after cortical cryolesion. (A) Lesion volumetry after 2,3,5-triphenyltetrazolium chloride staining of brain sections from male wild-type (WT) mice, FXII$^{-/-}$ mice, FXII$^{-/-}$ mice reconstituted with human FXII (FXII$^{-/-}$/hFXII), female wild-type (WT fem) mice, and female FXII$^{-/-}$ (FXII$^{-/-}$ fem) mice was performed on day 1 (d1) and d3 after focal brain trauma. Male and female FXII$^{-/-}$ mice show significantly reduced lesion volumes compared with WT mice. The beneficial effect of FXII deficiency can be reverted by administration of hFXII (n = 7 per group; *$p < 0.05$, **$p < 0.01$). (B) Serial coronal T2-weighted gradient-echo magnetic resonance images show hyperintense lesions on d1 and d7 after trauma induction in WT and FXII$^{-/-}$ mice. Hypointense areas indicating intracerebral hemorrhage are absent in both groups. The right panel shows 2 representative brain slices per group and time point. Magnetic resonance imaging–based lesion volumetry (left) confirms smaller lesions in FXII$^{-/-}$ mice (n = 8 or 9 per group; *$p < 0.05$). (C) Neuronal apoptosis is reduced in FXII$^{-/-}$ mice. The right panel shows representative brain sections from WT mice and FXII$^{-/-}$ mice on d1 after focal brain trauma, immunolabeled for the neuronal marker NeuN and subjected to TUNEL assay to detect apoptosis. Hoechst 33342 staining depicts cell nuclei. The left panel shows the number of TUNEL-positive neurons per brain slice in the injured hemisphere on d1 and on d3. The number of apoptotic neurons is significantly reduced in FXII$^{-/-}$ mice when compared with WT controls and FXII$^{-/-}$/hFXII mice (n = 4 per group; *$p < 0.05$, ***$p < 0.001$; scale bar represents 50μm).

