SUPPLEMENTAL MATERIALS

High fat diet delays plasmin generation in a thrombomodulin-dependent manner in mice

Adam Miszta, Anna K. Kopec, Asmita Pant, Lori A. Holle, James R. Byrnes, Daniel A. Lawrence, Kirk C. Hansen, Matthew J. Flick, James P. Luyendyk, Bas de Laat, Alisa S. Wolberg

SUPPLEMENTAL METHODS

Materials. Recombinant human tissue factor (TF, Innovin) was from Dade Behring. Recombinant human tissue plasminogen activator (rtPA, Actilyse) was from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Phospholipid vesicles (20 mol% phosphatidylserine, 60 mol% phosphatidylcholine, 20 mol% phosphatidylethanolamine) and human α_2-macroglobulin-plasmin complex (α_2M-Pm) were from Synapse Research Institute (Maastricht, the Netherlands). Thrombin fluorogenic substrate (Z-Gly-Gly-Arg-AMC) was from Diagnostica Stago (Parsippany, NJ). Plasmin fluorogenic substrate (Boc-Glu-Lys-Lys-AMC) was from Bachem (Bubendorf, Switzerland). Mouse α_2-antiplasmin was from Abcam (Cambridge, MA). Recombinant mouse plasminogen activator inhibitor-1 (PAI-1) produced in human cells was from Biorbyt (San Francisco, CA). Recombinant human PAI-1 containing four mutations (K154T, Q319L, M354I, and N150H) to stabilize the molecule in the active conformation[1] and human C-1 inhibitor was from Molecular Innovations (Novi, MI). Recombinant mouse thrombomodulin (rmTM) was from R&D System Inc (Minneapolis, MN). Potato tuber carboxypeptidase inhibitor (PTCI) was from Sigma Aldrich (Saint Louis, MO). Human thrombin activatable fibrinolysis inhibitor (TAFI) was from Haematologic Technologies (Essex Junction, VT). ELISAs to measure plasminogen (MBS564083) and α_2-antiplasmin (MBS135940) were from Mybiosource (San Diego, CA). Plasma PAI-1 antigen and activity were measured using a Luminex multiplex assay, as described.[1] ELISAs were also used to measure TAFI (SEA615Mu, Cloud-Clone Corp, Katy, TX) and fibrin degradation products (Asserachrom D-DI, Diagnostica Stago, France). Anti-thrombomodulin monoclonal antibodies (MTM-1701[2] and CTM1009[3]) were kind gifts from Dr. Charles T. Esmon, Oklahoma Medical Research Foundation (Oklahoma City, OK).

Mice. Mouse studies were approved by the University of North Carolina at Chapel Hill and Michigan State University Institutional Animal Care and Use Committees. Normal plasma used in assay development was from wild-type C57BL/6 mice. Genetically-engineered mice with plasminogen-

deficiency (*Plg*$^{-/-}$), fibrinogen-deficiency (*Fga*$^{+/-}$, *Fga*$^{-/-}$), mutated fibrinogen that cannot polymerize (*Fgn*^{AEK}), and factor XIII-deficiency (*F13a1*$^{+/-}$, *F13a1*$^{-/-}$) were described previously[4-7] and compared to littermate controls. Wild-type C57BL/6 mice (8-week old males) were fed a control diet (CD, 5% Teklad irradiated 22/5 rodent diet [8940]; Envigo, Saint Louis, MO) or high fat diet (HFD, 40% kcal from fat, mainly trans-fat; trans-oleic and trans-linoleic acids, supplemented with fructose [20% kcal] and cholesterol [2% w/w] [equivalent to Research Diets D09100301, Dyets, Inc. Bethlehem, PA])[8] for 12 or 16 weeks (2 cohorts); tissues and plasmas from these mice were used for experiments shown in Figures 2-5, 7, and Supplemental Figures 4-7. A separate cohort of male mice were fed a CD (13% kcal from fat, Laboratory Autoclavable Rodent Diet [5010]; LabDiet) or higher fat diet (60% kcal from fat, D12492; Research Diets Inc) for 16 weeks; plasmas from these mice were used for experiments shown in Figure 6 and Supplemental Figure 5.

Preparation of mouse plasmas. Blood was drawn into 3.2% citrate (10% vol/vol, final) from the inferior vena cava of mice anesthetized with 3%-3.5% isoflurane in 2% oxygen and processed to platelet-poor plasma by centrifugation (10 minutes, 4000*xg*). Normal, pooled mouse plasma was prepared in batches by mixing plasma from six C57BL/6 mice. Plasmas from genetically-engineered mice were studied individually.

Thrombin generation (TG) assay. TG was measured by calibrated automated thrombography, as described.[9] Briefly, 10 µL of trigger (1 pM TF, 4 µM lipids, final) was added to reaction wells and 10 µL of α_2-macroglobulin-thrombin complex to calibrator wells. Diluted plasma (40 µL) was then added to each well and plates were heated (10 minutes, 37°C). Reactions were initiated by adding 10 µL of substrate/calcium (FluCa) solution (0.416 mM Z-Gly-Gly-Arg-AMC substrate, 16.6 mM $CaCl_2$, 60 mg/mL bovine serum albumin in 20 mM HEPES, 0.02% NaN_3, pH 7.3) to the wells. Data were analyzed as described[10] to yield parameters: lagtime (time at 6 nM thrombin), time to peak (TtPeak), velocity (Peak/[TtPeak-lagtime]), peak, and endogenous thrombin potential (ETP, nmol/L thrombin × minute).

