SUPPLEMENTAL MATERIAL

DNase 1 protects from increased thrombin generation and venous thrombosis during aging: cross-sectional study in mice and humans

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Running Title: Thrombotic Potential during Aging

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Data S1. Supplemental Methods

Neutrophil Isolation

Mouse neutrophils were isolated from bone marrow using Histopaque® (Sigma-Aldritch, St. Louis, MO) density centrifugation (Swamydas M et al, 2015, Ref#68). Briefly, bone marrow suspended in HBSS buffer was layered over Histopaque-10771 and Histopaque-11191 and centrifuged at 850 *g* for 30 min at room temperature. Neutrophils isolated from the interface were washed first with RPMI media and was finally suspended in HBSS buffer.

Neutrophils from human blood were isolated by red blood cell sedimentation with dextran 3%, followed by Ficoll-Hypaque density gradient centrifugation and hypotonic erythrocyte lysis as described (Clark et al 2001, Ref#69). Briefly, citrated blood was mixed gently with equal volume of dextran/saline and incubated for 20 min at room temperature. Leukocyte-rich plasma (upper layer) was collected and centrifuged at 250 x g, 4 ^{o} C for 10 min; pellet was immediately resuspended in 10 mL of 0.9% saline followed by gentle addition of 10 mL Ficoll-Paque TM PLUS (GE Healthcare, Sweden) beneath cell suspension. The contents were centrifuged at 400 x g for 40 min without brake, neutrophil/RBC pellet were resuspended in 20 mL ice cold water for 30 seconds; 20 mL of 1.8% NaCl was added to restore isotonicity. Finally, neutrophils (pellet) were collected after centrifugation at 250 x g, 4 ° C for 6 min and resuspended in PBS.

Fluorescent Microscopy to Examine NETosis:

NETosis assay was performed as described previously (Fuch et al 2007, Ref#18) with minor modifications. Freshly isolated neutrophils were seeded on coverslips (3 x 10^5 cells/coverslip) and incubated with HBSS for 30 minutes in a CO_2 incubator at 37 ^{0}C to allow cells to adhere to coverslip. Neutrophils were activated with PMA (100 nM) for 4 hours in a CO_2 incubator at 37 ^{0}C. Cells were fixed in ice-cold PBS containing 4% paraformaldehyde at room temperature for 15 min, washed with ice-cold PBS and incubated with SYTOX green dye (1:500 dilution, Invitrogen) for 15 minutes at room temperature in the dark. Coverslips were washed with PBS and mounted onto glass slides using a drop of mounting medium (Fluoromount, Sigma), prior to fluorescence microscopy. Samples were analyzed using an Olympus BX61 microscope. A total of 200-300 neutrophils were counted in five fields at 40x magnification per mouse. Cells undergoing NETosis were identified as those either losing lobulated nuclear structure or releasing extracellular chromatin. The final data is presented as % of cells undergoing NETosis (% NETs release). To perform colocalization of DNA and H3Cit, cells were permeabilized using 0.5% Triton X-100 for 10 min at room temperature, washed twice with PBS and blocked with 5% BSA for an hour at room temperature. After blocking the cells were incubated with primary antibody against H3Cit (1:750, ab5103, AbCam) overnight at 4^{0}C and then with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000) for 2 hours at room temperature. The cells were washed with PBS twice and mounted on to the clean glass slides with a drop of mounting agent containing DAPI, which stains the nuclear DNA. The images were acquired on Olympus BX61 microscope.

Thrombin Generation Potential

To measure thrombin generation potential in platelet poor plasma (PPP), whole blood from human subjects was collected via venous puncture and from mice through cardiac puncture in 3.2% sodium citrate (9:1, v/v). Blood was centrifuged at 3000 *g* for 10 min at room temperature. The supernatant fraction was transferred to a separate tube and centrifuged again at 10,000 *g* for 10 min to obtain PPP. The PPP was aliquoted and stored frozen at -80˚C until assays were performed. To measure thrombin generation in PRP, mouse blood was collected in corn trypsin inhibitor (CTI, 50 µg/mL) to inhibit the contact activation pathway, centrifuged at 150 *g* for 15 min, and platelet count was adjusted to 500 x 10^6 cells/mL with PPP (Gould et al 2014, Ref#8).

