SUPPLEMENTARY MATERIAL

Fn-EDA in the plasma, but not endothelial cells, exacerbates stroke outcome by promoting post-ischemic thrombo-inflammation

Running title: Fn-EDA in the plasma exacerbates stroke outcome.

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Animals

Fn-EDA$^{fl/fl}$ mice have been described previously.[1] To generate endothelial-specific Fn-EDA deficient mice (*Apoe*$^{-/-}$*Fn-EDA*$^{fl/fl}$*Tie2*^{Cre}), *Apoe*$^{-/-}$*Fn-EDA*$^{fl/fl}$ mouse was crossed to *the Apoe*$^{-/-}$*Tie2*^{Cre} mouse. Similarly, to generate plasma-specific Fn-EDA deficient mice (*Apoe*$^{-/-}$*Fn-EDA*$^{fl/fl}$*Alb*^{Cre}), *Apoe*$^{-/-}$*Fn-EDA*$^{fl/fl}$ mouse was crossed to *Apoe*$^{-/-}$*Alb*^{Cre} mouse (Supplementary Figure 1). *Fn-EDA*$^{fl/fl}$*Tie2*$^{Cre-/-}$*Apoe*$^{-/-}$ and *Fn-EDA*$^{fl/fl}$*Alb*$^{Cre-/-}$*Apoe*$^{-/-}$ littermates were used as controls. Mice were genotyped by PCR according to protocols from the Jackson Laboratory, and as described previously.[1] Mice were kept in standard animal house conditions with controlled temperature and humidity and had *ad libitum* access to standard chow diet and water. All the mice used in the present study were on the C57BL/6J background. Both male and female mice (littermates of age approximately 10-12 weeks) weighing 20–26 grams were utilized. The University of Iowa Animal Care and Use Committee approved all the procedures and studies were performed according to the current Animal Research: Reporting of *In Vivo* Experiment guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

Quantification of cellular Fn-EDA and total fibronectin in plasma samples

Cellular Fn-EDA and total fibronectin levels in the plasma were measured by sandwich enzyme-linked immunosorbent assay (ELISA), as previously described.[2, 3]

Reverse transcription (RT) real-time polymerase chain reaction (PCR)

Lung endothelial cells were isolated by fluorescence-activated cell sorting (FACS, Becton Dickinson, Aria Fusion) using PE-labeled CD31 antibody (BD Pharmingen, Cat#553373). Total RNA from the endothelial cells and hepatocytes was isolated using the Rneasy mini kit (Qiagen, cat#74104). Total RNA (~400 ng) was reverse-transcribed using iScriptTM Reverse Transcription Reagent Kit (BIO-RAD, catalog# 1708840). Fn-EDA mRNA was quantitated via RT-PCR using primers in the exons flanking the EDA exon (FN2 Dir, 5 ACCATCACCCTGTATGCTGTCT-3 and EDA Rev, 5-CTATGAGTCCTGACACAATCAC-3).

Cerebral ischemia and reperfusion injury

Focal cerebral ischemia was induced by transiently occluding the right middle cerebral artery for 60 minutes, as described.[2, 4] Mice were anesthetized with 1–1.5% isoflurane mixed with medical air. After a midline incision, the right common carotid artery was temporarily clamped, and a filament (6.0 siliconized filament from Doccol Corp., catalog# 602256PK10) was inserted from the external carotid artery and advanced into the internal carotid artery up to the origin of the middle cerebral artery. Reperfusion was achieved by removing the filament after 60 mins and opening the common carotid artery. Throughout the surgery, the body temperature of the mice

was maintained at 37°C ± 1.0 using a heating pad. Buprenorphine (0.1 mg/kg, SC) was administered as an analgesic agent at every 6-12 hours for 48 hours post-surgery. Laser Doppler flowmetry (Perimed instruments, Sweden) was used for each mouse to confirm the successful induction of ischemia and reperfusion. Animals having more than 70% reduction in the regional cerebral blood flow (rCBF) and successful reperfusion (rCBF returns to 80-120% of baseline) were included in the study.

Embolic middle cerebral artery occlusions
To prepare the autologous embolus, arterial blood was supplemented with human fibrinogen (2 mg/ml, Sigma, Catalog#F3879) and immediately clotted in a PE-50 tube for four-hours at room temperature, followed by storage at 4°C overnight. Next day, 20 mm clot was washed in PBS by several passages from a PE-10 tube and transferred to a modified PE-10 catheter. Animals were anesthetized with 1–1.5% isoflurane during the surgery. The catheter containing a single 20 mm fibrin-rich clot was then introduced into the external carotid artery and advanced to the internal carotid artery. After the embolization, the catheter was removed, and the external carotid artery was blocked by cauterization. Laser Doppler flow monitoring (Perimed Instruments, Stockholm, Sweden) was used to confirm the induction of ischemia. The mice body temperature was maintained at 37 ± 1°C during the entire procedure. The right jugular vein was cannulated for the administration of rtPA (Cathflo from Genentech, 10 mg/kg, 10% volume by bolus and remaining slow infusion for 30 minutes, 60 minutes post-embolization). Buprenorphine (0.1 mg/kg, SC) was administered as an analgesic agent at every 6-12 hours for 48 hours post-surgery.

