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ORIGINAL RESEARCH

DNase 1 Protects From Increased Thrombin Generation and Venous Thrombosis During Aging: Cross-Sectional Study in Mice and Humans

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BACKGROUND: Human aging is associated with increased risk of thrombosis, but the mechanisms are poorly defined. We hypothesized that aging induces peroxide-dependent release of neutrophil extracellular traps that contribute to thrombin generation and thrombosis.

METHODS AND RESULTS: We studied C57BL6J mice and littermates of glutathione peroxidase-1 transgenic and wild-type mice at young (4 month) and old (20 month) ages and a healthy cohort of young (18–39 years) or middle-aged/older (50–72 years) humans. In plasma, we measured thrombin generation potential and components of neutrophil extracellular traps (cell-free DNA and citrullinated histone). Aged wild-type mice displayed a significant increase in thrombin generation that was decreased in aged glutathione peroxidase-1 transgenic mice. Both aged wild-type and aged glutathione peroxidase-1 transgenic mice demonstrated similar elevation of plasma cell-free DNA compared with young mice. In contrast, plasma levels of citrullinated histone were not altered with age or genotype. Release of neutrophil extracellular traps from neutrophils in vitro was also similar between young and aged wild-type or glutathione peroxidase-1 transgenic mice. Treatment of plasma or mice with DNase 1 decreased age-associated increases in thrombin generation, and DNase 1 treatment blocked the development of experimental venous thrombi in aged C57BL6J mice. Similarly, thrombin generation potential and plasma cell-free DNA, but not citrullinated histone, were higher in middle-aged/older humans, and treatment of plasma with DNase 1 reversed the increase in thrombin generation.

CONCLUSIONS: We conclude that DNase 1 limits thrombin generation and protects from venous thrombosis during aging, likely by hydrolyzing cell-free DNA.

Key Words: age ■ aging ■ extracellular traps ■ oxidative stress ■ thrombosis

Human aging is associated with an increased risk of thrombotic complications,[1–3] but the mechanisms of thrombus development are not fully elucidated. Emerging data suggest that plasma from patients with thrombotic events such as myocardial infarction,[4] acute ischemic stroke,[5] or deep vein thrombosis[6] exhibits increased potential for thrombin generation, which can be quantitated as endogenous thrombin potential (ETP), when exposed to an extrinsic trigger such as tissue factor. Increased ETP has also been reported in conditions such as diabetes,[7] sepsis,[8,9] and cancer-associated thrombosis.[10,11] Studies have suggested that human aging is associated with an increase in markers of thrombin generation even in

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CLINICAL PERSPECTIVE

What Is New?

- Aging increases circulating cell-free DNA in a NETosis-independent pathway.
- DNase 1 protects from age-induced increase in endogenous thrombin potential and venous thrombosis.

What Are the Clinical Implications?

- Cell-free DNA and endogenous thrombin potential may serve as the biomarkers for a prothrombotic state during aging.
- Therapeutic potential of DNase 1 for the treatment of age-associated thrombotic events should be considered.

Nonstandard Abbreviations and Acronyms

cfDNA	cell-free DNA
ETP	endogenous thrombin potential
Gpx1 Tg	glutathione peroxidase-1 transgenic
H3Cit	citrullinated histone
NETs	neutrophil extracellular traps
PMA	phorbol myristate acetate
PPP	platelet poor plasma
WT	wild type

the absence of any known thrombotic risk factors or a clinical thrombotic event.[12–14] It is likely that the subset of older healthy subjects exhibiting increased potential for thrombin generation is more vulnerable to a future thrombotic event. Therefore, better understanding of the early mechanisms leading to increased potential for thrombin generation in aging may lead to the development of diagnostics for identifying an underlying subclinical prothrombotic state.

Using experimental models of thrombosis, we[15] and others[16,17] have demonstrated that aged mice display increased susceptibility to arterial and venous thrombosis. We have further observed that aged mice overexpressing the antioxidant glutathione peroxidase-1 (Gpx1; an enzyme that converts peroxides to water) are protected from increased thrombotic susceptibility.[15] However, it is not known whether, like humans, aged mice also display increased potential for thrombin generation and whether it is modulated by peroxide. It has been demonstrated that hydrogen peroxide (H_2O_2) mediates activation of neutrophils in vitro, and subsequent release of neutrophil extracellular traps (NETs), in a process called NETosis.[18] NETosis involves chromatin decondensation that occurs through peptidylarginine deiminase 4–mediated deimination of nuclear histones.[19] NETs contain cell-free DNA (cfDNA), histones, citrullinated histone H3 (H3Cit), elastase, and myeloperoxidase. The cfDNA and histones are pro-thrombotic in nature.[20] Extracellular traps are classically defined to be released from neutrophils,[21] but recently other cells such as eosinophils,[22] mast cells,[23] and basophils[24] also have been documented to release extracellular traps. Components of NETs have been identified in plasma and tissues during aging. For example, tissues of elderly patients with severe vasculitis exhibit dense infiltration of H3Cit-positive neutrophils,[25,26] and elevated plasma cfDNA has been observed with human aging in the absence of any vascular risk factors.[27] One study also observed NETosis in conjunction with cardiac and lung fibrosis in murine aging.[28] Furthermore, levels of reactive oxygen species are known to increase with human aging in several tissues, including neutrophils,[29] and reactive oxygen species such as H_2O_2 are known to release NETs in vitro.[18] It is not known, however, whether increased NETosis in aging leads to increased thrombin generation potential in a peroxide-dependent manner or whether it contributes to age-associated thrombosis.

Components of NETs such as cfDNA and histones can contribute to increased thrombin generation,[8] and NETs have been proposed to promote the development of venous thrombosis in mice.[30] We hypothesized that aging induces peroxide-dependent release of NETs that contribute to thrombin generation and venous thrombosis. Herein, using mice overexpressing Gpx1 and their wild-type (WT) littermates, and plasma samples from healthy young and middle-aged/older human subjects, we demonstrate that both murine and human aging is associated with increased potential for thrombin generation, which is likely mediated through cfDNA. Surprisingly, the aging-related prothrombotic effects of cfDNA were independent of peroxide and NETosis, suggesting an alternative mechanism for generation of cfDNA during aging. Finally, our data in mice demonstrated that treatment with DNase 1 protects aged C57BL6J mice from increased susceptibility to venous thrombosis.

METHODS

The data that support the findings are available from the corresponding author upon reasonable request.

Mice

C57BL6J mice and mice overexpressing Gpx1 (Gpx1 transgenic [Gpx1 Tg] mice)[31] and their WT littermates were included in the study design. Genotyping for the Gpx1 transgene was performed using real-time

polymerase chain reaction.[32] All animal protocols were approved by the University of Iowa Animal Care and Use Committee. Male and female mice were examined at 4 and 20 months of age. Blood samples were collected in 3.2% Na-citrate through heart puncture. For treatment of mice with DNase 1 followed by thrombin generation assay, mice were infused with either 1 μg/g of DNase 1 or heat-inactivated DNase 1 (free of other proteases, LS006344 DPRFS; Worthington Biochemicals, NJ) retro-orbitally, 60 minutes before blood collection.