Plasmin generation (PG) assay. Plasmin was measured using a method similar to calibrated automated thrombography. The assay consisted of two measurements: one in which endogenous PG took place, and one in which calibrator with a known α_2-macroglobulin-plasmin complex (α_2M-Pm) activity was added. Since plasma color can influence fluorescence intensity, each plasma was compared to its own calibrator measurement. Briefly, 10 µL of trigger solution (TF at the concentrations indicated, phospholipids [4 µM, final], and rtPA at the concentrations indicated) was added to reaction wells and 10 µL of α_2-

macroglobulin-plasmin to calibrator wells. Diluted plasma (40 µL) was then added to each well and plates were heated (10 minutes, 37°C). Reactions were initiated by adding 10 µL of substrate/calcium solution (0.5 mM Boc-Glu-Lys-Lys-AMC substrate, 16.6 mM $CaCl_2$, 60 mg/mL bovine serum albumin in 20 mM HEPES, 0.02% NaN_3, pH 7.3) to the wells and plates were mixed by shaking for 10 seconds. Reactions were monitored every 20 seconds with a fluorometer (Fluoroskan Ascent, Thrombinoscope, Maastricht, the Netherlands) equipped with a dispenser and 390/460 filter set (excitation/emission). Data were analyzed as in the TG assay to yield parameters analogous to TG parameters: lagtime, TtPeak, velocity, peak, and endogenous plasmin potential (EPP). For reactions with low rtPA, we calculated velocity as the slope in the first 60 seconds after the lagtime. Intra-assay variation was the ratio of standard deviation to mean values obtained from individual experiments performed on a single day. Inter-assay variation was the ratio of standard deviation to mean values from experiments performed on 5 different days.

PG measurements by subsampling. Diluted plasma (40 µL of 1:3 dilution) was clotted with 20 µL of 1 pM TF, 4 µM phospholipids, 1.25 µg/mL rtPA, and 16.6 mM $CaCl_2$ (final concentrations) in a 96-well plate. Fluorogenic substrate (0.5 mM, final) was added to each well individually at 15-second intervals and fluorescence signal (rate of substrate cleavage by plasmin) was measured.

Fibrin formation and lysis. TF, phospholipids, and rtPA (10 µL of 1 pM, 4 µM, and 0.31 or 1.25 µg/mL, final, respectively) were added to a 96-well plate. Diluted plasma (40 µL of 1:3 dilution) was then added and reactions were initiated by addition of 10 µL of $CaCl_2$ (16.6 mM, final). Clot formation and lysis were monitored at 405 nm (SpectraMax 384Plus plate reader, Molecular Devices, Sunnyvale, CA) for 1 hour at 37°C. Data were analyzed as described.[11] Clot lysis time was defined as the time from 50% of the turbidity increase to 50% of the turbidity decrease.

Tissue Staining. Formalin-fixed, paraffin-embedded livers were sectioned (5-µm) and stained with hematoxylin and eosin and Sirius red by the Michigan State University Investigative Histopathology Laboratory, as described.[12]

Mass spectrometry (MS). Plasma (10 µL) was diluted 10X in phosphate-buffered saline (pH 7.4) and 10 µL diluted plasma was mixed with 10 µL of 10% SDS. Samples were processed on S-Trap Micro spin columns (Protifi, Huntington, Huntington, NY) per manufacturer's protocol. After digestion, peptides were desalted (C18 stop-and-go-extraction tips, StageTips) and analyzed on an Orbitrap Fusion

mass spectrometer coupled to an Easy-nLC 1200 system (Thermo Fisher Scientific, Waltham, MA) through a nanoelectrospray ion source. Peptides were separated on a C-18 analytical column (100 μm internal diameter, 20 cm length) packed with 2.7 μm CORTECS particles. After equilibration (3 μL 5% acetonitrile/0.1% formic acid), peptides were separated using a 120-minute linear gradient (6-42% acetonitrile with 0.1% formic acid, 400 nL/min). Liquid chromatography (LC) mobile phase solvents and sample dilutions used 0.1% formic acid in water (Buffer A) and 0.1% formic acid in 80% acetonitrile (Buffer B) (Optima™ LC/MS, Fisher Scientific, Pittsburgh, PA).

Data acquisition was performed using Xcalibur v4.1 software. Survey scans covering the mass range 350-1800 were performed in the Orbitrap by scanning from m/z 300-1800 with a resolution of 120,000 (at m/z 200), S-Lens RF Level of 30%, maximum injection time of 50 milliseconds, and automatic gain control target value of $4x10^5$. For MS2 scan triggering, monoisotopic precursor selection was enabled, charge state filtering was limited to 2–7, an intensity threshold of $2x10^4$ was employed, and dynamic exclusion of previously selected masses was enabled for 45 seconds with a tolerance of 10 ppm. MS2 scans were acquired in Orbitrap mode with a maximum injection time of 35 milliseconds, quadrupole isolation, isolation window of 1.6 m/z, higher-energy collisional dissociation with collision energy of 30%, and automatic gain control target value of $5x10^4$.

MS/MS spectra were extracted from raw data files and converted into mascot generic format (.mgf) files using Metamorpheus (v0.0.30). Converted files were searched against the mouse Uniprot database and matched between runs for area under the curve quantification. Mass tolerances were ±10 ppm for MS peaks and ±20 ppm for MS/MS fragment ions. Trypsin specificity was used, allowing for 1 missed cleavage. Methionine oxidation, protein N-terminal acetylation, and peptide N-terminal pyroglutamic acid formation were allowed as variable modifications, while carbamidomethyl of cysteine was set as a fixed modification. Additional searches were performed with Byonic for glycan identification using the above parameters. Quantification of detected proteins was performed by summing the area under the curve measurements for each peptide from a given parent protein. This allowed for relative comparison of individual protein levels across samples.