Functional assessment of neurological outcome:
Bederson Scale: The Bederson scale is a global neurological assessment that was developed to measure neurological impairments following stroke.[5] Neurological outcomes were assessed by an observer blinded to the treatment groups and was scored on a four-point scale as previously described:[2] 0, no observable neurological deficit (normal); 1, failure to extend left forepaw on lifting the whole body by tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, leaning to the contralateral side at rest (severe); 4, no spontaneous motor activity. **Modified Neurological Severity Score (mNSS):** The mNSS rates neurological functioning with a minimum neurological score of 3, and a maximum of 18 (higher score indicates a better outcome). The mNSS includes a composite score of six different tests, which are: spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception and responses to vibrissae touch, as previously mentioned.[6] We evaluated mNSS on day-1, day-3, and day-7, post-stroke. **Cylinder test:** The cylinder test[7] was used to assess forelimb use and rotation asymmetry. Mice were placed in a clear cylinder videotaped for 5 minutes. Forelimb use of the first contact against the wall after rearing and during lateral exploration was analyzed. The final score = (nonimpaired forelimb movement - impaired forelimb movement) / (nonimpaired forelimb movement + impaired forelimb movement + both movement). On day-1 and day-3 post-stroke, we could not detect much vertical exploration, and hence we analyzed data only for day-7. **Accelerated rotarod test:** The accelerated rotarod (Harvard Apparatus, model#LE8205) was used to access post-stroke motor coordination. For this, mice were trained for 3-5 days on rotarod rotating at 4rpm such that animals may walk forward to keep balance. Training is considered complete when mice can stay on the rod rotating at 4rpm for at least 1 minute. On the test day, mice were placed on the rod rotating at 4rpm and then rotation was set in acceleration mode (4-40 rpm in 5 minutes). Latency to fall was recorded for each mouse on day-1, day-3, and day-7, post-stroke.

Treatment with an anti-fibronectin cellular antibody
Mice were infused with either anti-cellular fibronectin antibody (100 µg/mouse, monoclonal Fn-3E2; Sigma, catalog# SAB4200784) or with control Ig isotype (100 µg/mouse, Rockland antibodies and assays, catalog# 610-4107-0500) intravenously, 1 hour after embolization, just before rtPA infusion.

Immunohistochemistry
Brain cortical sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval as described.[2] Briefly, sections were blocked with 5% serum at room temperature (RT), from the species in which the secondary antibody was raised. Endogenous peroxidase activity was quenched with 0.1% hydrogen peroxide in methanol for 15 min. Sections were stained with primary antibodies for neutrophil (rat anti-mouse Ly6B.2; 1:100; Bio-rad), in the presence of 5% rabbit serum. After overnight incubation at 4˚C, slides were washed with PBS for 5 minutes and incubated with biotinylated secondary antibody for 1 hour at RT. Slides were then incubated with streptavidin-HRP for 40 minutes at RT, washed and incubated with DAB substrate until color develops. Slides were then washed and counter-stained with hematoxylin, mounted using an aqueous mounting medium and examined under a light microscope (Olympus). Incubation without primary antibodies and with isotype-matched immunoglobulins was used as a negative control for immunostaining. **Quantification**: In four different regions of the infarct and surrounding area extravascular neutrophils (400X magnification) were quantified by counting the immunoreactive cells (brown color staining). NIH Image J software (with the plugin for individual cell analysis) was used for neutrophil quantification. Each data represents a mean of 16 fields from 4 serial sections (separated by 30 µm).

Immunofluorescence
All sections were subjected to heat-induced antigen retrieval. Sections were blocked with 5% normal goat serum in Tris-buffered saline at room temperature (RT). Sections were washed thrice with PBS for 5 minutes and incubated overnight with primary antibodies for platelet (rat anti-mouse CD41; Bio-rad, catalog# MCA2245T), anti-fibrin(ogen) (1:400, Acris Antibodies, Catalog# AP00766PU-N), Fibronectin-EDA+ (FN-3E2, 1:100, Sigma, catalog# 6140) and endothelial cells (rat anti-mouse CD31; Bio-rad, catalog# 550274) at 4°C. After washing, sections were labelled with appropriate secondary antibodies [goat anti-rabbit IgG Alexa flour-546 (1:400, Invitrogen, catalog# A11010), goat anti-mouse IgG Alexa flour-546 (1:400, Invitrogen, catalog# A11003), and goat anti-rat IgG Alexa flour-488 (1:400, Invitrogen, catalog# A11006)]. Nuclei were stained using DAPI. Isotype-matched immunoglobulins were used as a negative control. Images were taken using Nikon Eclipse Ti-U inverted fluorescent microscope equipped with a 40x/0.75 and 20x/0.8 Plan Apo lens, cooled CCD camera and a Nis Elements imaging software (Nikon). ImageJ software (NIH ImageJ, USA) was used for all the quantifications. The thrombotic index was calculated by dividing occluded vessels from a total number of vessels.