Human Subjects

Stored plasma samples utilized in this study were available from healthy human subjects recruited under 2 Institutional Review Board–approved protocols: a randomized clinical study (IRB# 201201739, NCT01775865),[33] and a study of platelets in aging (IRB#201309851). From NCT01775865, only the baseline samples were used before any intervention. Samples from a total of 27 young (age 18–39 years) and 28 middle aged/older subjects (age 50–72 years) were analyzed. The proportion of men and women was similar between groups, and all subjects were non-Hispanic White individuals. Healthy subjects were recruited from the Iowa City community through flyers and email advertisements to participate in a single visit, cross-sectional study. Subjects were free of cardiovascular and metabolic disease, were not taking any medications for a known chronic condition, and were ambulatory and independent. All blood samples were collected in the morning after an overnight fast from an antecubital intravenous catheter 15 minutes after catheterization or via butterfly needle into tubes containing 3.2% Na-citrate. All participants gave written informed consent. Recruitment and blood collection protocol was approved by Institutional Review Board at the University of Iowa.

Thrombin Generation

We measured thrombin generation using platelet poor plasma (PPP) or platelet rich plasma in a Calibrated Automated Thrombogram (CAT, Diagnostica Stago, Inc, Parsippany, NJ). The details of methods are provided in Data S1.

Prothrombin, DNase 1, and Components of NETs

Plasma levels of prothrombin were measured using murine and human specific enzyme-linked immunosorbent assay kits (Molecular Innovations, MI). Plasma levels of DNase 1 were quantified using a commercially available kit (from LifeSpan BioSciences, Inc. for mouse and Cloud Clone Corp. for human). Quantification of circulating cfDNA in plasma samples was performed using the Qubit dsDNA HS Assay kit (Invitrogen, Life Technologies, Carlsbad, CA) according to manufacturer's instructions. The levels of H3Cit were measured using a commercially available kit (Caymen Chemical, MI).

Experimental Venous Thrombosis

Mice anesthetized with isoflurane underwent a stasis model of experimental deep vein thrombosis,[15] where inferior vena cava and all other visible side and back branches were ligated. Mice received retro-orbitally either 2.5 μg/g DNase 1 or heat-inactivated DNase 1 (Pulmozyme, Genentech Inc., CA), 30 minutes before experimental thrombosis. Thereafter, the same amount was injected intraperitoneally every 12 hours. This protocol was adapted from previous reports[30,34] with minor modifications. After 48 hours of ligation, mice were euthanized, and thrombus developed in inferior vena cava was harvested for measurement of the length and weight.

Statistical Analysis

All data were analyzed using GraphPad Prism software. Normality testing was performed using D'agostino-Pearson omnibus. A 2-way ANOVA with the Tukey test for multiple comparisons was performed in mice for measures of thrombin generation, cfDNA, and H3Cit and a 3-way ANOVA with Šídák's multiple comparisons test was used in studies using DNase 1. Kruskal–Wallis with the Dunn's test for multiple comparisons was used to analyze data for experimental venous thrombosis. In human plasma samples, mean and SD were reported for continuous measures stratified by the young versus middle-aged/older groups; count and percentage were reported for categorical measures stratified by the young versus middle-aged/older groups. Continuous measures between the young versus middle-aged/older groups were compared using unpaired *t* test. Linear regression analysis was performed to determine effects of age and cfDNA on thrombin generation. Statistical significance was defined as a value of $P<0.05$.

RESULTS

Murine Aging Is Associated With Increased Thrombin Generation Potential That Is Moderately Reversed With Gpx1 Overexpression

To determine whether thrombin generation potential is increased during aging and whether this effect is modulated through peroxide, we first performed a fluorometric-based thrombin generation assay in PPP collected from young and aged WT and Gpx1 Tg mice. For ETP, which is

measured as area under the thrombin generation curve, 2-way ANOVA indicated a significant main effect for age (P<0.01) but not for genotype (P=0.68). However, there was a significant interaction between age and genotype (P<0.05), reflecting a loss of age-related increase in ETP in Gpx1 Tg mice (Figure 1A, Table S1). Accordingly, the ETP was significantly increased in aged WT mice compared with young WT mice (P<0.001), whereas ETP was not significantly different in aged Gpx1 Tg mice compared with either young WT mice (P=0.069) or young Gpx1 Tg mice (P=0.066). These findings suggest that peroxide contributes to aging-associated increases in thrombin generation potential. A similar pattern was observed for thrombin peak, with a significant main effect for age (P<0.01) but not for genotype (P=0.69) or interaction between age and genotype (P=0.07) (Figure 1B, Table S1). Aged WT mice displayed a significantly higher thrombin peak than young WT mice (P<0.05), but the thrombin peak in aged Gpx1 Tg mice was not significantly different from young Gpx1 Tg mice (P=0.8) or aged WT mice (P=0.7). The lag time, which represents the time to onset of thrombin generation, did not differ significantly among the groups (Figure 1C, Table S1). Plasma prothrombin levels were also not significantly different among the groups (Figure S1, Table S1).

Aged Mice Display Elevated Levels of cfDNA Regardless of Gpx1 Overexpression

Since NETs are known to contribute to thrombin generation, we next tested whether there was an increase in components of NETs in the plasma of aged mice and whether they are modulated by peroxide. We measured levels of cfDNA and H3Cit in plasma samples from WT and Gpx1 Tg mice in both age groups. Two-way ANOVA indicated a significant main effect for age on cfDNA (P<0.0001, Figure 2A, Table S1), but the effects for genotype (P=0.16) or the interaction of age and genotype (P=0.6) on cfDNA were not significant. A significant increase in the levels of cfDNA in both aged WT mice (P<0.0001 versus young WT mice) and aged Gpx1 Tg mice (P<0.0001 versus young Gpx1 Tg mice) was observed and the levels of cfDNA in aged WT mice were similar to those in aged Gpx 1 Tg mice (P=0.5). These findings suggest that age-dependent elevation in cfDNA is not modulated by peroxide. In contrast to cfDNA, H3Cit levels did not differ significantly among the groups (main effects of age, P=0.7, genotype, P=0.7, and interaction, P=0.6, Figure 2B, Table S1). Regression analysis revealed a positive linear relationship between cfDNA and both ETP and thrombin peak (P<0.05 for both, Figure 2C and 2D). The relationship between cfDNA and H3Cit was not significant (data not shown). The absence of an increase in H3Cit in aged WT mice suggests that age-associated changes in cfDNA and ETP may not be mediated via NETosis.