Fibrinogen and thrombomodulin measurements. Fibrinogen and thrombomodulin were detected by immunoblot in which 10 μL diluted plasma (3.5 μL plasma mixed with 55 μL of 6X reducing SDS sample buffer) was loaded on 10% gels and transferred to nitrocellulose. Fibrinogen was detected with rabbit anti-human fibrinogen primary (Dako, Carpinteria, CA) and goat anti-rabbit-horseradish peroxidase-conjugated secondary (Jackson ImmunoResearch, West Grove, PA) antibodies. Thrombomodulin was detected with anti-thrombomodulin monoclonal (CTM1009) and donkey anti-

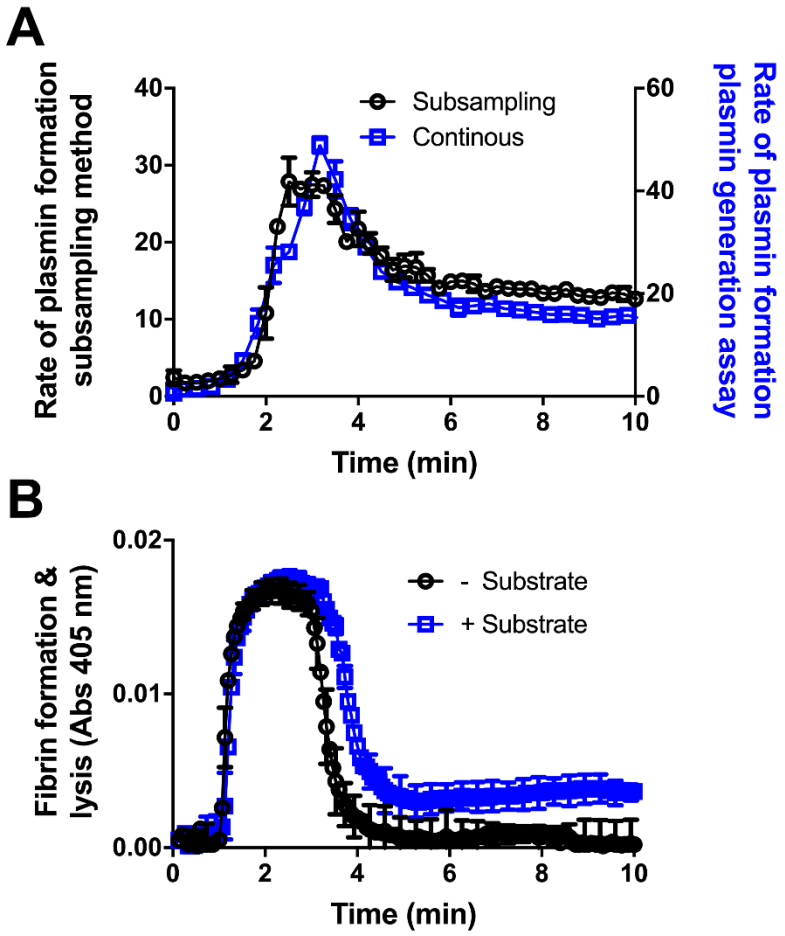
goat secondary (LI-COR, Lincoln, NE) antibodies. Bands were visualized by fluorescence using an Odyssey Imaging System (LI-COR) and quantified by densitometry (ImageJ version 1.51j8, National Institutes of Health, USA).

Statistical analysis. Experiments with two groups and normally distributed data (by Lilliefors test) were compared by Student t test for equal or unequal variances, as appropriate. Experiments with more than two groups were compared by 1-way ANOVA with Dunnett's post-hoc testing. Correlations were performed using nonparametric Spearman correlation. $P<0.05$ was considered statistically significant. For proteomic analyses, identifications were filtered using a decoy search strategy employing a 1% false discovery rate cutoff. Protein groups were constructed based on global peptide Q-Values; only peptides below a global cutoff of 0.01 were used for protein group construction. The kNN method was used for imputation of missing values; samples were normalized by sum and log transformed for comparison. Clustering was performed using the Euclidean distance measure with the Ward Algorithm. Univariate and multivariate statistical calculations were performed using Metaboanalyst.[13,14]