Western blot
Brain cortical tissue was collected (23 hours post-reperfusion) from the infarcted and surrounding areas and homogenized in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40) containing 0.1% SDS and 4% proteinase inhibitor (complete protease inhibitor cocktail, Roche, catalog# 11836153001). Samples were sonicated for a total 30 seconds with 10 seconds gap. Tissue lysates were centrifuged at 14000×g for 20 min at 4°C and supernatants were used for the determination of protein content (by Lowry method) and subsequent Western blot analysis. Total lysates were mixed with sample loading buffer (Novex by Life Technologies, catalog# NP0007) and heated at 95°C for 5 min. 20 µg of total protein was loaded per well, electrophoresed and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking

for 60 min with blocking buffer (5% nonfat dry milk, 50 mM Tris-HCl pH 7.5, 0.05% Tween-20), membranes were incubated with: anti-fibrin(ogen) (1:5000, Acris Antibodies, catalog# AP00766PU-N), phospho-NF-κB p65 (Ser536) (1:1000, Cell Signaling Technologies, catalog#3033S) and NF-κB p65 (1:1000, Cell Signaling Technologies, catalog#4764S) at 4°C overnight: followed by appropriate secondary antibodies (polyclonal goat anti-rabbit IgG, Dako, catalog# P0448) conjugated to horseradish peroxidase (HRP). Enhanced chemiluminescence kit (Thermo Scientific, catalog# 34580) was used for Western blots. All blots were stripped and reanalyzed for the β-actin (anti-beta actin antibody from Abcam, catalog# ab8226) as a loading control. The intensity of the bands was measured by densitometry and quantified using NIH-Image J software

ELISA assay for TNF-α and IL-1β
After 23 hours of reperfusion, cortical brain tissue was collected from the infarcted and surrounding areas and lysed in tissue lysis buffer (20 mM Tris-Cl, pH 8.0, 10 mM NaCl, 2% Triton X-100). Supernatants from brain homogenates were used for determination of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) with commercially available mouse ELISA kits (TNF-α catalog# MTA00B, IL-1β catalog#MLB00C, both from R&D Systems) according to the manufacturer's instructions.

Flow cytometry
24 hours after MCAO, mice were perfused with 20 mL of 0.9% saline. The ipsilateral hemispheres from were, minced with scissors and cells were dissociated using a Dounce tissue grinder (Wheaton). Samples were then passed through a sterile 18 g syringe several times in order to obtain a single-cell suspension. Samples were then washed and then mixed with a 30% Percoll solution, and centrifuged at 1,400 × g at 4°C for 20 minutes. Cells were then washed twice in FACS buffer (0.5% FBS, 0.1% sodium azide in PBS), and then 100,000 cells were distributed into each tube for antibody staining. Cells were blocked for 60 minutes on ice with Fc-block (BD Biosciences), and then fluorescently-labeled antibodies (Ly6G-1A8 (FITC), CD11b (e450), Ly6C(PercPcy5.5); BD Biosciences) were added for 45 minutes on ice in the dark. Samples were washed in FACS buffer and analyzed using a Becton Dickenson LSRII.

Bone marrow transplantation
Bone marrow transplantation (BMT) was performed in $Apoe^{-/-}Fn\text{-}EDA^{fl/fl}Tie2^{Cre}$ mice at 7-8 weeks of age as reported.[2] Recipient mice were irradiated with 2 doses of 6.5-Gy at an interval of 4 hours between the first and second irradiations. Bone marrow cells were aseptically extracted from excised femurs and tibias of euthanized $Apoe^{-/-}Fn\text{-}EDA^{fl/fl}Tie2^{Cre}$ and $Apoe^{-/-}Fn\text{-}EDA^{fl/fl}$ mice. All mice were on the C57BL/6J background. Bone marrow cells (1×10^7) were resuspended in sterile PBS and injected into the retro-orbital plexus of lethally irradiated recipient mice. After transplantation, mice were maintained in sterile cages and fed autoclaved food and water ad libitum. BMT success was analyzed after 4 weeks by PCR to check the presence of the genomic DNA (of the respective donor mice) in peripheral blood mononuclear cells from transplanted mice. Complete blood counts were obtained using automated veterinary hematology analyzer (ADVIA) to ascertain that BMT did not affect the number of BM-derived blood cells.

References:

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Tables

	$Apoe^{-/-}Fn$-$EDA^{fl/fl}$	*$Apoe^{-/-}Fn$-$EDA^{fl/fl}Tie2^{Cre}$*	*$Apoe^{-/-}Fn$-$EDA^{fl/fl}Alb^{Cre}$*
Plasma total cholesterol (mg/dL)	422±63.8	424.6±89.7	485.4±39.5
Plasma Triglycerides (mg/dL)	211±25.8	191.5±7.9	235.1±33.7
Body weight (g)	24.9±0.7	25.2±0.7	24.8±0.5

Online supplementary Table I. Total plasma cholesterol, plasma triglycerides concentrations were measured from each mouse using enzymatic colorimetric assays according to the manufacturers' instructions. Values are expressed as mean ± SD. N= 3-6 mice/group. P= Non-significant.