We next utilized in vitro approaches to directly test the susceptibility of neutrophils from young or aged mice to undergo NETosis and whether this response is modulated by peroxide. We isolated neutrophils from mice and seeded on coverslips. Neutrophils were isolated from young or aged WT or Gpx1 Tg mice, and release of NETs with phorbol myristate acetate (PMA) stimulation was monitored using fluorescent microscopy. The percentage of neutrophils releasing NETs in response to PMA were comparable between the groups (Figure S2); neither the main effect for age (P=0.58) nor genotype (P>0.99) nor the interaction of age and genotype (P=0.52) were significant. Next, neutrophils from young WT, aged WT, or aged Gpx1 Tg mice were suspended in pooled mouse plasma and incubated with either the vehicle buffer (control) or

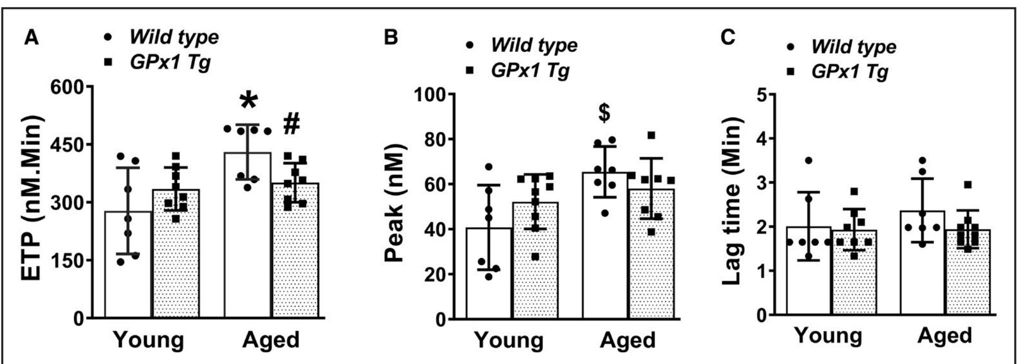

Figure 1. Murine aging is associated with increased thrombin generation, which is moderately reversed with Gpx1 overexpression.
Thrombin generation was measured using calibrated automated thrombogram in platelet poor plasma in young and aged wild-type mice as well as littermates overexpressing Gpx1 (glutathione peroxidase-1 transgenic [Gpx1 Tg]). **A**, Endogenous thrombin potential (ETP). **B**, Peak thrombin generation. **C**, Lag time. Data expressed as mean±SD. *P<0.001 vs young wild type, $^{\#}P$<0.05 vs aged wild type, and $^{\$}P$<0.05 vs young wild type (2-way ANOVA with Tukey test). N=7 to 8 in each group.

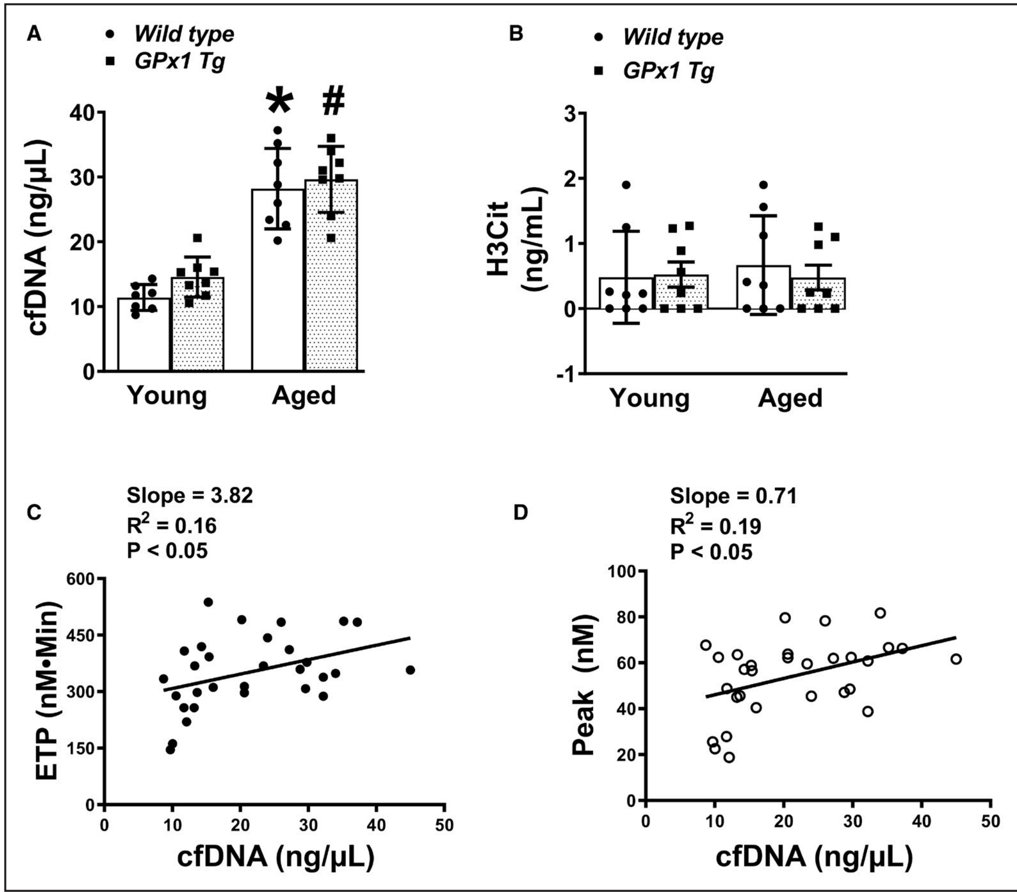

Figure 2. Aged mice display elevated levels of cfDNA regardless of Gpx1 overexpression and cfDNA has a positive effect on thrombin generation.

Components of neutrophil extracellular traps were measured in plasma from young and aged wild-type mice and littermates overexpressing Gpx1 (glutathione peroxidase-1 transgenic [Gpx1 Tg]). **A**, Plasma cell-free DNA (cfDNA). **B**, Plasma citrullinated histone (H3Cit). Data for (**A** and **B**) are expressed as mean±SD. **C**, Linear regression analysis between cfDNA and endogenous thrombin potential (ETP). **D**, Linear regression analysis between cfDNA and thrombin peak. **P*<0.0001 vs young wild type and #*P*<0.001 vs young GPx1 Tg mice (2-way ANOVA with Tukey test). N=7 to 8 in each group.

PMA. Compared with vehicle, stimulation with PMA increased levels of cfDNA, H3Cit, and ETP (the main effect for PMA was significant, with *P*<0.05, *P*<0.001, and *P*<0.001, respectively, Figure S3). Within the PMA-treated groups, however, no further elevations in cfDNA, H3Cit, or ETP were observed in aged WT or aged Gpx1 Tg mice compared with young WT mice. These findings suggest that (1) neutrophils from young and aged mice release similar levels of NETs and produce similar increases in ETP after PMA activation, and (2) peroxide does not modulate the in vitro release of NETs from neutrophils upon PMA stimulation, even in aged mice.