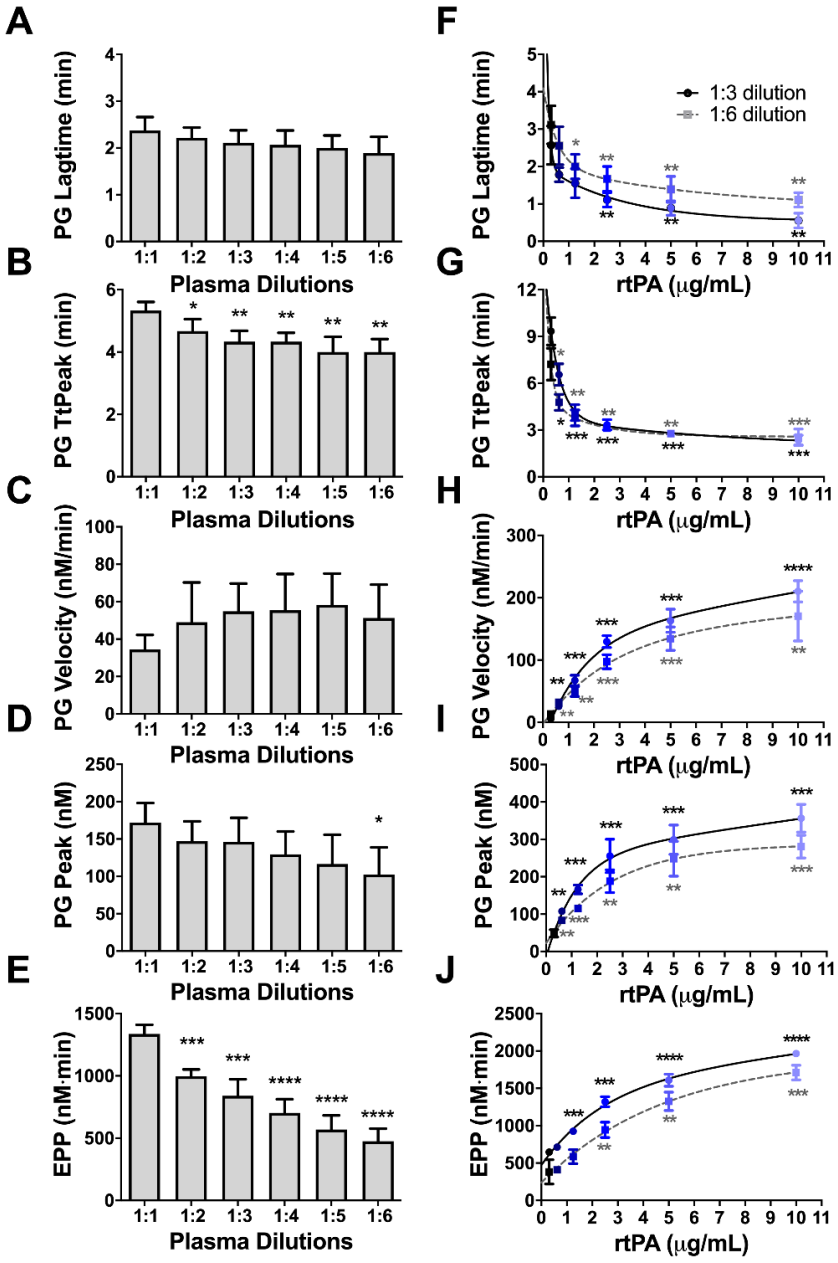
SUPPLEMENTAL REFERENCES

1. Courey AJ, Horowitz JC, Kim KK, et al. The vitronectin-binding function of PAI-1 exacerbates lung fibrosis in mice. *Blood.* 2011;118(8):2313-2321.
2. Zheng X, Li W, Gu J-M, et al. Effects of membrane and soluble EPCR on the hemostatic balance and endotoxemia in mice. *Blood.* 2007;109(3):1003-1009.
3. Fukudome K, Ye X, Tsuneyoshi N, et al. Activation mechanism of anticoagulant protein C in large blood vessels involving the endothelial cell protein C receptor. *J Exp Med.* 1998;187(7):1029-1035.
4. Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes Dev.* 1995;9(7):794-807.
5. Bugge TH, Kombrinck KW, Flick MJ, Daugherty CC, Danton MJS, Degen JL. Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. *Cell.* 1996;87(4):709-719.
6. Prasad JM, Gorkun OV, Raghu H, et al. Mice expressing a mutant form of fibrinogen that cannot support fibrin formation exhibit compromised antimicrobial host defense. *Blood.* 2015;126(17):2047-2058.
7. Carmeliet P, Kieckens L, Schoonjans L, et al. Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J Clin Invest.* 1993;92(6):2746-2755.
8. Kopec AK, Abrahams SR, Thornton S, et al. Thrombin promotes diet-induced obesity through fibrin-driven inflammation. *J Clin Invest.* 2017;127(8):3152-3166.
9. Tchaikovski SN, Van Vlijmen BJM, Rosing J, Tans G. Development of a calibrated automated thrombography based thrombin generation test in mouse plasma. *J Thromb Haemost.* 2007;5(10):2079-2086.
10. Hemker HC, Kremers R. Data management in thrombin generation. *Thromb Res.* 2013;131(1):3-11.
11. Gidley GN, Holle LA, Burthem J, Bolton-Maggs PHB, Lin F-C, Wolberg AS. Abnormal plasma clot formation and fibrinolysis reveal bleeding tendency in patients with partial factor XI deficiency. *Blood Adv.* 2018;2(10):1076-1088.
12. Joshi N, Kopec AK, Ray JL, et al. Fibrin deposition following bile duct injury limits fibrosis through an αMβ2-dependent mechanism. *Blood.* 2016;127(22):2751-2762.
13. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Curr Prot Bioinf.* 2019;68(1):e86.
14. Chong J, Xia J. MetaboAnalystR: an R package for flexible and reproducible analysis of metabolomics data. *Bioinformatics.* 2018;34(24):4313-4314.