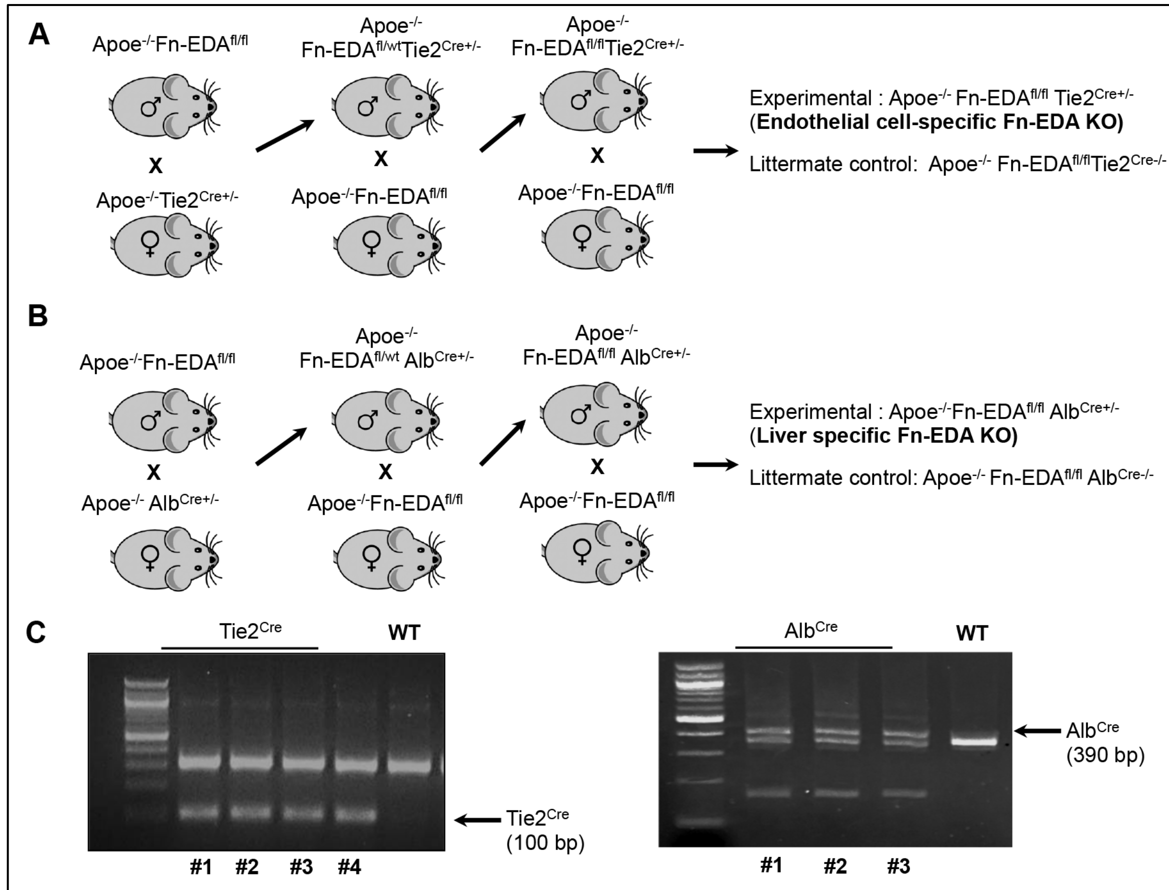
	$Apoe^{-/-}Fn$-$EDA^{fl/fl}$	*$Apoe^{-/-}Fn$-$EDA^{fl/fl}Tie2^{Cre}$*	*$Apoe^{-/-}Fn$-$EDA^{fl/fl}Alb^{Cre}$*
WBC (10^3/uL)	16.6±1	15±1.6	14.8±1.1
RBC (10^6/uL)	8.3±0.1	8.6±0.2	8.8±0.2
HGB (g/dL)	15.2±0.5	14.4±0.4	14.8±0.4
HCT (%)	46.8±0.6	45.6±0.2	44.8±0.8
PLT (10^3/uL)	1124±39.6	1012±66.3	1075±42.9
Neutrophil (10^3/uL)	0.6±0.1	0.5±0.1	0.5±0.1

Online supplementary Table II: Complete blood counts from 8- 10 weeks old mice were obtained using automated veterinary hematology analyzer (Advia). Value are expressed as mean ± SEM. N= 5-6 mice/group. P= Non-significant versus control $Apoe^{-/-}Fn$-$EDA^{fl/fl}$ mice.

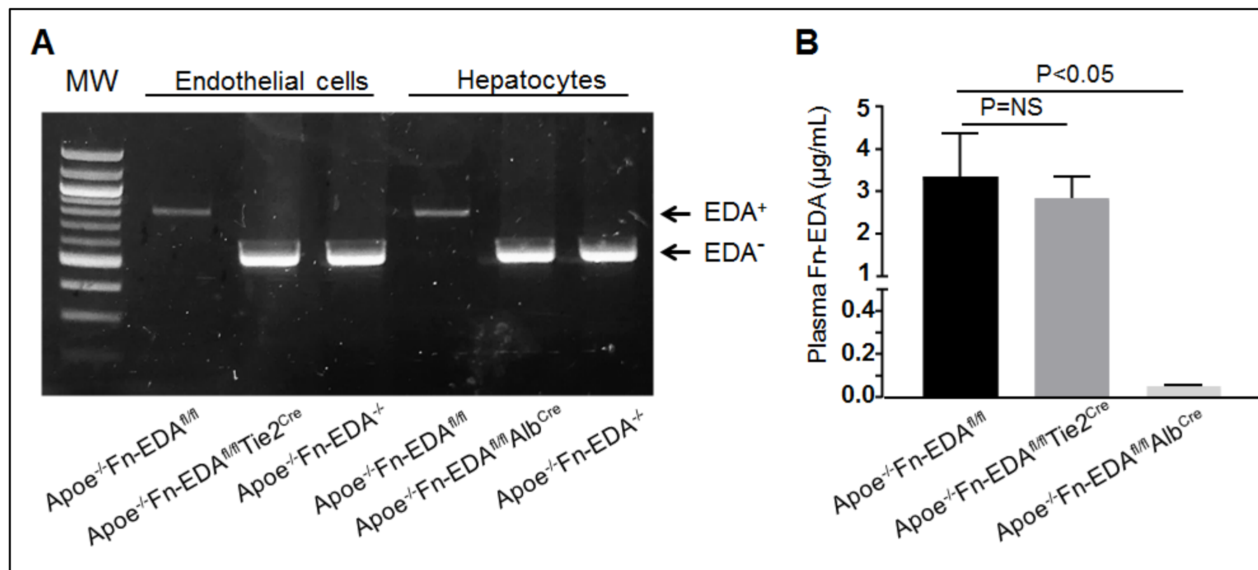
	$Apoe^{-/-}Fn$-$EDA^{fl/fl}$	*$Apoe^{-/-}Fn$-$EDA^{fl/fl}Tie2^{Cre}$*	*$Apoe^{-/-}Fn$-$EDA^{fl/fl}Alb^{Cre}$*
Ischemia LDF (% of baseline)	16.3±2.8	14.8±3.3	17.8±2.75
Reperfusion LDF (% of baseline)	86.8±7.2	82.6±6.9	93.7±9.3