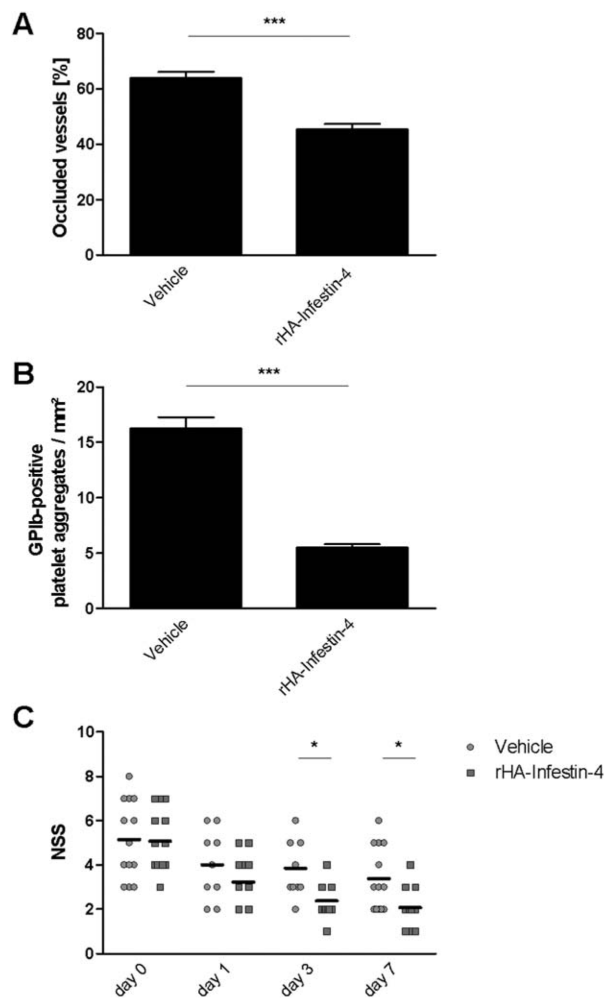

FIGURE 6: Pharmacological inhibition of activated factor XII (FXIIa) with rHA-Infestin-4 reduces intracerebral thrombosis and improves outcome after weight-drop brain trauma. (A) Calculating the thrombosis index from brain sections stained with hematoxylin and eosin on day 7 showed a highly significant decrease of occluded vessels in mice treated with rHA-Infestin-4 (n = 5 per group; ******p* **< 0.001). (B) Analysis of immunofluorescence staining using glycoprotein Ib (GPIb) and CD31 antibodies reveals marked reduction in intravascular platelet depositions on day 7 after injury induction in mice treated with rHA-Infestin-4 (n = 4 per group; ******p* **< 0.001). (C) Mice treated with rHA-Infestin-4 had a significantly lower Neurological Severity Score (NSS) than vehicle-treated (Vehicle) mice on days 3 and 7 after brain trauma. At 1 hour (day 0) and 1 day after brain trauma, animals had similar neurological deficits (n = 13 per group; ****p* **< 0.05).

showed that fewer thrombi occluded cerebral microvessels in rHA-Infestin-4–treated mice compared with vehicle-treated mice in both TBI models (Figs 6A, B and 7A, B).

The diminished thrombus formation in rHA-Infestin-4–treated mice was associated with better neurological outcome. Although the initial severity of neurological deficits (1 hour and day 1) after weight-drop injury was comparable between the treatment groups ($p > 0.05$), rHA-Infestin-4–treated mice had recovered significantly better than vehicle-treated mice 3 and 7 days after trauma (day 3: median NSS [25th percentile, 75th percentile]: 2.0 [2.0, 3.0] in rHA-Infestin-4–treated mice vs 3.0 [3.0, 3.0] in vehicle-treated mice, $p < 0.05$; day 7: median NSS [25th percentile, 75th percentile]: 2.0 [1.5, 2.5] in rHA-Infestin-4–treated mice vs 3.0 [2.0, 5.0] in vehicle-treated mice, $p < 0.05$; see Fig 6C). After cryolesion, lesion volume and neurodegeneration on days 1 and 3 were reduced in rHA-Infestin-4–treated mice compared with vehicle-treated mice. For both readouts, the protective effect of rHA-Infestin-4 was even more pronounced on day 3 than on day 1 after cryolesion (see Fig 7C, D). In summary, pharmacological inhibition of FXIIa results in reduced microvascular thrombosis and a better outcome after experimental TBI.

FXII Deficiency or Inhibition Does Not Increase the Risk of Intracerebral Hemorrhage

To prove whether blockade of FXII activity may be associated with an increased risk for cerebral hemorrhage after brain trauma, we used repeated MRI monitoring on days 1 and 7 after weight-drop injury. These analyses using bleeding-sensitive MRI sequences detected no signs of hemorrhages in the contusioned brain parenchyma of $FXII^{-/-}$ mice (Table 2, Fig 8). In contrast, bleedings were evident in the traumatized brain tissue of mice deficient for FXI, the primary substrate of FXIIa in the intrinsic coagulation cascade, which is in line with the observation that $FXI^{-/-}$ humans have a mild to moderate bleeding phenotype.[31] The $FXI^{-/-}$ mice also showed increased hemoglobin content in the lesioned brain hemispheres after cryolesion, whereas no increase was detected in the brain tissue of $FXII^{-/-}$ or wild-type mice treated with rHA-Infestin-4 compared with sham-operated control mice. In accordance, MRI hypointense areas in brain tissue, indicating intracerebral hemorrhage after focal cryolesion, were absent in both wild-type and $FXII^{-/-}$ mice (see Fig 5B).