The Calibrated Automated Thrombogram (CAT, Diagnostica Stago, Inc, Parsippany, NJ) method, including the Thrombinoscope software, Fluoroskan Ascent, and CAT reagents were used to perform the thrombin generation assay (Hemker et al, 2005, Ref#70). Briefly, in the assay measuring platelet-independent thrombin generation, either 20 µl of mouse PPP ±± 60 µl of HEPES buffered saline (HBS) ±± 20 µl of PPP Reagent LOW or 80 µl of human PPP ±± 20 µl of PPP Reagent LOW, was incubated in round-bottom 96-well Immulon plates for 10 min at 37° C inside the instrument per the instructions from the Thrombinoscope software. PPP Reagent LOW contains a final concentration in the well of 1 pM tissue factor (TF) and 4 µM phospholipids. For platelet-dependent thrombin generation assay, 20 µl mouse PRP ±± 60 µl HBS was incubated for 10 min at 37° C inside the instrument with 20 µl PRP Reagent (Hemker et al, 2005, Ref#70). The PRP Reagent contains 1 pM TF (final concentration) and no phospholipids, as the phospholipids in the assay to support thrombin generation are

provided by the platelets from the sample. The FluCa fluorogenic substrate (Z-GGR-AMC) buffer solution containing $CaCl_2$ was added by the instrument, and thrombin generation was measured for 60 min. Data for lag time (min), thrombin peak (nM), and endogenous thrombin potential (ETP; nM.Min) were determined. In some experiments, PPP was treated with 20 µg/ml DNase 1 containing 1 mM $CaCl_2$ (Worthington Biochemicals, NJ) or equal amount of heat-inactivated DNase 1 (control) at 37 ˚ C for 60 min prior to running the assay.

To measure neutrophil-dependent thrombin generation, a total of 1 x 10^5 murine or human neutrophils was added to the pooled mouse or human plasma respectively. Samples were incubated with 100 nM phorbol myristate acetate (PMA) or vehicle buffer at 37 ˚ C for 60 min prior to running the assay (Gould et al 2014, Ref#8).

Table S1: Plasma levels of cell-free DNA (cfDNA), citrullinated histones (H3Cit), DNase 1, and parameters of thrombin generation potential in mouse plasma.

Mouse samples	**Young**		**Aged**	
Mouse PPP samples	**WT**	**Gpx1 Tg**	**WT**	**Gpx1 Tg**
ETP (nM.Min)	277.9±111.5	345.8±88.6	430.2±26.7***	353.6±19.6*
Peak (nM)	40.8±18.8	52.2±12.9	65.4±4.2$^{\$}$	58.1±4.7
Lag time (Min)	2.1±1	2.1±0.6	2.6±0.4	1.99±0.2
cfDNA (ng/µL)	11.4±2	14.6±3.1	28.2±2.2***	30.3±2.6$^{\$\$\$}$
H3Cit (ng/mL)	0.5±0.73	0.53±0.5	0.54±0.2	0.48±0.2
DNase 1 (ng/mL)	0.82±0.2	0.74±0.1	0.84±0.2	0.74±0.1
Prothrombin (µg/mL)	186.2±38.1	164±31.6	190.6±55.6	182.6±46.6
Mouse PRP samples				
ETP (nM.Min)	490±117.7	478.1±67.8	548.7±52.4	523.1±47.3
Peak (nM)	36.1±8.1	30.4±6.5	35.2±6.2	38.1±3.9
Lag time (Min)	5.9±1.6	6.2±0.7	5.1±0.5	5.9±0.3
Mouse PPP samples treated with Heat inactivated DNase1				
ETP (nM.Min)	285.6±81.9	337.9±84.3	410.9±102.7	346.2.8±123.2
Mouse PPP samples treated with DNase1				
ETP (nM.Min)	212.2±70.9	252.8±87.9	271.1±66.1	229.7±78.9
PPP samples collected from Mouse treated with Heat inactivated DNase1				
ETP (nM.Min)	445.9±72.8	434.8±84.5	506.4±125.9	454.9±130.3
cfDNA (ng/µL)	12.9±4.4	14.3±2.6	22.7±3.7$^{@@}$	20.3±4.9
PPP samples collected from Mouse treated with DNase1				
ETP (nM.Min)	343.9±68.8	317.9±76.4	361.8±94.8	329.5±40.7
cfDNA (ng/µL)	7.1±4.3	9.1±3.9	12.2±4.6$^{\#\#}$	13.1±2.8

Data are reported as mean± SD. N = 5-9 in each group. ETP, endogenous thrombin potential, PPP, Platelet Poor Plasma, PRP, Platelet Rich Plasma. *P<0.05 vs aged WT mice and ***P<0.001 vs young WT mice. $^{\$}$P<0.05 vs young WT mice and $^{\$\$\$}$P<0.001 vs young Gpx1 Tg mice. $^{@@}$P<0.01 vs young WT mice treated with heat inactivated DNase 1. $^{\#\#}$P<0.01 vs aged WT mice treated with heat inactivated DNase 1.