Online supplementary Table III: Laser Doppler Flowmetry (LDF) was similar among groups during and after ischemia. Values are expressed as mean ± SEM. N= 4-5 mice/group.

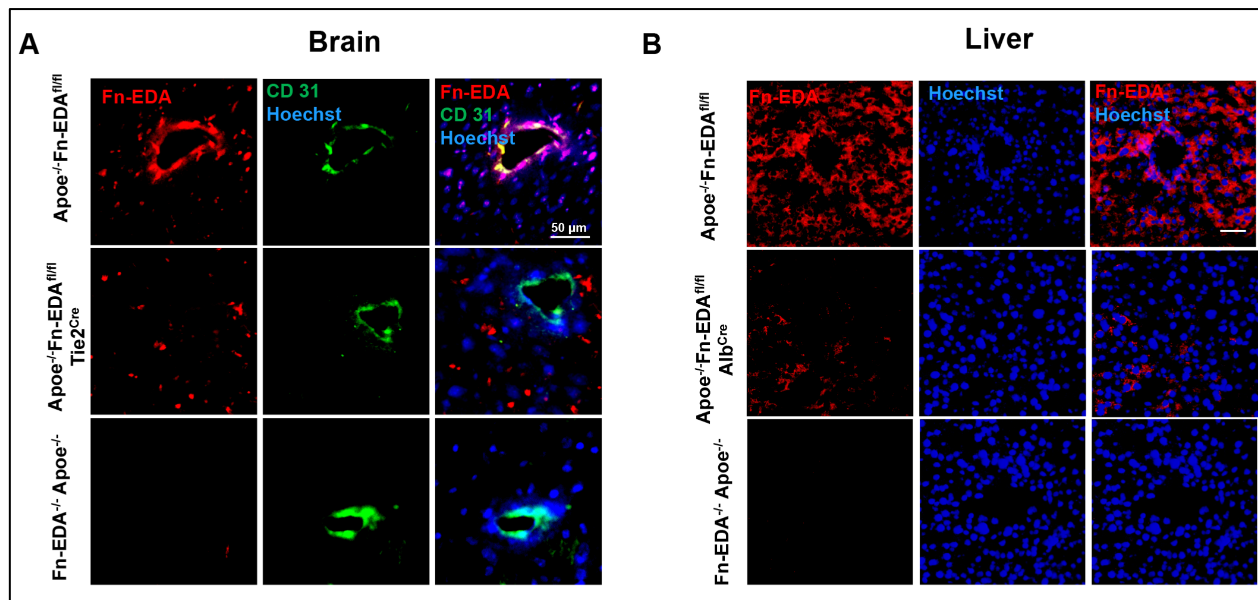
Figures



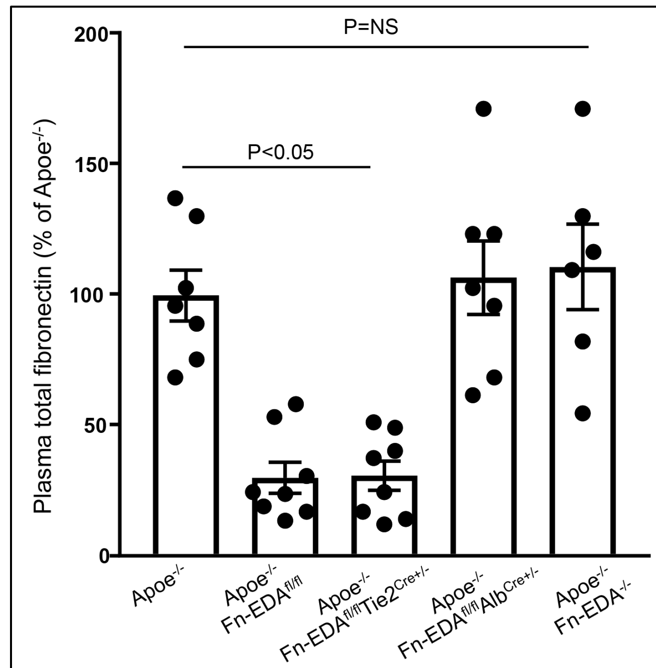
Online Supplementary Figure I. **A& B**. Schematic showing the strategy to generate endothelial-specific and liver-specific Fn-EDA deficient mice. **C.** Genomic PCR confirming the presence of *Tie2^{Cre}* and *Alb^{Cre}* gene in *Fn-EDA$^{fl/fl}$Apoe$^{-/-}$* mice.