Platelet-Dependent Thrombin Generation Is Not Altered by Murine Aging

Since histones can promote thrombin generation through platelet activation,[35] we next tested whether platelet-dependent thrombin generation is increased with age and whether mice overexpressing Gpx1 are protected. Platelet-dependent thrombin generation was measured in platelet rich plasma. In contrast to findings with PPP, no significant main effects of age, genotype, or the interaction of age and genotype on ETP or peak thrombin generation were observed in platelet rich plasma (Figure S4, Table S1); these findings suggest that platelet-dependent thrombin generation does not change with age in this model.

Incubation of Plasma Samples With DNase 1 Decreases Thrombin Generation Potential in Mice

First, to determine whether levels of endogenous DNase 1 are affected by age, we measured DNase 1 antigen levels in plasma samples (Figure S5, Table S1). The DNase 1 levels were not found to be altered because of age (*P*=0.9) or genotype (*P*=0.6). Next, to assess the

potential mechanistic role of cfDNA in driving thrombin generation during aging, we treated PPP samples from young or aged mice with either DNase 1 or heat-inactivated DNase 1 (as a control) before measurement of thrombin generation. By 3-way ANOVA, the main effect of treatment was significant (*P*<0.001, Figure 3, Table S1). However, the main effect of age or genotype was not significant. Also, there were no significant interactions between age, genotype, or treatment. We observed 31% and 26% reductions in ETP in young WT and young Gpx1 Tg mice, respectively, and 43% and 39% reductions in aged WT and aged Gpx1 Tg mice, respectively, with DNase 1 treatment. Also, within the DNase 1–treated groups, we did not observe any differences in ETP between young or aged mice or mice with Gpx 1 overexpression. These findings indicate that DNase 1 treatment normalized the differences in ETP between young and aged mice.

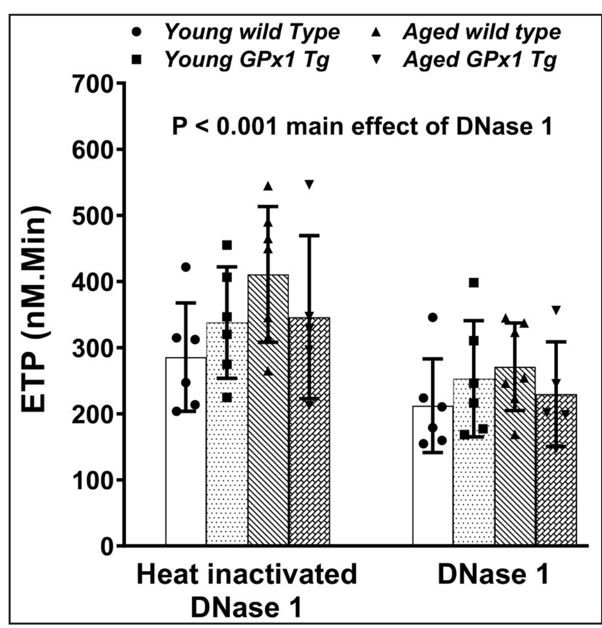

Figure 3. In vitro treatment of plasma with DNase 1 lowered age-associated increase in thrombin generation potential in mice regardless of genotype.
Platelet poor plasma from young and aged wild-type mice or glutathione peroxidase-1 transgenic (Gpx1 Tg) littermates were treated with either 20 μg/mL of DNase 1 or same dose of heat-inactivated DNase 1 as control for 60 minutes, and thrombin generation (endogenous thrombin potential [ETP]) was measured. Data are expressed as mean±SD. Three-way ANOVA with Šídák's multiple comparisons. N=5 to 7 in each group.

Treatment of Aged Mice With DNase 1 Lowers Plasma cfDNA Burden and Protects From Increased Thrombin Generation Potential Ex Vivo and Susceptibility to Venous Thrombosis In Vivo

To determine whether lowering of cfDNA in vivo would decrease thrombin generation potential and protect aged mice from increased susceptibility to thrombosis, we infused mice retro-orbitally with DNase 1 or heat-inactivated DNase 1. First, we performed a dose–response study in young C57BL6J mice and observed that 1 mg/kg DNase 1 significantly decreased levels of circulating cfDNA 60 minutes postinfusion (Figure S6). Compared with mice infused with heat-inactivated DNase 1, infusion of DNase 1 significantly lowered cfDNA and ETP (main effect for treatment was *P*<0.0001 and *P*<0.01, respectively, by 3-way ANOVA, Figure 4A and 4B, Table S1). We also observed a significant main effect of age (*P*<0.0001 for cfDNA, and *P*<0.01 for ETP), but not of genotype and there were no significant interactions between age, genotype, or treatment. Post DNase 1 infusion, the levels of cfDNA or ETP were similar in all 4 groups of mice, suggesting that infusion of DNase 1 normalized differences between young and aged mice regardless of genotype. Accordingly, while the linear regression analysis within the heat-inactivated DNase 1 infusion group revealed a positive relation between cfDNA and ETP (slope=10, R^2=0.25 and *P*<0.05, Figure 4C), this relationship was lost after DNase 1 treatment (slope=−6.8, R^2=0.11 and *P*=0.11, Figure 4D).

Finally, in a murine experimental deep vein thrombosis model, we tested the effect of elevated cfDNA on venous thrombosis in aged mice. Since overexpression of Gpx1 did not alter NETosis, cfDNA levels, or the effect of DNase 1 treatment on ETP, we chose to study the role of cfDNA in venous thrombosis by infusing DNase 1 in young and aged WT mice rather than Gpx1 Tg mice. We infused young and aged C57BL6J mice before and every 12 hours during the 48 hours of inferior vena cava ligation with either DNase 1 or heat-inactivated DNase 1. We compared the length and weight of thrombi that developed at 48 hours after ligation. The main effect of treatment for both length and weight of thrombi was significant (*P*<0.01), and there was also a significant interaction between age and treatment for the length (*P*<0.01), but not weight (*P*=0.1) of the thrombus. Aged control mice developed longer-length thrombi than young mice (*P*<0.05, Figure 5A), and treatment with DNase 1 significantly reduced the thrombus length and weight in aged mice (*P*<0.01 and *P*<0.001, respectively, Figure 5A and 5B).

Together, these findings suggest that DNase 1 protects from age-associated increased thrombin generation potential and susceptibility to venous thrombosis in mice.