Table S2: Human subject characteristics

	Young	Middle-Aged/Older	P value
N	27	28	
Males (%)	13 (48.1)	14 (50.0%)	0.89
Age (years)	26.6 ± 5.3	58.5 ± 6.6	<0.0001
Body mass index (kg/m2)	24.8 ± 3.13	28.5 ± 4.32	0.40
Systolic BP	114± 11.4	122 ± 17.3	0.20
Diastolic BP	67 ± 8	74 ± 9	0.05

Data represented as mean ± SD; N, number of subjects; BP, Blood Pressure; Unpaired t-test.

Table S3: Plasma levels of cell-free DNA (cfDNA), citrullinated histones (H3Cit), DNase 1 and thrombin generation potential in humans.

Human samples	**Young**	**Middle-aged/Older**
ETP (nM.Min)	1401±274.5	1894.2±100.6****
Peak (nM)	211.9±75.9	298.6±20.8**
Lag time (Min)	5.9±1.1	6.1±0.28
cfDNA (ng/µL)	12.2±1.2	13.4±1.2***
H3Cit (ng/mL)	1.0±0.9	0.96±1.0
DNase 1 (ng/mL)	31.5±5.4	31.2±8.8
Prothrombin (µg/mL)	156.2±88.4	165.9±59.1
Human plasma samples treated with Heat inactivated DNase 1		
ETP (nM.Min)	1435±272.6	1820.5±685.5$^{\$}$
Human plasma samples treated with DNase 1		
ETP (nM.Min)	773.8±125.3$^{\$\$}$	831.5±64.8$^{\$\$}$

Data are reported as mean ± SD. ETP, endogenous thrombin potential. ****P<0.0001, ***P<0.001 and **P<0.01 vs young humans. $^{\$}$ P < 0.01 vs heat inactivated group of young humans. $^{\$\$}$ P < 0.0001 vs heat inactivated DNase 1 for same age group.