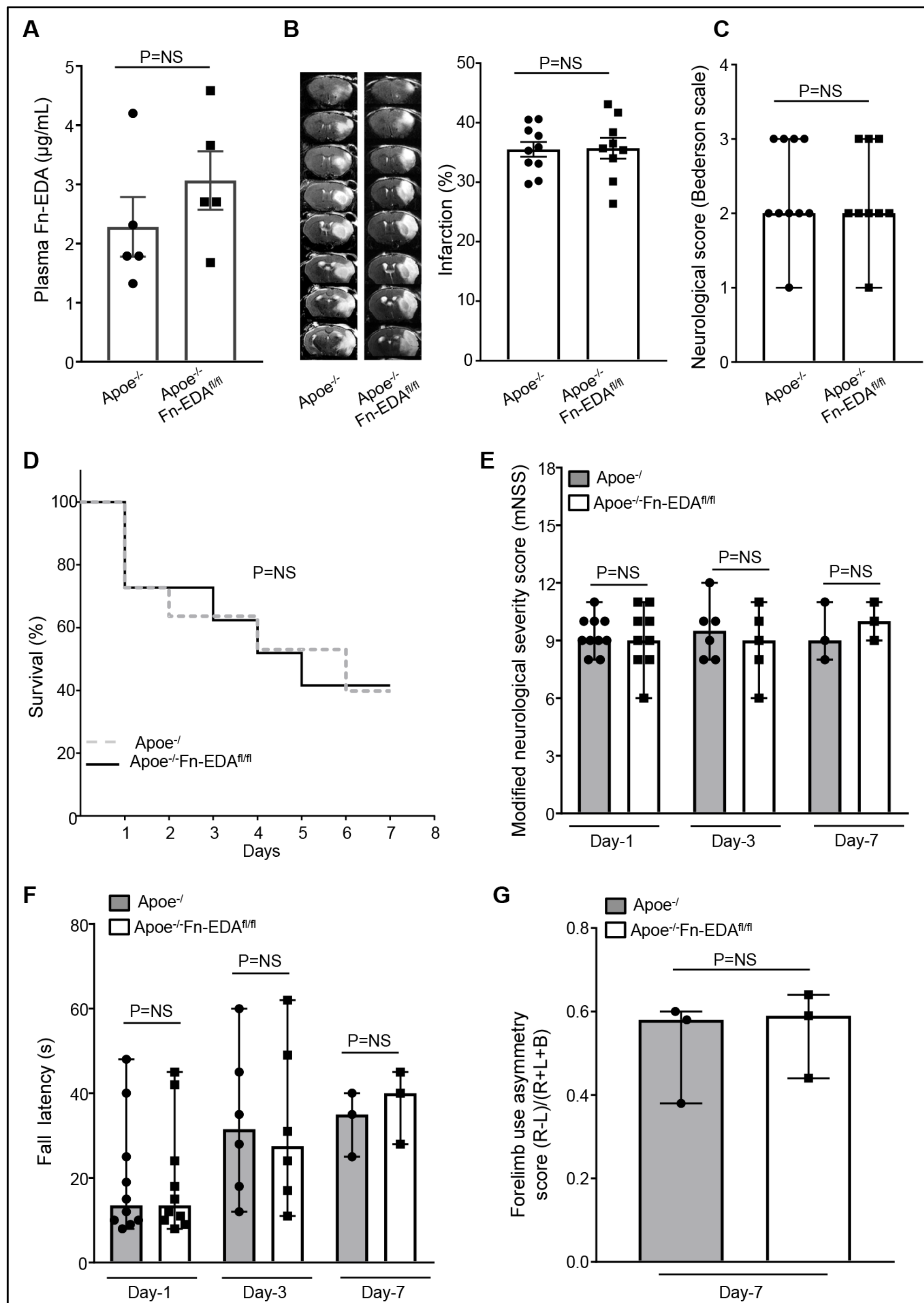
Online Supplementary Figure II: **A.**RT-PCR showing the absence of Fn-EDA mRNA in the endothelial cells of *Apoe*$^{-/-}$*Fn-EDA*$^{fl/fl}$*Tie2*^{Cre} and hepatocytes of *Apoe*$^{-/-}$*Fn-EDA*$^{fl/fl}$*Alb*^{Cre} mice. *Apoe*$^{-/-}$*Fn-EDA*$^{-/-}$ mouse was used as a positive control. **B.** Quantification of plasma Fn-EDA levels by ELISA (n=6 mice/group). Data are presented as mean ± SEM



Online Supplementary Figure III: **A**. Immunofluorescence staining showing an absence of Fn-EDA in the brain vasculature of *Apoe$^{-/-}$Fn-EDA$^{fl/fl}$Tie2^{Cre}* mice. **B.** Immunofluorescence staining showing reduced Fn-EDA expression in the liver of *Apoe$^{-/-}$Fn-EDA$^{fl/fl}$Alb^{Cre}* mice. *Apoe$^{-/-}$ Fn-EDA$^{-/-}$* mouse was used as a negative control.