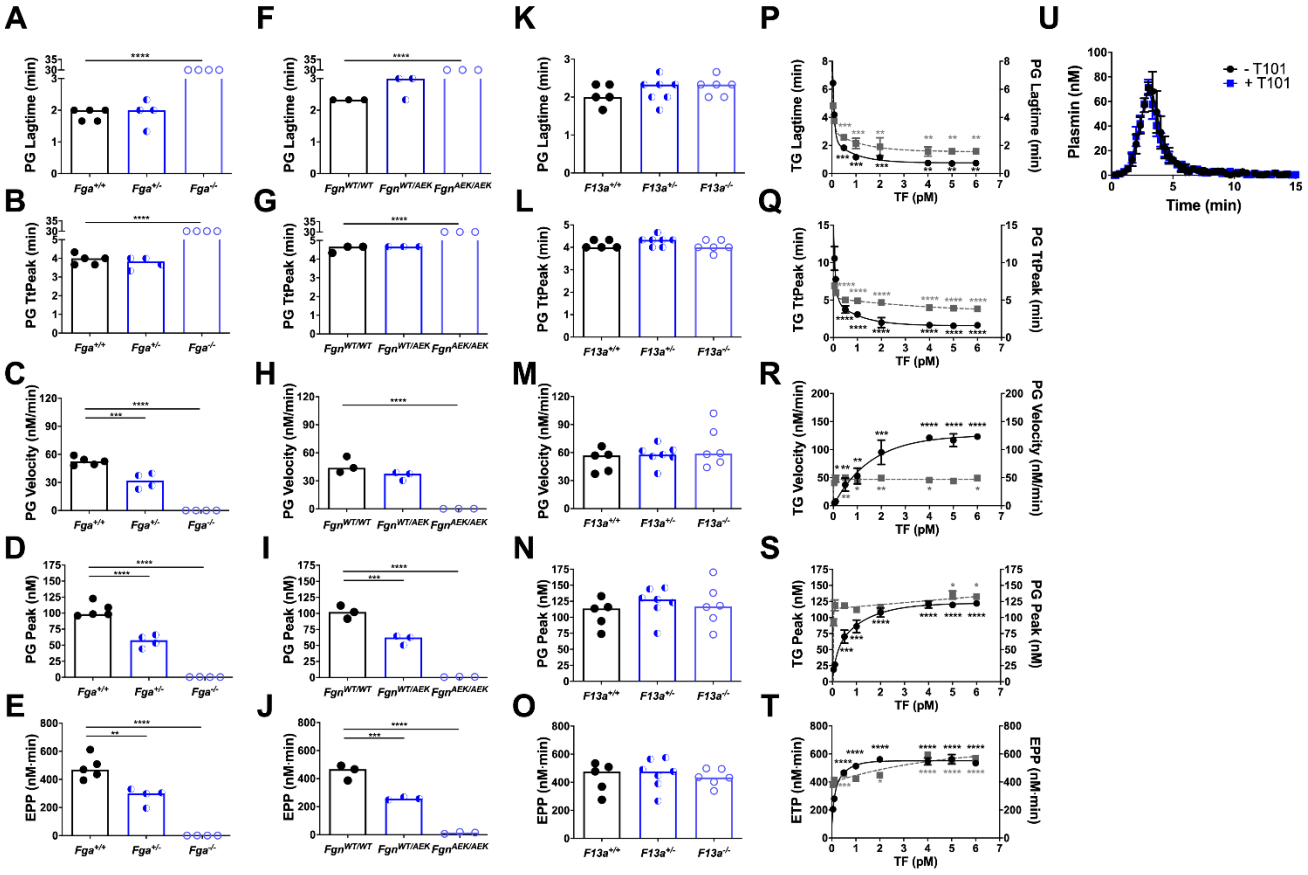
SUPPLEMENTAL FIGURES



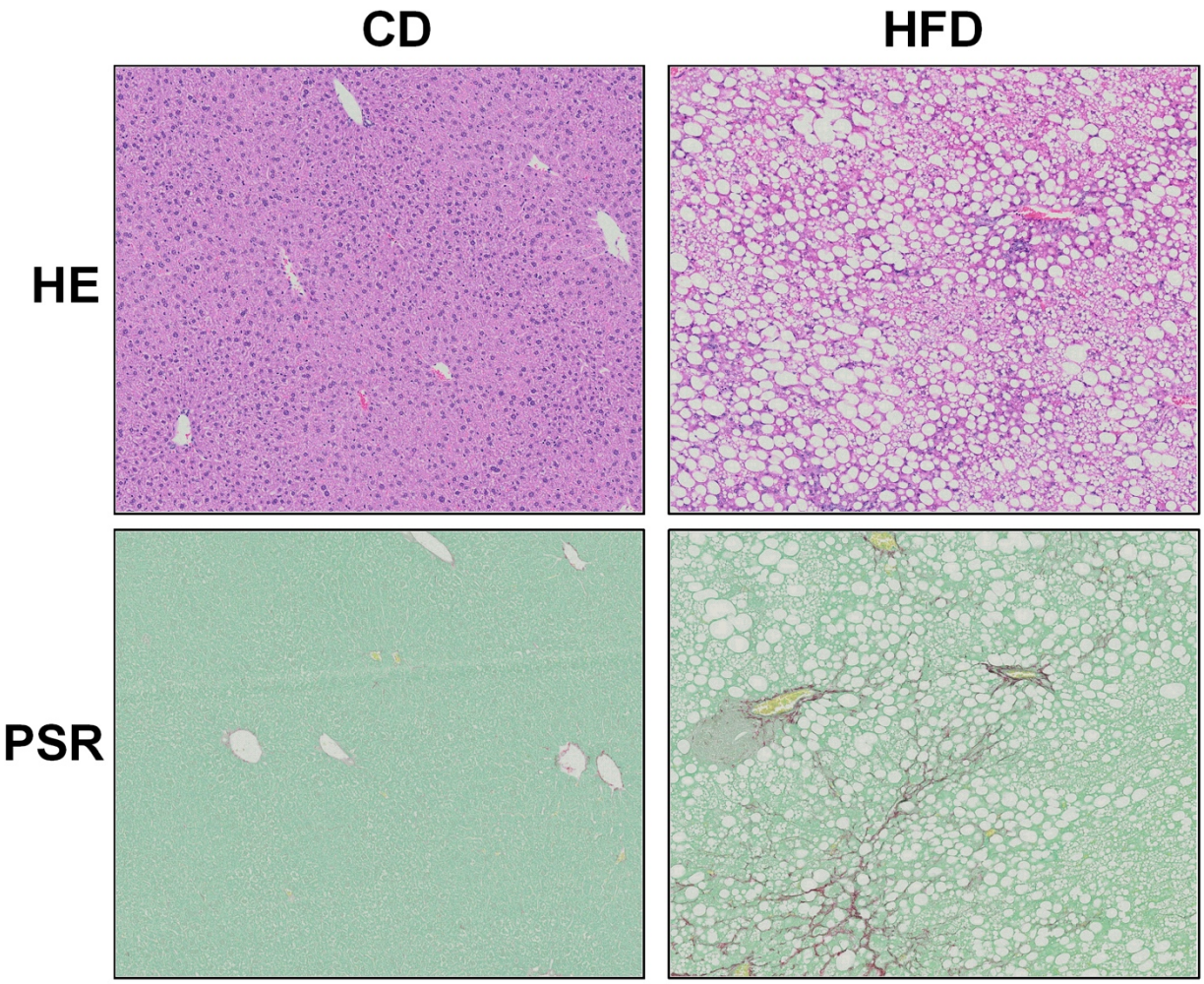
Supplemental Figure 1. The plasmin fluorogenic substrate does not alter plasmin generation, fibrin formation, or fibrinolysis. (A) Plasmin was measured in 1:3 diluted plasma by subsampling or continuous read, as described in the methods. Results are presented as a rate of substrate cleavage by plasmin detected by subsampling (black circles) and the plasmin generation assay (blue squares). (B) Fibrin formation and lysis were measured by turbidity in the absence (black circles) and presence (blue squares) of 0.5 mM plasmin substrate. Curves show mean and standard deviation (N=3/group).