Human Aging Is Associated With Increased Thrombin Generation Potential and Elevated cfDNA

To examine how the aforementioned findings in aged mice are translated into human aging, we examined

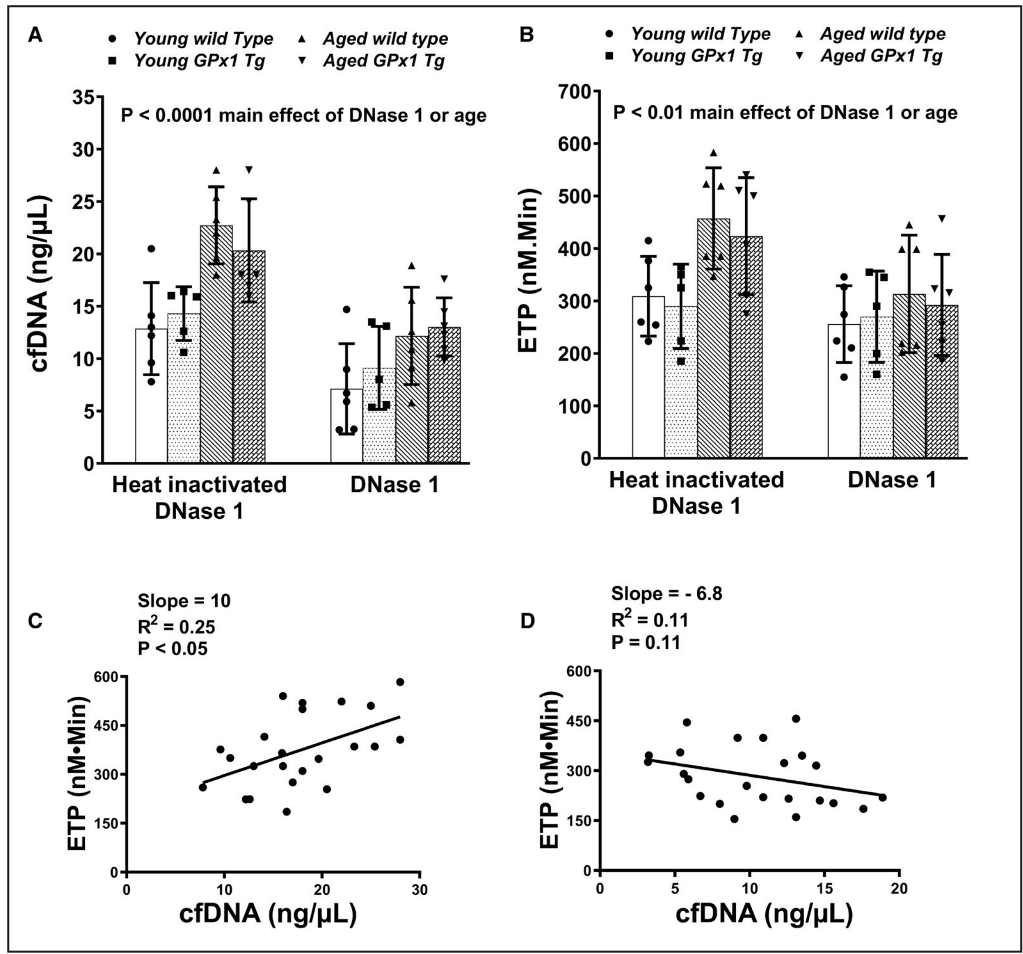

Figure 4. In vivo treatment with DNase 1 lowered potential of plasma to generate thrombin ex vivo in aged mice regardless of genotype.

Young and aged wild-type mice or glutathione peroxidase-1 transgenic (Gpx1 Tg) littermates were treated retro-orbitally with either 1 mg/kg of DNase 1 or heat-inactivated DNase 1. Blood was collected 60 minutes postinfusion, and plasma cell-free DNA (cfDNA) (**A**) and endogenous thrombotic potential (ETP) (**B**) were measured. Data are expressed as mean±SD and analyzed with 3-way ANOVA with Šídák's multiple comparisons. N=5 to 6 in each group. Linear regression analysis for cfDNA and ETP was performed in mice infused with heat-inactivated DNase 1 (**C**) or DNase 1 (**D**).

PPP samples from a healthy cohort of young and middle-aged/older humans. The baseline characteristics of the human subjects are described in Table S2; body mass index and blood pressure were within the clinically normal range for all the subjects. The ETP was higher in middle aged/older subjects compared with young subjects ($P<0.0001$, Figure 6A, Table S3). Regression analysis also showed a positive relationship between age and ETP (slope=13.8, $R^2=0.23$, $P<0.001$, Figure 6B). Likewise, peak thrombin generation was higher in middle aged/older subjects compared with young subjects ($P<0.01$, Figure 6C, Table S3), and was positively associated with age (slope=2.36, $R^2=0.15$, $P<0.01$, Figure 6D). The lag time was not different between the 2 age groups ($P=0.3$, Figure 6E, Table S3), and regression analysis also did not show significant association with age (Figure 6F). Plasma levels of prothrombin were similar in both age groups (Figure S7).

Similar to our findings in aged mice, we observed an elevation in plasma levels of cfDNA in middle-aged/older human subjects compared with young subjects ($P<0.001$, Figure 7A, Table S3) and linear regression analysis revealed a positive relationship between cfDNA and age (slope=0.036, $R^2=0.47$, $P<0.001$, Figure 7B). Plasma levels of endogenous DNase 1 did not differ with age ($P=0.9$, Figure S8, Table S3).

In contrast to cfDNA levels, plasma levels of H3Cit were not found to be altered with age in humans (Figure 7C and 7D). In regression analysis, we observed that the elevation in plasma cfDNA with age was also positively associated with ETP ($P<0.05$, Figure S9). We next utilized an in vitro approach to directly test the susceptibility of neutrophils from young and middle-aged/older subjects to undergo NETosis.

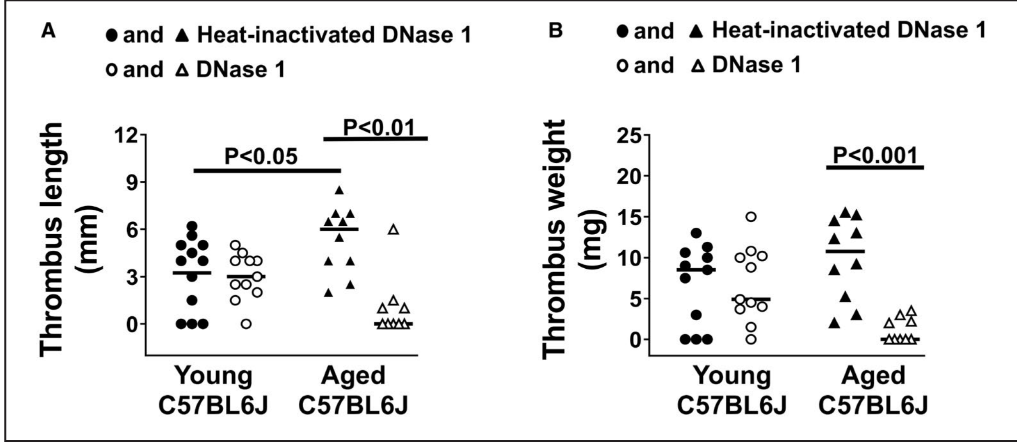

Figure 5. Treatment of aged C57BL6J mice with DNase 1 blocked development of venous thrombi in vivo.
Young and aged C57BL6J mice were treated with either DNase 1 or heat-inactivated DNase 1 control, during experimental venous thrombosis. Inferior vena cava was ligated in a stasis model and 48 hours later venous thrombus was harvested. **A**, Thrombus length. **B**, Thrombus weight. Kruskal–Wallis with Dunn's test for multiple comparison. N=9 to 12 in each group.