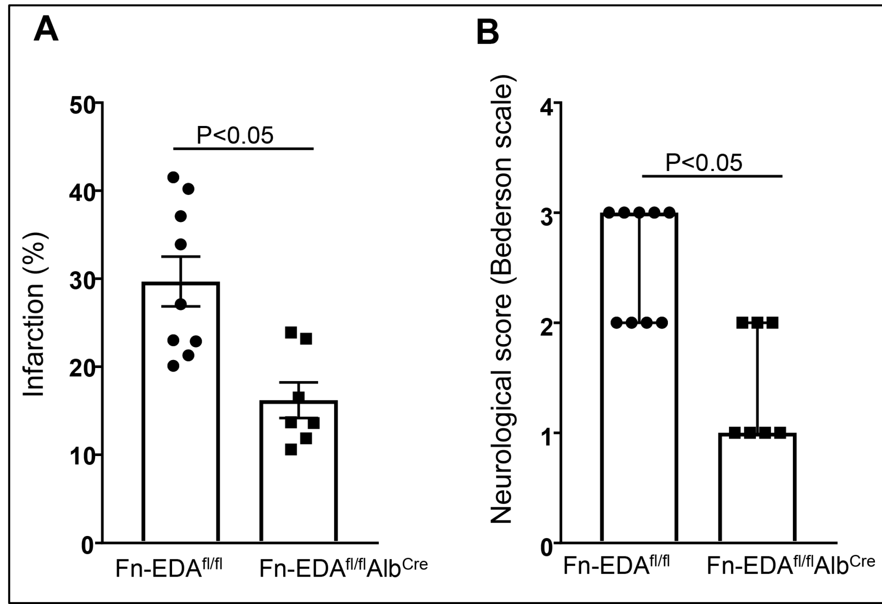


Online Supplementary Figure IV. Quantification of total plasma fibronectin levels by ELISA (n=6-7 mice/group). Data are presented as mean ± SEM.

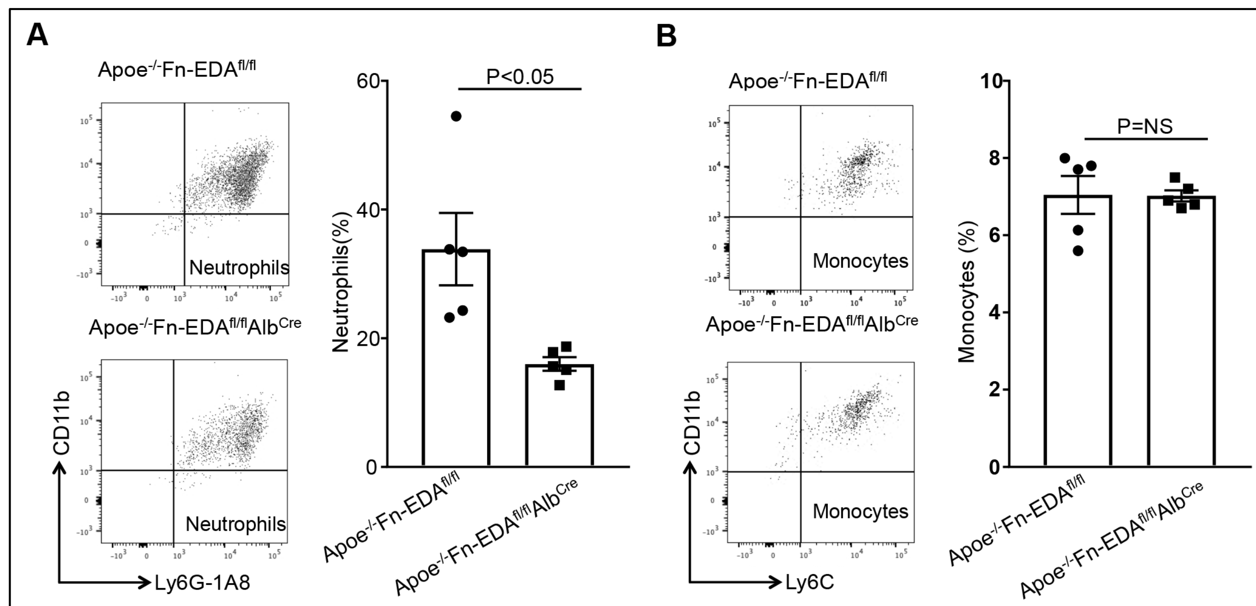


Online Supplementary Figure V. *Apoe$^{-/-}$Fn-EDA$^{fl/fl}$* and *Apoe$^{-/-}$* mice display similar stroke severity and post-stroke sensorimotor behavioral recovery. A. Plasma Fn-EDA levels by ELISA. **B.** The left panels show representative MRI from one mouse of each genotype on day 1. White is the infarct area. The right panels show corrected mean infarct volumes (N=9/group). Data are mean ± SEM. Statistical analysis: Data were analyzed by unpaired t-test. **C.** The neurological outcome as assessed before sacrifice on day 1 (depicted as scatter plots including median). Analysis of variance on ranks was applied to test for significant differences in the neurological score. **D.** Mortality rate between day 0 and day 7 after 60 minutes transient ischemia (N = 9/ group). **E.** Modified Neurological Severity Score at day 1, 3 and 7 based on spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing,