Supplemental Figures

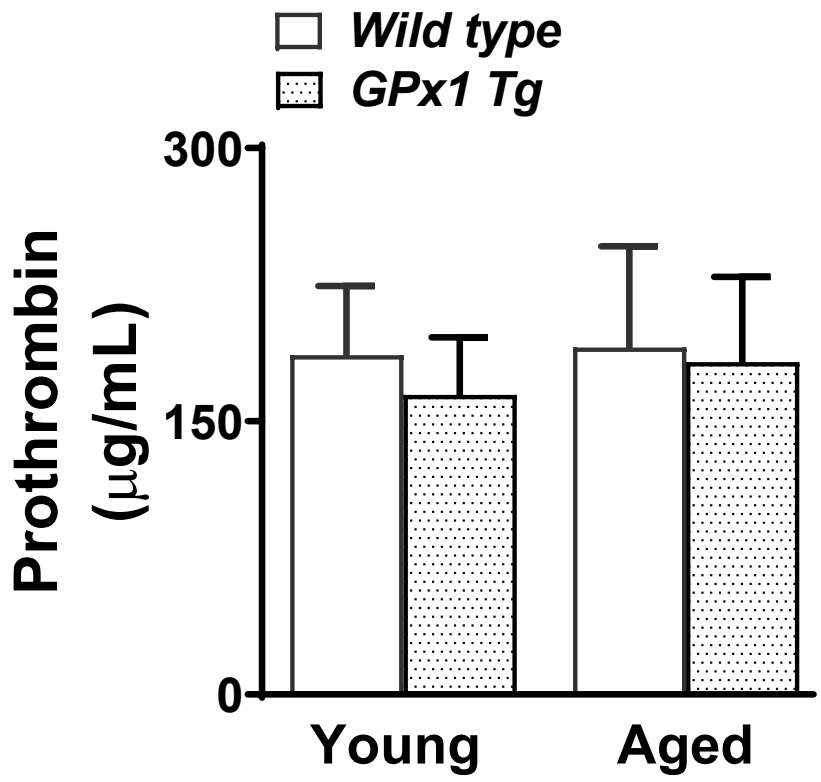
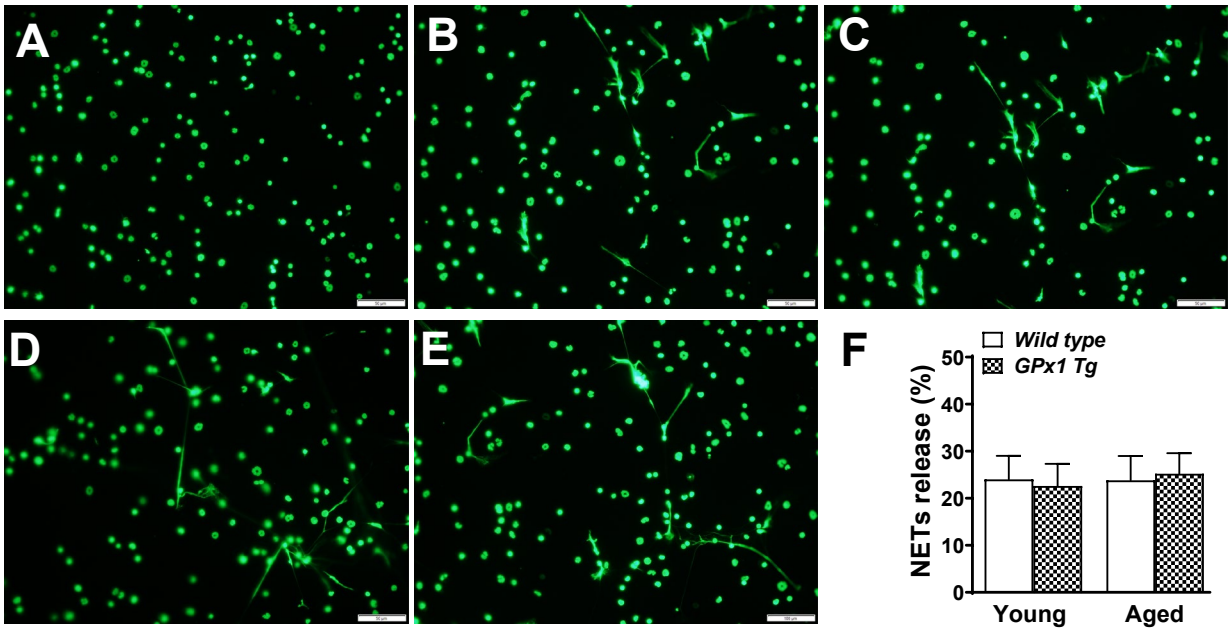


Figure S1: Plasma prothrombin levels in mice. Prothrombin level was measured in plasma from young (4 month) and aged (20 month) wild type mice and their littermates overexpressing Gpx1. N = 7-8 mice in each group. Data expressed as mean ± SD. Two-way ANOVA.