Neutrophils from each group were added to pooled human plasma and incubated with either the vehicle buffer (control) or PMA at 37 °C for 60 minutes to activate neutrophils to release NETs. Similar to findings in mice, we observed PMA-dependent increases in levels of cfDNA, H3Cit, and ETP in both age groups (main effect of PMA treatment was $P<0.05$ for H3Cit and $P<0.01$ for cfDNA and ETP, Figure S10). No age-dependent increase was observed for any of these parameters upon PMA treatment (main effect of age was $P=0.4$ for cfDNA, $P=0.9$ for H3Cit, and $P=0.3$ for ETP).

Incubation of Human Plasma Samples With DNase 1 Decreased Thrombin Generation Potential

Finally, to determine the role of cfDNA in driving thrombin generation potential ex vivo during human aging, we treated PPP samples with DNase 1 or control (heat-inactivated DNase 1) before measurement of thrombin generation (Figure 8, Table S3). The comparison for main effect of treatment, age, and the interaction of age and treatment was significant ($P<0.0001$, $P<0.01$, and $P<0.05$ respectively). Furthermore, we observed that treatment with DNase 1 led to 43% reduction in ETP compared with heat-inactivated DNase 1 for young subjects ($P<0.01$) and 52% reduction for middle-aged/older subjects ($P<0.0001$). These findings suggest that the DNase 1 treatment normalized the differences in ETP between young and middle/aged older mice.

DISCUSSION

In the present study, we addressed whether NETs contribute to age-associated increased potential for thrombin generation and thrombosis. Our study reveals 3 major findings. First, we observed that both human and murine aging are associated with an increase in thrombin generation potential as well as elevation in cfDNA, but not in H3Cit, a specific marker of NETosis. Second, we demonstrated that overexpression of Gpx1 provided only modest protection of aged mice from developing increased thrombin generation potential, and the protective effect of Gpx1 was independent of cfDNA. Finally, we observed that treatment with DNase 1 reduced thrombin generation potential in aged mice and in human plasma and blocked the development of venous thrombi in aged mice. Taken together, these findings suggest that aging leads to elevation in cfDNA in a NETosis- and peroxide-independent manner, and treatment with DNase 1 is sufficient to decrease ETP and thrombosis burden.

Aging is a major risk factor for clinical thrombotic events.[1–3] It is associated with altered levels of several procoagulant factors[36–38] that may potentially contribute to a prothrombotic state. In the present study, we measured thrombin generation potential in plasma samples from aged mice and humans. The thrombin generation assay measures the potential of plasma to generate thrombin in response to an extrinsic trigger (tissue factor); the area under the thrombin generation curve over its entire time course is quantitated as the ETP by means of a fluorescently labeled synthetic substrate. In our study, we observed an increase in thrombin generation potential with both human and murine aging, consistent with prior reports in aged humans.[13,14] One limitation of our human samples is that the sample size was inadequate to assess for potential differences in ETP that may occur over a broad age range. This will

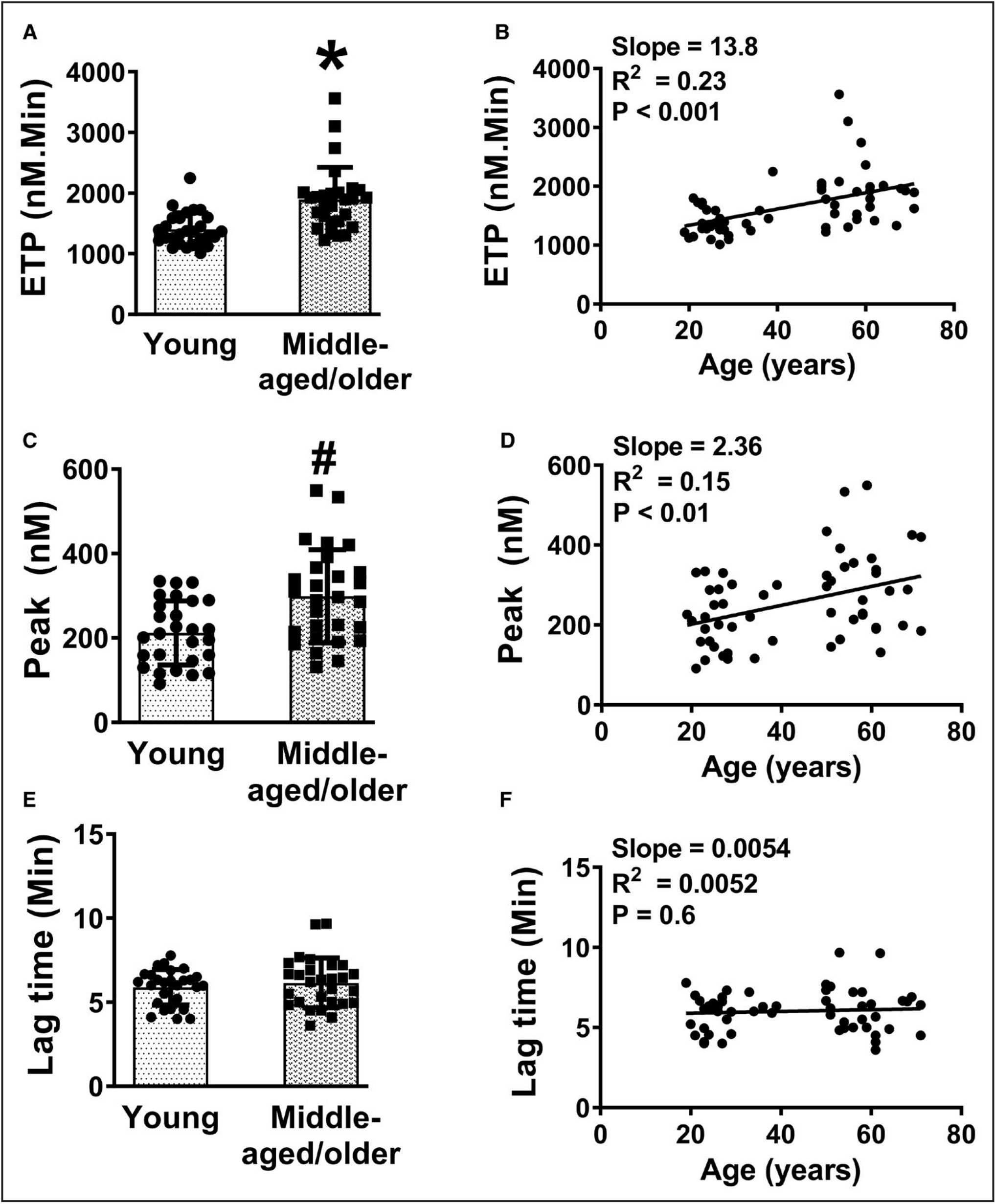

Figure 6. Human aging is associated with increased thrombin generation.
Thrombin generation was measured using platelet poor plasma from young and middle-aged/older subjects. **A**, Endogenous thrombin potential (ETP). **B**, Linear regression analysis of ETP with age. **C**, Peak thrombin generation. **D**, Linear regression analysis of peak thrombin generation with age. **E**, Lag time. **F**, Linear regression analysis of lag time with age. Data for (**A**, **C**, and **E**) are expressed as mean±SD *P<0.0001 and $^{\#}P$<0.01 vs young subjects. Unpaired t test for (**A**, **C**, and **E**) and linear regression for (**B**, **D**, and **F**). N=27 to 28 in each group.

be an important question for future study given that the risk of thrombosis increases with every decade past 50 years of age.[39,40] Another limitation is that we could not determine effects of race/ethnicity on thrombin generation in our aged cohort.