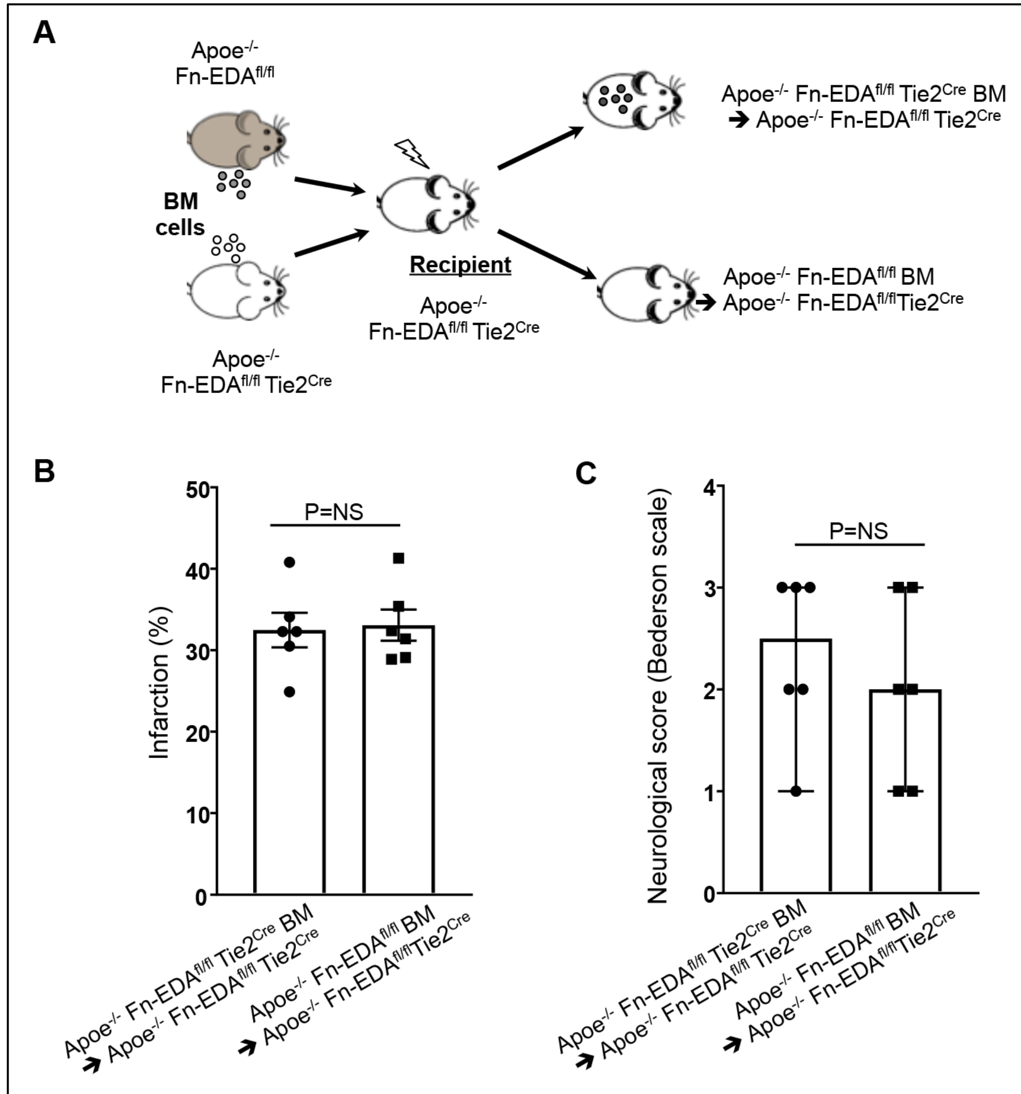
body proprioception and responses to vibrissae touch (higher score indicates a better outcome). Data were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test. **F.** Post-stroke motor function as analyzed by accelerated rotarod on day-1, 3 and 7. Data were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test. **G.** Post-stroke sensorimotor behavioral recovery as analyzed by cylinder test on day 7. Data were analyzed by unpaired t-test.



Online Supplementary Figure VI. *Fn-EDA$^{fl/fl}$Alb^{Cre}* mice exhibit improved stroke outcome.
A. Corrected mean infarct volumes of each group (N=7-9/group). Data are mean ± SEM. Statistical analysis: Data were analyzed by unpaired t-test. **B.** Neurological outcome assessed prior to sacrifice on day 1 (depicted as scatter plots including median). Analysis of variance on ranks was applied to test for significant differences in the neurological score.



Online Supplementary Figure VII. Plasma *Fn-EDA*$^{-/-}$ exhibit reduced neutrophil but not monocytes infiltration in post-stroke brain samples. Following 23 hours after the reperfusion, perfused ipsilateral hemispheres were harvested for further analysis by flow cytometry. **A**. Left panels show representative dot plots displaying neutrophils in isolated ipsilateral hemispheres from each genotype. The right panel shows quantification. **B**. Left panels show representative dot plots displaying monocytes in isolated ipsilateral hemispheres from each genotype. The right panel shows quantification. Data are mean ± SEM. Statistical analysis: Data were analyzed by unpaired t-test.



Online Supplementary Figure VIII. Fn-EDA derived from bone marrow cells does not contribute to stroke severity. A. Schematic diagram of the bone-marrow transplant (BMT) protocol. **B**. Corrected mean infarct volumes of each group (N=6/group). Data are mean ± SEM. Statistical analysis: Data were analyzed by unpaired t-test. **C.** Neurological outcome assessed prior to sacrifice on day 1 (depicted as scatter plots including median). Analysis of variance on ranks was applied to test for significant differences in the neurological score.

*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:

Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	N/A
Type and methods of randomization have been described:	N/A
Methods used for allocation concealment have been reported:	N/A

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	N/A
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	N/A

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	Yes

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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