Discussion

In view of the heterogeneous nature of TBI, we used 2 complementary mouse models to show that specific inhibition of FXIIa improves outcome after TBI in mice by reducing intracerebral thrombosis, but without increasing the risk of abnormal hemorrhage. In patients with TBI, anticoagulant treatment is met with controversy. Some studies support a net beneficial effect of anticoagulation following TBI achieved by reducing the risk of thromboembolic events[13,32] and improving outcome parameters,[16,33] whereas other studies report a detrimental effect of anticoagulation[17] due to an increased risk of

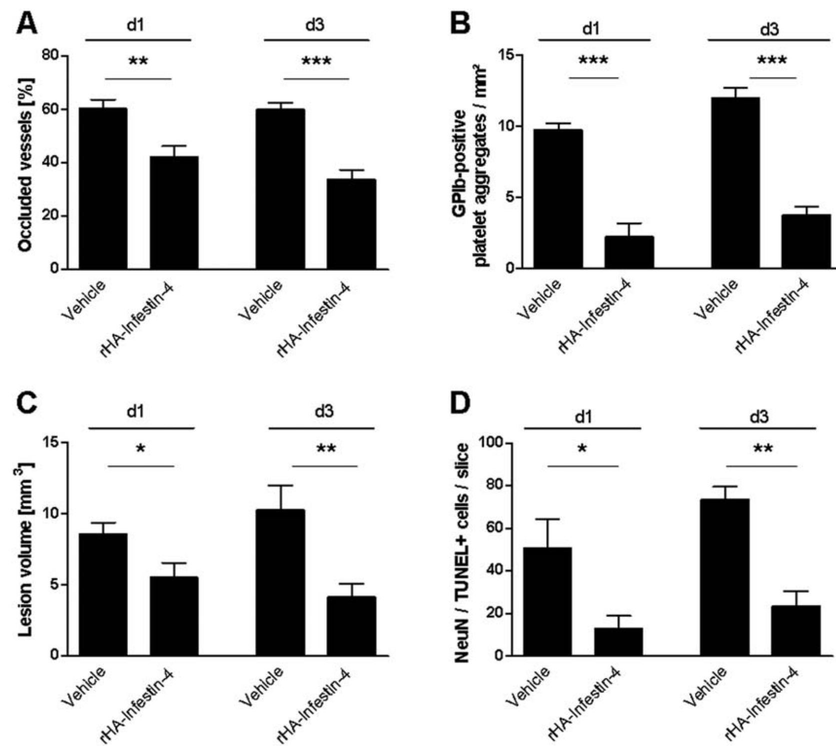

FIGURE 7: Pharmacological inhibition of activated factor XII (FXIIa) with rHA-Infestin-4 reduces intracerebral thrombosis and provides protection from focal brain trauma. (A) Occluded vessels are more abundant in vehicle-treated animals (Vehicle) compared with rHA-Infestin-4–treated mice, as determined by calculating the thrombosis index on day 1 (d1) and on d3 after injury induction (n = 4 per group; **p < 0.01, ***p < 0.001). (B) Analysis of immunofluorescence stainings using glycoprotein Ib (GPIb) and CD31 antibodies reveals a marked reduction in intravascular platelet aggregation on d1 and d3 after injury induction in mice treated with rHA-Infestin-4 (n = 4 per group; ***p < 0.001). (C) Lesion volumetry after 2,3,5-triphenyltetrazolium chloride staining of brain sections of vehicle-treated mice and mice treated with rHA-Infestin-4 was performed on d1 and d3 after focal brain trauma. Mice treated with rHA-Infestin-4 are substantially protected from brain trauma as indicated by the reduction in lesion volume (n = 7 per group; *p < 0.05, **p < 0.01). (D) The number of TUNEL-positive neurons per brain slice was assessed after immunolabeling for the neuronal marker NeuN and subjected to TUNEL assay to detect apoptosis. The number of apoptotic neurons is significantly diminished in rHA-Infestin-4–treated mice compared with vehicle-treated mice on d1 and d3 (n = 4 per group; *p < 0.05, **p < 0.01).

intracranial hemorrhage that also occurs frequently after TBI. In this context, FXII could be an interesting target for anticoagulant treatment that is devoid of hemorrhagic risk.