A
B
C
D
E
F
Wild type
GPx1 Tg
NETs release (%)
50
40
30
20
10
0
Young
Aged

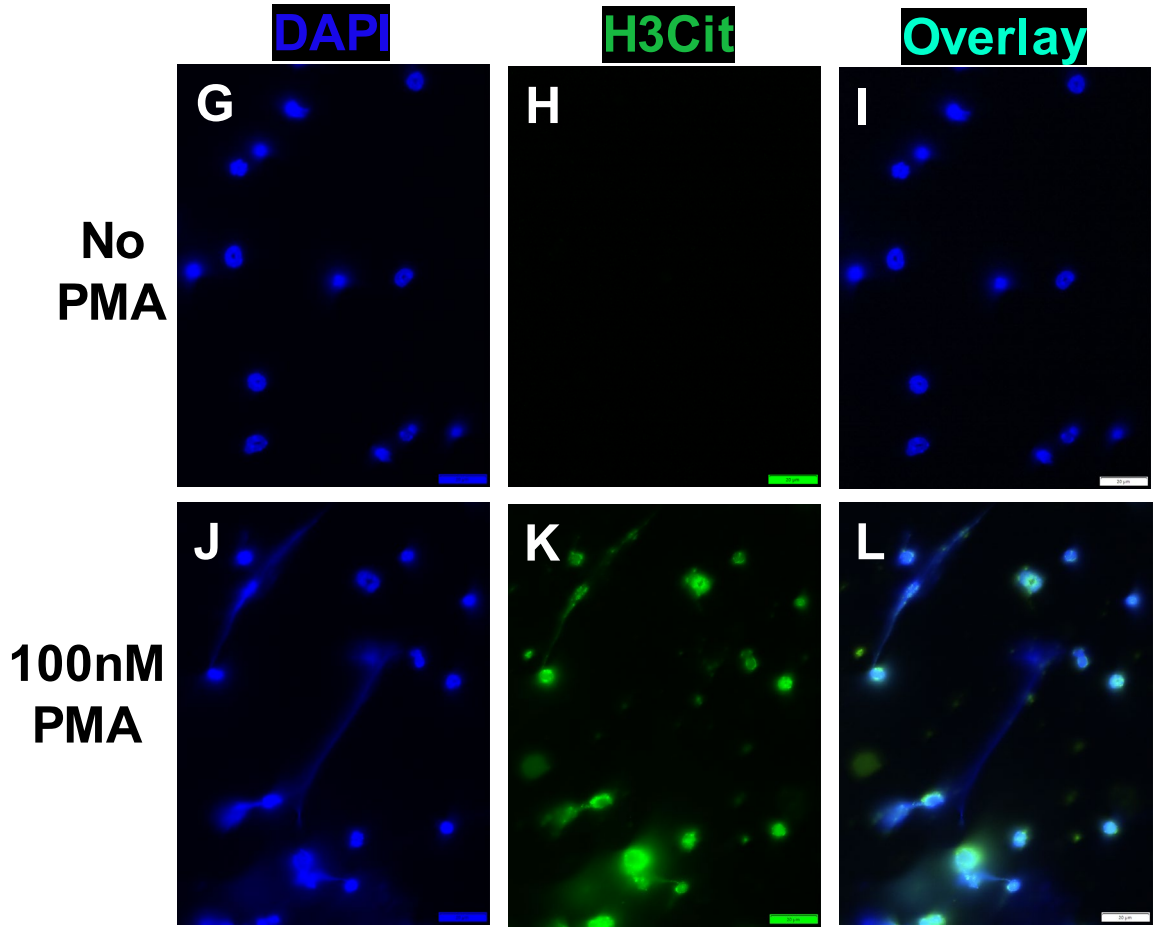

DAPI
H3Cit
Overlay
G
H
I
No PMA
J
K
L
100nM PMA

Figure S2: Aging or overexpression of Gpx1 do not influence extent of NETosis. Neutrophils were isolated from young and aged wild type (WT) or Gpx1 Tg littermates and activated with 100 nM PMA for 4 hours. Staining of extracellular nuclear material was performed either using SYTOX green to stain DNA (**A-E,** scale bar = 50 μM) or was double stained using DAPI and anti-H3Cit (**G-L,** scale bar = 20 μM). Representative images of NETs from **A.** Control neutrophils (no PMA, image from aged WT mouse [control images were similar in all groups], or **B-E.** PMA treated cells, **B.** Young WT, **C.** Young Gpx1 Tg, **D.** Aged WT, and **E.** Aged Gpx1 Tg mice. **F.** Bar graph of cells undergoing NETosis that were quantified in 5 different non-overlapping fields and is presented as % NETs release (counted as average number of cells releasing NETs). N = 5 mice in each group. Data expressed as mean ± SD. Two-way ANOVA. **G-L.** Images of either DAPI (showing as blue staining for DNA), anti-H3Cit (showing as bright green staining for cells undergoing NETosis) or overlay (showing as neon fluorescent cells undergoing NETosis) of control or PMA treated neutrophils from an aged WT mouse.