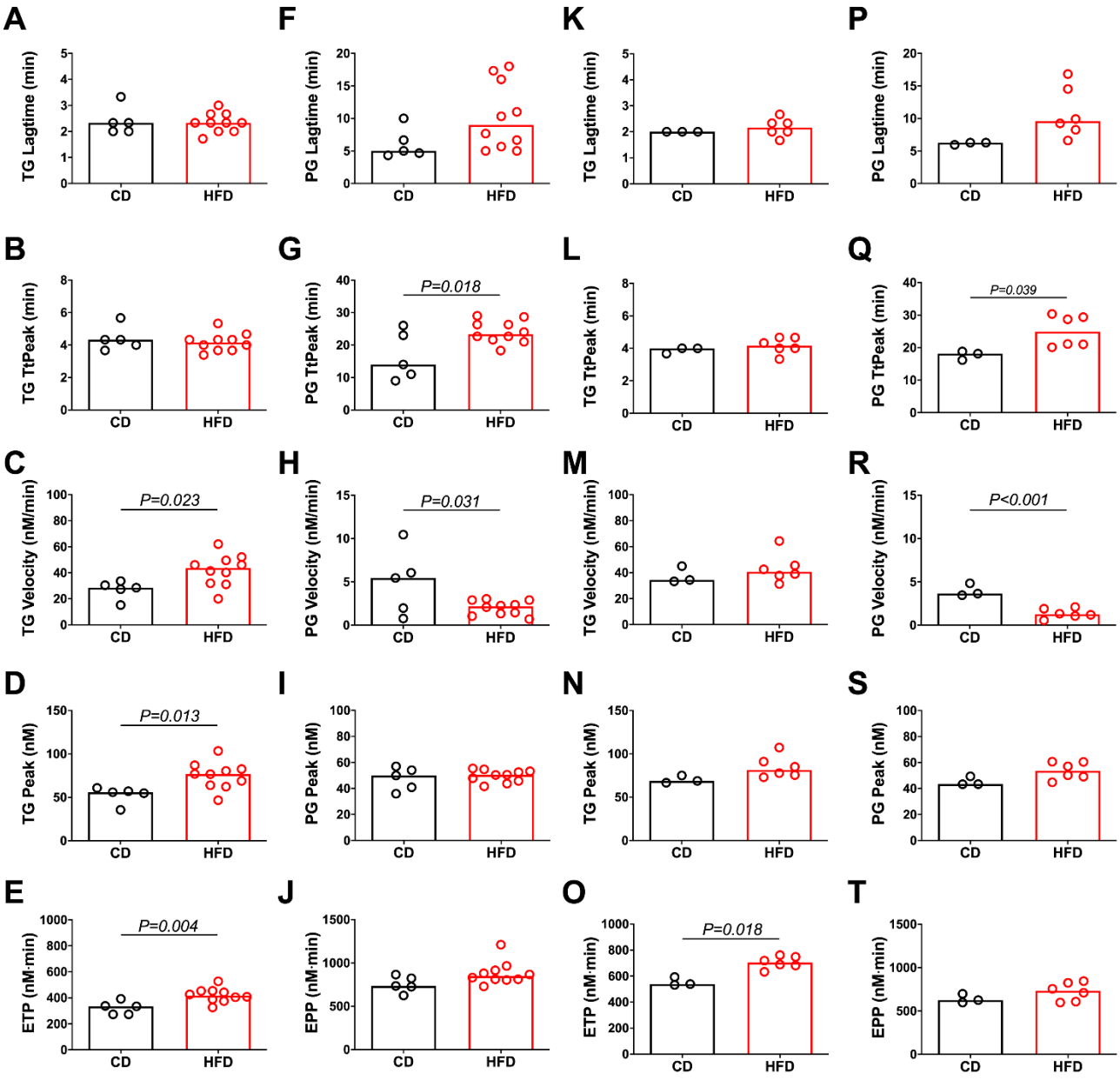
Supplemental Figure 2. Both plasma dilution and rtPA concentration influence plasmin generation (PG). (A-E) Normal, pooled plasma was diluted in HEPES-buffered saline, as indicated. PG was measured in the presence of 1 pM TF and 1.25 μg/mL rtPA. (F-J) PG parameters from normal, pooled plasma diluted 1:3 (solid line) or 1:6 (dashed line) and triggered with 1 pM TF and indicated concentrations of rtPA. Statistical comparisons were performed by ANOVA with Dunnett's post-hoc testing, using 1:1 dilution or 0.31 μg/mL rtPA, for A-E and F-J. respectively, as the index condition. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001. Plots show the mean and standard deviations (N=4 experiments).