TABLE 2. Contingency Table

	No Bleedings	Bleedings	Row Totals
Wild-type	5	0	5
FXII$^{-/-}$	5	0	5
FXI$^{-/-}$	1	2	3
Column totals	11	2	13 (grand total)

Bleeding tendencies after weight-drop injury in wild-type, factor XII (FXII)-deficient, and FXI-deficient mice were analyzed using bleeding-sensitive magnetic resonance imaging sequences.

For decades, FXII has been considered to have no coagulation function in vivo. In recent years, however, it has been shown that FXII$^{-/-}$ mice are resistant to experimentally induced thrombosis[34,35] and ischemic brain injury.[19] As deficiency of FXII is not associated with abnormal hemorrhaging from injury sites (hemostasis) either in patients or in animals,[19,34] inhibition of activated FXII prevented arterial thrombosis and ischemic brain injury[20,36] without affecting hemostasis.[20,37] Our current study shows that FXII is a promising antithrombotic target for the acute treatment of TBI, without increasing the risk of intracranial hemorrhage.

Our results confirm that thrombus formation in the cerebral microvasculature is a common pathological feature in TBI. We observed thrombus formation in the cerebral vessels of patients with TBI but not in control patients. A similar pattern of pathological thrombus formation in cerebral microvessels in human TBI was also detected in 2 experimental models of brain injury. When we evaluated the impact of FXII deficiency, we found

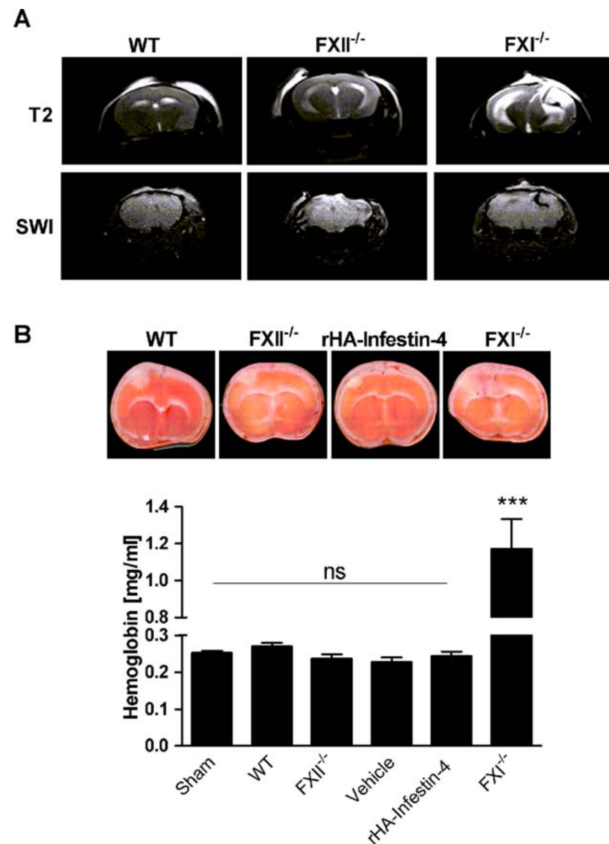

FIGURE 8: Genetic deficiency or pharmacological inhibition of factor XII (FXII) does not increase the risk of intracranial hemorrhage. (A) Representative magnetic resonance images from T2-weighted gradient-echo constructed interference in steady state and susceptibility-weighted imaging sequences on day 1 after weight-drop. No bleedings were observed in wild-type (WT) mice and $FXII^{-/-}$ mice (n = 5/group), whereas $FXI^{-/-}$ mice (n = 3) showed parenchymal bleedings. (B) The upper panel shows representative brain slices stained with 2,3,5-triphenyltetrazolium chloride from sham-operated mice, $FXII^{-/-}$ mice, mice treated with rHA-Infestin-4, and $FXI^{-/-}$ mice after cortical cryolesion. The lower panel shows the concentration of hemoglobin in the lesioned hemispheres of sham-operated mice (Sham), WT mice, $FXII^{-/-}$ mice, vehicle-treated (Vehicle) mice, mice treated with rHA-Infestin-4, and $FXI^{-/-}$ mice 1 day after trauma induction. Hemoglobin concentrations in the groups with FXII inhibition remain at the level of sham-operated animals; $FXI^{-/-}$ mice show highly significantly increased amounts of hemoglobin (n = 4 or 5 per group; ***$p < 0.001$). ns = not significant.