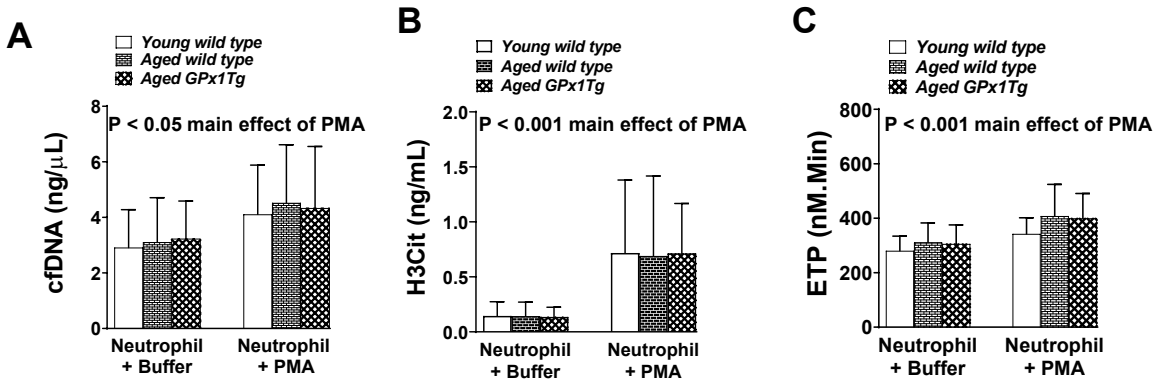


Figure S3: Neutrophils from young or aged mice display similar potential to release NETs and increase thrombin generation potential. Neutrophils were isolated from young and aged wild type mice or aged Gpx1 Tg littermates. A total of 1 x 10^5 cells was added to pooled mouse plasma and incubated with 100 nM PMA or control buffer for 60 min, followed by measurement of cfDNA (**A**), H3Cit (**B**), and endogenous thrombin potential i.e. ETP (**C**). N = 6-7 mice in each group. Data expressed as mean ± SD. Two-way ANOVA (2 x 3) showing main effect of PMA is significant for A, B and C.

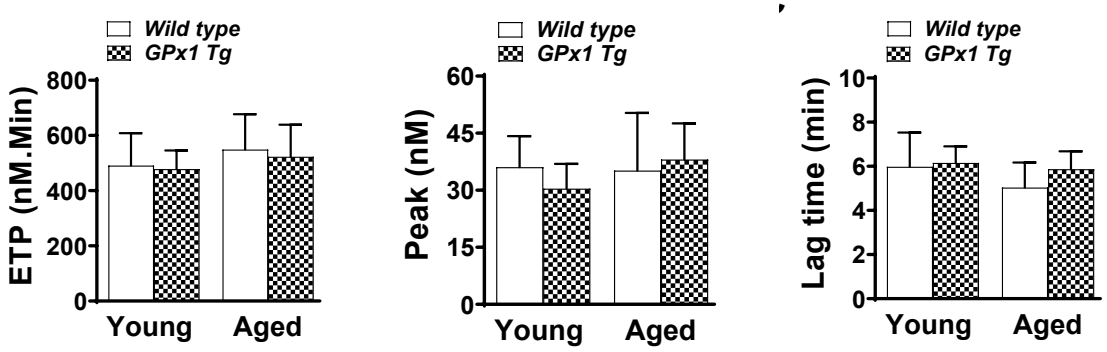


Figure S4: Murine aging does not increase platelet-dependent thrombin generation. (A) endogenous thrombotic potential, i.e. ETP, **(B**) thrombin peak and **(C)** lag time in PRP of young and aged wild type mice as well as littermates overexpressing Gpx1 (Gpx1 Tg). Data expressed as mean ± SD. N = 5 mice in each group. Two-way ANOVA.

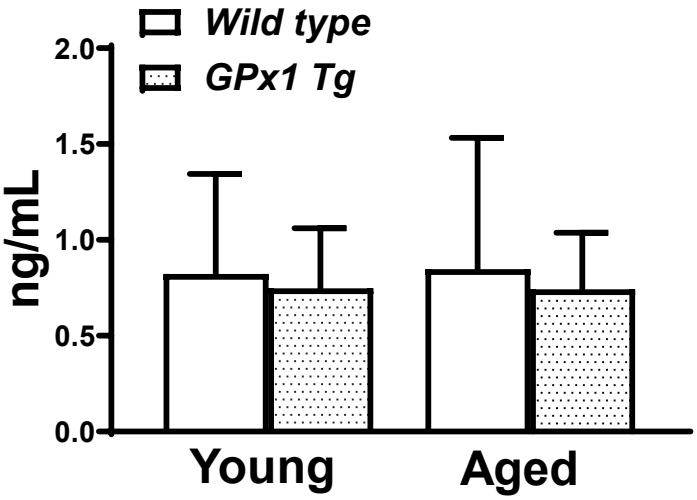


Figure S5: Plasma DNase 1 levels in mice. Antigen levels of DNase 1 were measured in plasma from young (4 month) and aged (20 month) wild type mice and their littermates overexpressing Gpx1. N = 6-8 mice in each group. Data expressed as mean ± SD. Two-way ANOVA.