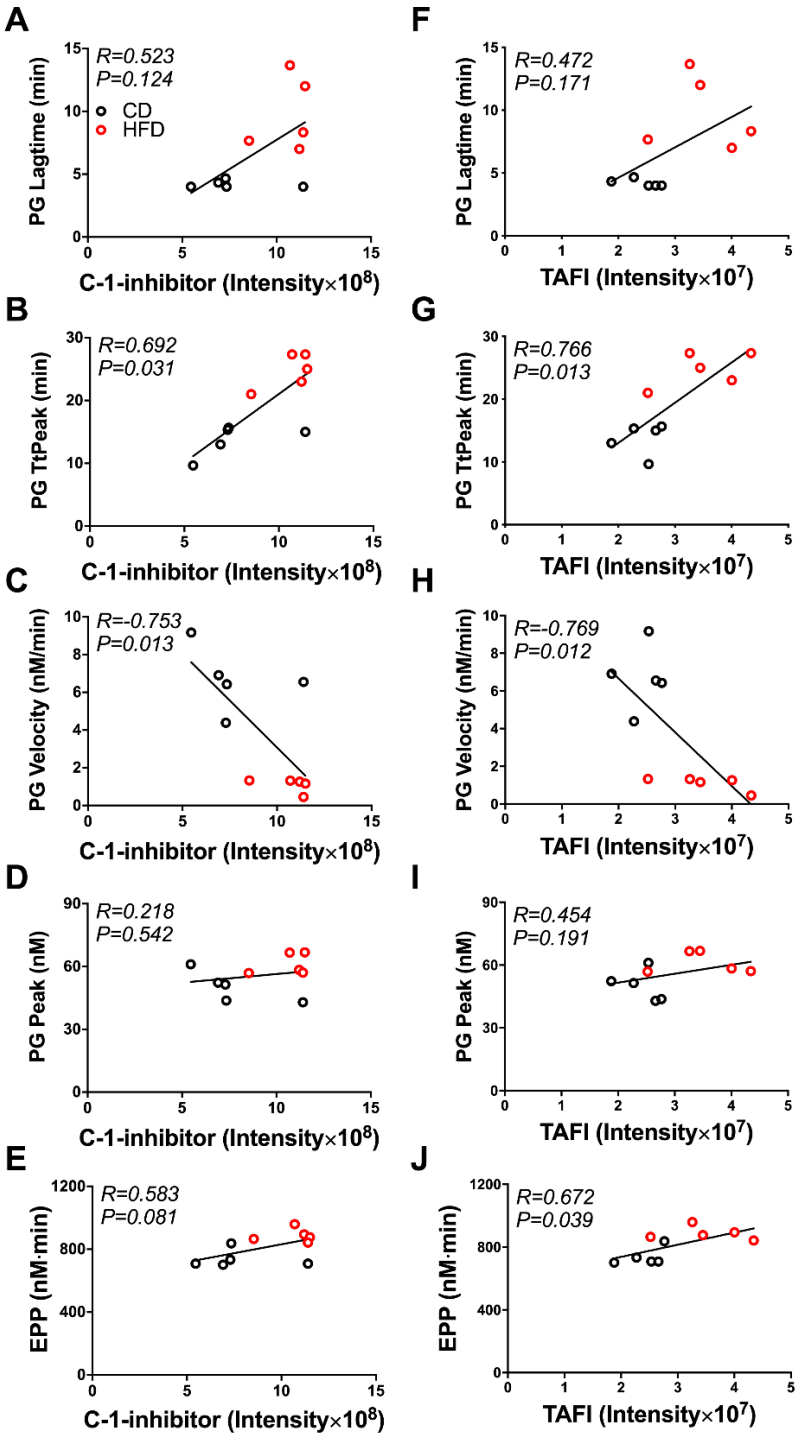
Supplemental Figure 3. Plasmin generation (PG) depends on fibrin formation, but not fibrin crosslinking. (A-E) PG parameters obtained for plasmas from wild-type ($Fga^{+/+}$) and fibrinogen-deficient mice ($Fga^{+/-}$, $Fga^{-/-}$), (F-J) mice expressing mutated fibrinogen that cannot polymerize (Fgn^{AEK}), (K-O) factor XIII-deficient mice ($F13a1^{+/-}$, $F13a1^{-/-}$). Bars indicate medians. Each dot represents a separate mouse. Reactions included 1 pM TF and 1.25 µg/mL rtPA. (P-T) Thrombin generation (TG, solid lines) and PG (dashed lines) parameters for plasmas from wild-type mice triggered with 1.25 µg/mL rtPA and the concentrations of tissue factor (TF) indicated (N=4 mice per point, averages are shown). (U) PG in the absence and presence of the factor XIII inhibitor T101 (N=2 experiments, averaged curves are shown). Statistical comparisons were performed by ANOVA with Dunnett's post-hoc testing, using wild-type mice or 0.05 pM TF, for A-O or P-T, respectively, as the index condition. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared to wild-type mice or 0.05 pM TF (black stars for TG, gray stars for PG).