One interesting observation in our murine studies was that age-induced increases in thrombin generation potential were moderately decreased by overexpression of Gpx1. This suggests that the age-associated increase in thrombin generation is mediated, at least in part, by peroxides. Peroxide levels are known to increase with aging, which is mainly attributable to a decline in anti-oxidant systems.[41,42] These findings are in accordance with our previous work demonstrating that aging is accompanied by peroxide-dependent increases in experimental thrombosis.[15] The effect of Gpx1 overexpression on thrombin generation was modest, however, and it is likely that peroxides contribute to

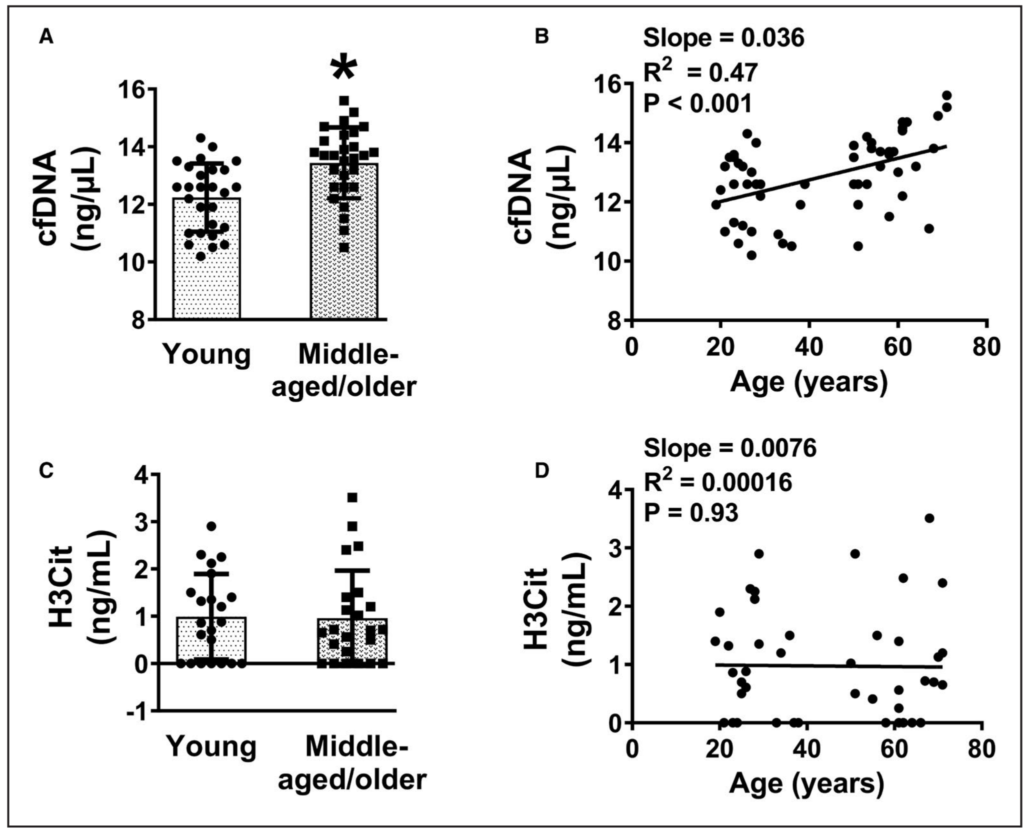

Figure 7. Human aging is associated with elevated levels of cfDNA.
Components of neutrophil extracellular traps were measured in plasma from young and middle-aged/older subjects. **A**, Plasma cell-free DNA (cfDNA). **B**, Linear regression analysis of cfDNA with age. **C**, Plasma citrullinated histone (H3Cit). **D**, Linear regression analysis of H3Cit with age. Data for (**A** and **C**) are presented as mean±SD. **P*<0.001 vs young subjects. Unpaired *t* test was used for (**A** and **C**) and linear regression analysis for (**B** and **D**). N=27 to 28 for (**A**), 22 to 23 for (**C**), 55 for (**B**), and 45 for (**D**).

aging-related thrombosis primarily via effects on platelet activation rather than thrombin generation.[15]

Recently, NETs have emerged as major mediators of increased thrombotic susceptibility in animal models,[30,34] and increased markers of NETs have been reported in human patients with thrombosis.[43–46] Histones and cfDNA have been proposed to enhance thrombin generation through platelet-dependent and -independent mechanisms, respectively.[8,35] While histones contribute to thrombin generation by activating platelets through TLR2 and TLR4 leading to surface phosphatidyl serine exposure, cfDNA is known to activate coagulation through the contact activation pathway. In vivo, we observed a low level of plasma H3Cit, a specific marker of NETosis, in young mice or humans, and it did not increase with age. In contrast, we observed age-dependent increases in plasma cfDNA in both mice and humans. The lack of effect of aging on plasma H3Cit may explain the absence of platelet-dependent increase in thrombin generation potential with age in mice. In contrast, the profound increase in ETP in PPP in aged mice or humans is consistent with the elevation in cfDNA.

The lack of change in plasma levels of H3Cit with age in our study was unexpected, since recently a group has reported increased NETosis with age in mice[28] or in aged individuals with severe vasculitis.[25,26] We considered the possibility that the assay we used may have limited sensitivity to detect small changes in plasma H3Cit. Therefore, since neutrophils are the source of NETs, we used direct in vitro approaches to determine whether neutrophils from aged mice have higher potential to undergo NETosis and whether overexpression of Gpx1 protects from this effect of aging. Upon PMA activation of neutrophils isolated from young and aged WT or Gpx1 Tg mice and seeded on coverslips in the presence of HBSS media, we observed a similar extent of NETosis when visualized by fluorescent microscopy. Furthermore, when neutrophils were suspended in plasma and activated with PMA, we observed a similar elevation in cfDNA, H3Cit, and ETP regardless of age or genotype. Neutrophils from young or aged humans suspended in plasma also showed similar increases in cfDNA, H3Cit, and ETP in both age groups upon PMA activation. These findings suggest

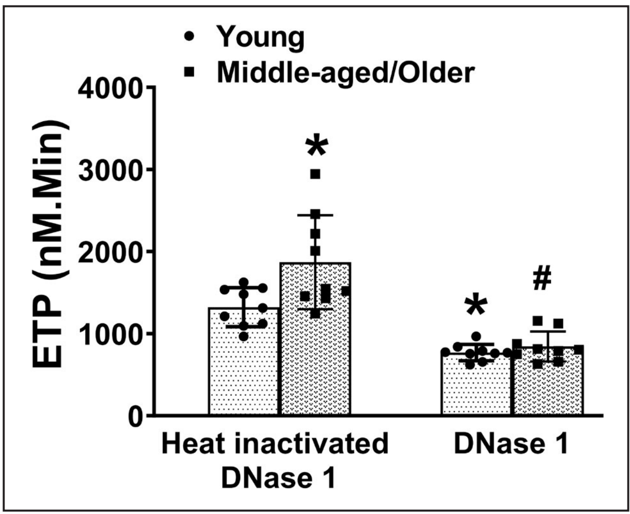

Figure 8. Increased thrombin generation potential in aged humans is likely mediated through cfDNA.