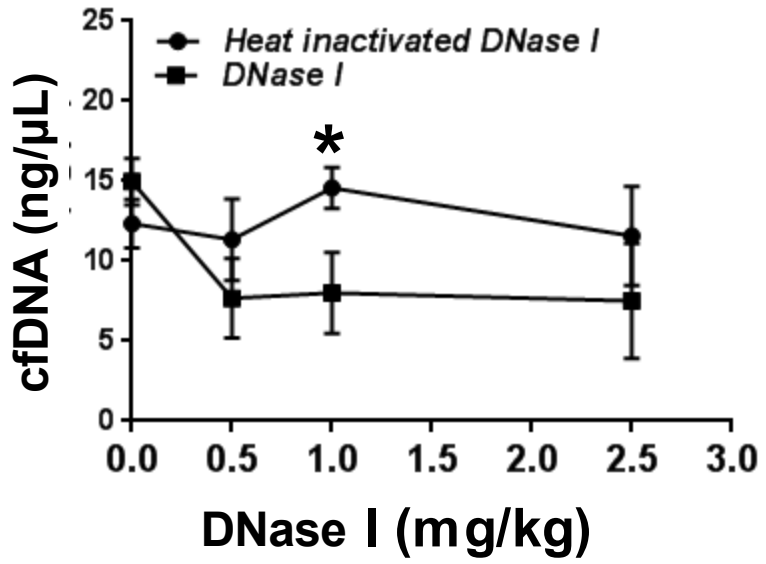


Figure S6: Dose dependence of DNase 1 infusion on plasma cfDNA. Wild type mice were treated retro-orbitally with either DNase 1 or heat-inactivated DNase 1, blood was collected 60 minutes post-infusion and cfDNA was measured. Data expressed as mean ± SEM. N = 4 mice in each group. *P < 0.05 vs. DNase 1, Two-way ANOVA.

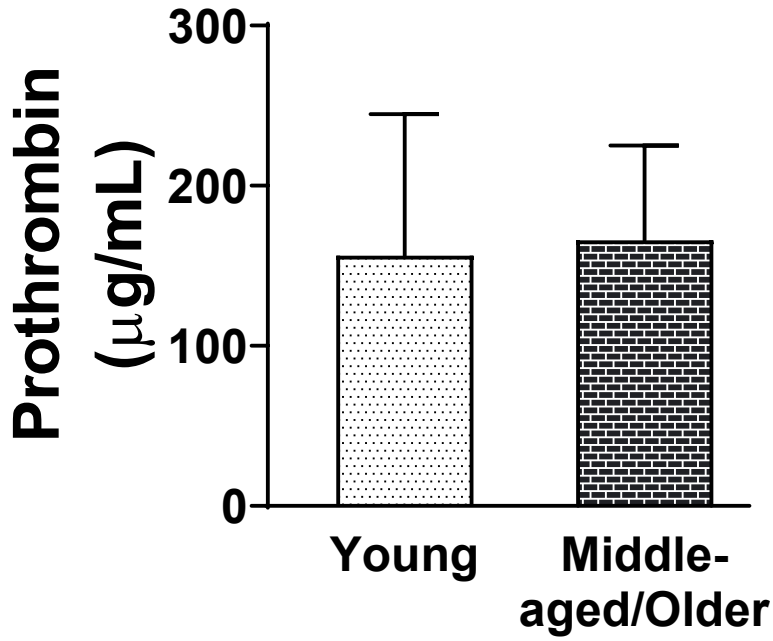


Figure S7: Plasma prothrombin levels in humans. Prothrombin level was measured in plasma from healthy young (18-39 years) and Middle-aged/older (50-71 years) subjects. N = 17-18 subjects in each group. Data expressed as mean ± SD. Unpaired t-test.

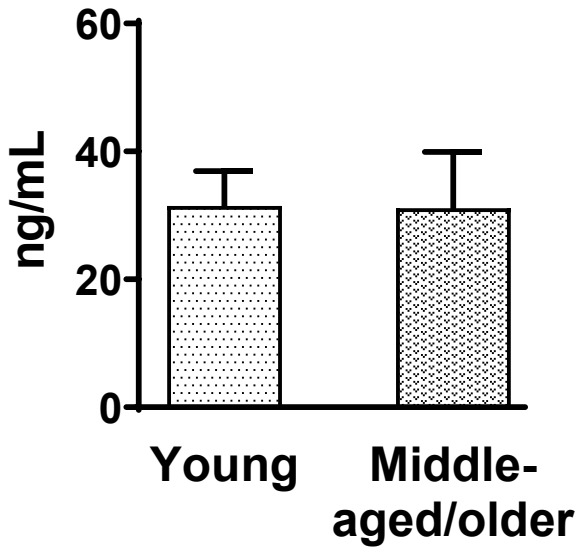


Figure S8: Plasma DNase 1 levels in humans. Antigen levels of DNase 1 were measured in plasma from healthy young (22-38 years) and Middle-aged/older (51-71 years) subjects. N = 12-15 subjects in each group. Data expressed as mean ± SD. Unpaired t-test.