significantly fewer thrombi in the cerebral microvessels of $FXII^{-/-}$ mice in comparison with wild-type mice. Corroborating these data, platelet accumulation was less in injured brains from $FXII^{-/-}$ mice compared with wild-type mice. Importantly, we further observed that the injury-induced microvascular thrombosis, brain damage, and functional deficits could be recovered in $FXII^{-/-}$ mice by the administration of hFXII but not by the administration of the recombinant FXII variant hFXII-3xAla, which does not possess procoagulant activity. This proves, for the first time, that activation of the intrinsic coagulation pathway by FXII plays a key role in post-traumatic cerebral thrombus formation. Interestingly, in thrombus formation on atherosclerotic plaques, the extrinsic coagulation pathway triggered by the release of tissue factor contributes to the initial thrombus generation, whereas activated FXII ensures stability of the thrombus in later phases.[38] Considering that TBI immediately results in massive release of tissue factor (as reviewed by Maegele[12]), a similar scenario might apply during cerebral thrombus formation in TBI.

Cerebral thrombus formation in our study was associated with a reduction in pericontusional blood flow. This may contribute to secondary brain damage, as reported previously both in humans[9,39,40] and in experimental TBI.[5–7,10,29,41–43] Low post-traumatic cerebral blood flow is a known predictor of worse outcome in pediatric severe TBI[44] and in adult diffuse TBI.[45] Prevention of pathological thrombus formation in the cerebral microcirculation in $FXII^{-/-}$ mice or after acute treatment with rHA-Infestin-4 was also associated with a better neurological outcome in the present study.

In conclusion, genetic deficiency or selective inhibition of FXII in mice results in less cerebral thrombus formation and a better outcome in experimental TBI, without increasing the risk of abnormal hemorrhaging.

Despite intensive research, specific therapeutic interventions are still lacking for TBI. Challenging existing pathophysiological concepts and exploring innovative ideas might overcome this roadblock. The pathophysiological role of microvascular thrombosis in TBI has been neglected by previous research in the TBI field even though the presence of occluded cerebral vessels in patients with TBI was reported 1995.[4] Our study confirms and extends the previous clinical findings in a larger, well-characterized patient cohort and provides preclinical evidence that microvascular thrombosis might contribute to secondary brain damage. Moreover, it was shown for the first time that targeting FXII prevents thrombus formation and has a beneficial effect on outcome after TBI, indicating that it might be a novel treatment option to decrease ischemic injury after TBI.

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Author Contributions

S.H. and C.A.-W. designed and performed experiments, analyzed and interpreted data, and drafted the manuscript and the figures. S.M., M.K.S., B.N., C.-M.M., I.A., N.M., P.M.S., M.B., M.W.N., and C.S. provided specific input on the experimental design, and data acquisition and analysis, and contributed to manuscript writing. A.-L.S. and C.K. conceived and designed the study and critically revised the manuscript. All authors have seen and agree with the content of the manuscript. S.H. and C.A.-W. contributed equally to this work.

Potential Conflicts of Interest

P.M.S. is an employee of CSL Limited and M.W.N. is an employee of CSL Behring. C.K. received research funding from CSL Behring. A patent application has been filed by CSL Behring to protect the intellectual property rights of FXIIa inhibitors for use in traumatic brain injury (WO 2015/193457 A1; M.W.N., C.K., A.-L.S., C.A.-W., S.H.).

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