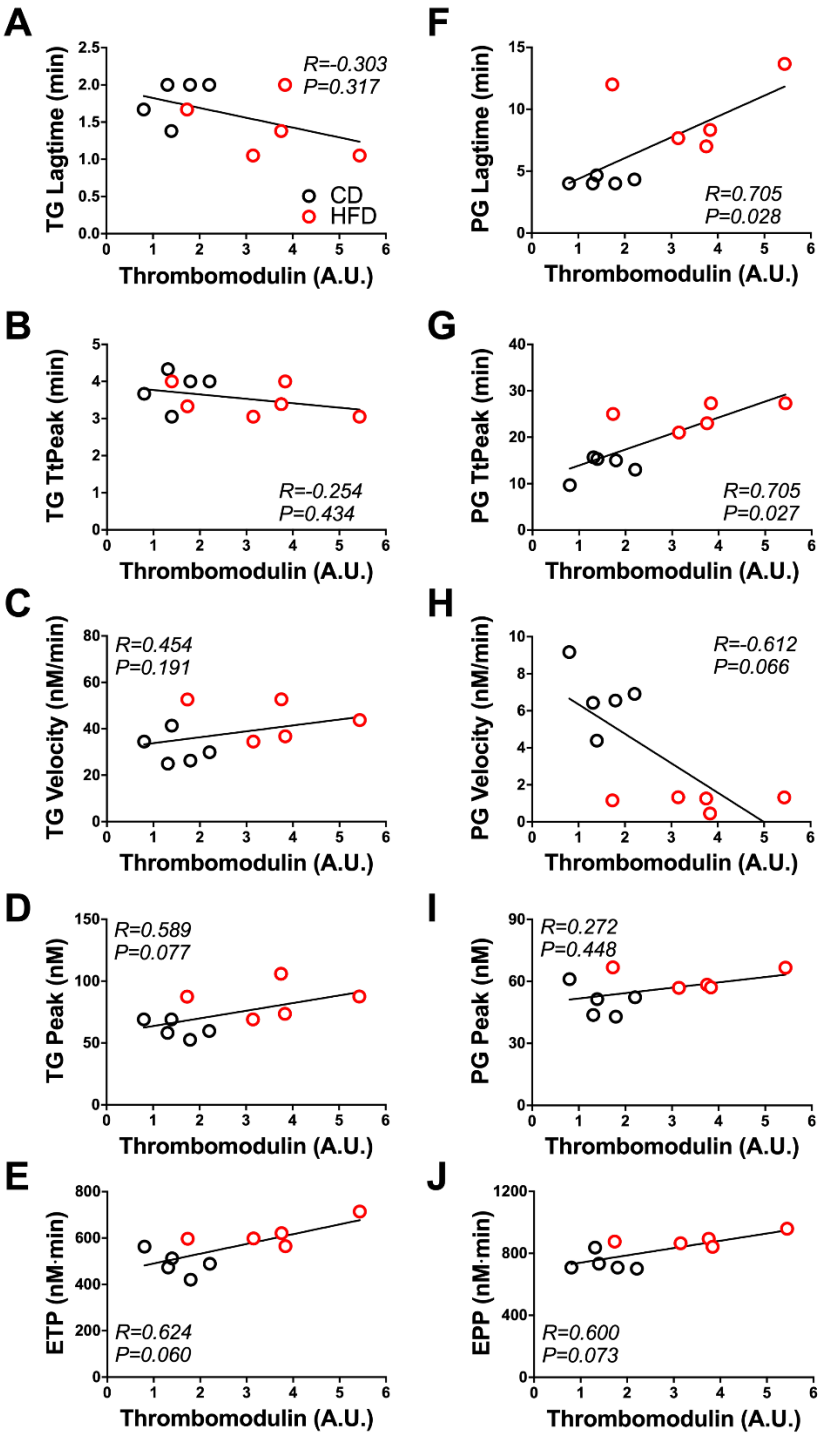
Supplemental Figure 4. Histological evidence of steatohepatitis and fibrosis in HFD-fed mice. Representative hematoxylin and eosin (HE), Picro Sirius Red (PSR) immunohistochemistry-stained liver from mice fed CD or HFD for 12 weeks.



Supplemental Figure 5. Effects of high fat diet (HFD) on thrombin generation (TG) and plasmin generation (PG) are also seen in plasma from mice fed 45% or 60% kcal from fat for 16 weeks. Mice were fed for 16 weeks with a control diet (CD, 13% kcal from fat) or HFD (45 or 60% kcal from fat). TG and PG were measured at 0.5 pM tissue factor and 0.31 µg/mL rtPA. (A-E) TG for CD and 45% kcal from fat. (F-J) PG for CD and 45% kcal from fat. (K-O) TG for CD and 60% kcal from fat. (P-T) PG for CD and 60% kcal from fat. Bars indicate medians. Each dot represents a separate mouse.



Supplemental Figure 6. PG correlates with C-1-inhibitor and thrombin-activatable fibrinolysis inhibitor (TAFI). PG parameters in mice fed control diet (CD, black circles) or high fat diet (HFD, red circles) for 12 weeks were correlated with (A-E) C-1-inhibitor (intensity from mass spectrometry analysis) and (F-J) TAFI determined by ELISA. Each dot represents a separate mouse.



Supplemental Figure 7. Plasmin generation (PG) correlates with soluble thrombomodulin. (A-E) TG and (F-J) PG in plasma from mice fed control diet (CD, black circles) or high fat diet (HFD, red circles) for 12 weeks. Each dot represents a separate mouse.

SUPPLEMENTAL TABLE

Supplemental Table 1. Complete list of plasma proteins identified by mass spectrometry (false discovery rate < 0.005) from mice fed control diet (CD) or high fat diet (HFD) for 12 weeks (separate file).