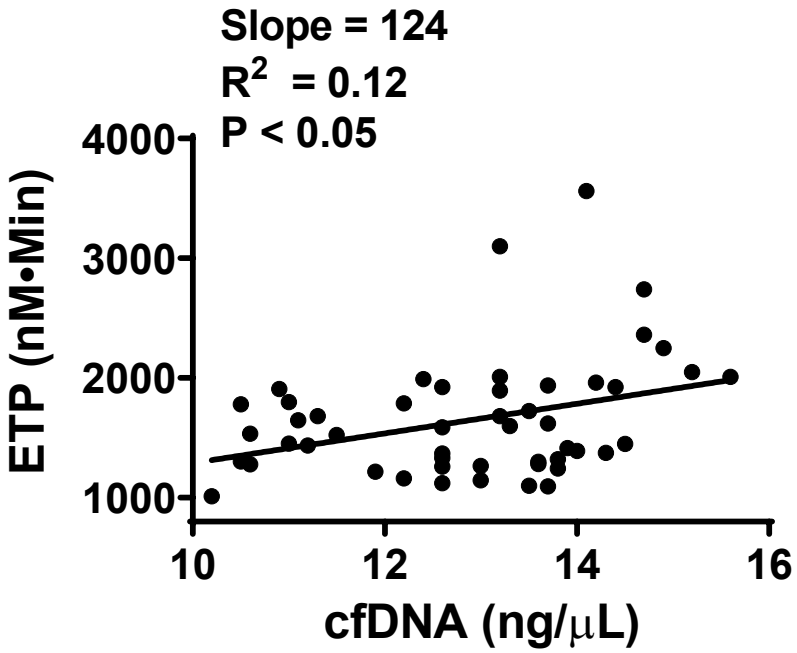


Figure S9: Elevated plasma cfDNA level in humans is associated with increased thrombin generation. Plasma cfDNA and endogenous thrombin potential (ETP) was measured using platelet poor plasma from young (18-39 years) and middle-aged/older subjects (50-72 years). N = 55. Data set was analyzed using simple linear regression.

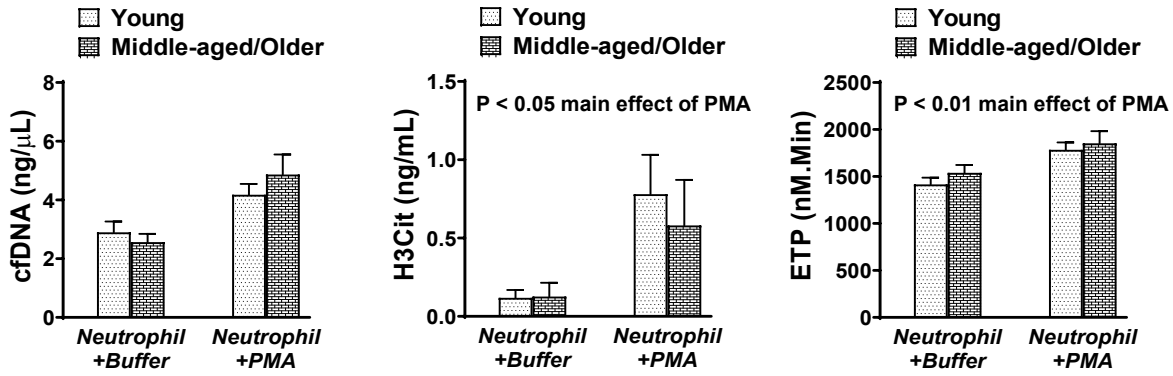


Figure S10: Activation of neutrophils from either young or middle-aged/older humans with PMA produces similar increases in plasma cfDNA, H3Cit and thrombin generation. Neutrophils were isolated from healthy young and middle-aged/older humans. A total of 1 x 10^5 cells was added to the pooled human plasma and incubated with 100 nM PMA or control buffer for 60 min, followed by measurement of cfDNA (**A**), H3Cit (**B**), and endogenous thrombin potential i.e. ETP (**C**). N = 5 subjects in each group. Data expressed as mean ± SD. Two-way ANOVA, showing main effect of PMA is significant for cfDNA, H3Cit as well as ETP.