Platelet poor plasma from young and middle aged/older subjects was treated with either 20 µg/mL of DNase 1 or same dose of heat-inactivated DNase 1 as control for 60 minutes and endogenous thrombin potential (ETP) was measured. Data are presented as mean±SD. *P<0.01 vs young subjects treated with heat-inactivated DNase 1, #P<0.0001 vs middle aged/older subjects treated with heat-inactivated DNase 1 (2-way ANOVA, with Tukey test). N=9 in each group. cfDNA indicates cell-free DNA.

that age in the absence of an underlying disease condition is not a major mediator of NETosis in our model. The observed differences in findings may have resulted from the use of somewhat older C57BL6J mice (24–27 months old) in the previous report[28] compared with our use of slightly younger mice (up to 20 months). We have observed that C57BL6J mice beyond 20 months of age often develop inflammatory health conditions, including dermatitis and spontaneous neoplasms that have also been reported by others,[47] so we excluded mice >20 months of age. Recent studies have reported alternative mechanisms of release of extracellular traps, other than reactive oxygen species or histone citrullination, some of which could potentially be important in aging and need to be explored in future study design.[48–50]

A previous report has suggested that neutrophils are activated by hydrogen peroxide and release NETs in vitro.[18] However, similar release of NETs from neutrophils in WT or mice overexpressing Gpx1 in our study may suggest that endogenous peroxides do not have a major role in release of NETs during aging. Overall, our findings of elevations in plasma cfDNA with age in the absence of any alterations in plasma H3Cit or PMA-driven in vitro NETosis suggest that increases in plasma cfDNA in aging may have occurred because of NETosis-independent mechanisms. We considered the possibility that the changes in endogenous DNase 1 levels with age may mediate the changes in the levels of circulating cfDNA. However, our findings did not suggest any alterations in DNase 1 levels in either mice or humans with age. It may be that the cellular stress mechanisms such as senescence, necrosis, apoptosis, or increased cellular turnover that occur with age[51] may contribute to elevated circulating cfDNA in our model. It is also important to note that the Qubit cfDNA assay we utilized detects both histone-free and histone-bound cfDNA. It may be that the elevation in histone-free cfDNA could be partly mitochondrial in origin[52] and mitochondrial and nuclear DNA may exert differential procoagulant activities. A limitation of our study therefore is that we could not identify the source of cfDNA, and future studies focusing on distinguishing the origin of cfDNA may aid in developing targeted therapies.

cfDNA contributes to thrombin generation by activating FXI and FXII.[8] In vitro treatment with DNase 1 lowers the ETP in plasma samples from patients with sepsis.[8] Similarly, in vivo treatment of mice with DNase 1 lowers thrombin generation potential[53] and protects from thrombotic consequences such as stroke,[54] atherosclerosis,[55] and venous thrombosis.[30,34] Therefore, we sought to determine whether the elevated plasma cfDNA observed in aged mice and humans modulates increases in ETP and thrombosis. We observed that treatment of plasma with DNase 1 in vitro significantly decreased ETP in both young and middle aged/older humans, and that either in vitro treatment of plasma or in vivo treatment of mice with DNase 1 decreased ETP in all groups of mice. In these assays, thrombin generation was initiated via a low concentration of tissue factor (1 pmol/L), which triggers the extrinsic pathway of coagulation. Under these conditions, continued thrombin generation is dependent on thrombin-mediated FXI activation through a feed-forward mechanism.[56] We speculate, therefore, that elevated plasma cfDNA in aging leads to enhanced thrombin generation by promoting thrombin-dependent activation of FXI, similar to other negatively charged endogenous surfaces such as polyphosphate.[57] The observation that DNase 1 treatment lowered thrombin generation and normalized the differences between the young and aged cohorts suggests that cfDNA is a likely mediator of increased thrombin generation in aging. A complementary approach in future to consider would be to study mouse model lacking and/or overexpressing DNase 1 under conditions of aging.

Finally, we observed that treatment with DNase 1 also decreased the increased susceptibility to venous thrombosis in aged mice. These findings corroborate the clinical findings where patients with FXI deficiency have lower incidence of deep vein thrombosis and cardiovascular events,[58–60] and FXI is currently a target for novel anticoagulant therapies in development.[61] The

fact that after DNase 1 treatment aged mice developed substantially smaller-sized thrombi than younger mice treated with DNase 1 suggests that venous thrombosis is not only exacerbated by cfDNA during aging but also rather is dependent on it. This differential requirement for cfDNA in young versus aged mice in mediating venous thrombosis in our model is interesting and should be explored in future studies. Some of these effects can also be driven by a potential role of cfDNA in modulating clot structure and delaying fibrinolysis, since in the setting of elevated cfDNA in a cell-free system, DNase 1 is shown to protect from formation of dense clot structure and in potentiating fibrinolysis.[9] Alternatively, DNase 1 may also exert a protective effect independent of cfDNA in aged mice.[62]

With regard to our in vivo findings with DNase 1 treatment, it is important to mention that a study reported that NETs itself is not prothrombotic,[20] but its components such as cfDNA and histones are. This study also showed that degradation of NETs by DNase 1 would release cfDNA and histones to augment thrombin generation. Based on these findings, in our aged mice, if NETosis was indeed occurring in vivo and expelling NETs actively, then treatment with DNase 1 would have worsened the effects on venous thrombosis by releasing procoagulant histones. In fact, we observed that treatment with DNase 1 instead protected from developing venous thrombi in aged mice and is consistent with elevation in circulating cfDNA but not H3Cit in our aging models. Together these findings support the idea that in our model, NETosis is not the mediator of venous thrombosis. Our findings are also consistent with a report where Jiménez-Alcázar and colleagues,[63] in a prospective cohort study, observed that cfDNA but not NETs was associated with the extent of venous thromboembolism in humans aged >65 years.

In summary, these findings suggest that aging leads to cfDNA-associated increases in thrombin generation and venous thrombosis. Our findings also suggest that plasma cfDNA and ETP may be considered early markers of a prothrombotic state during aging. Given the emerging evidence that use of DNase 1 is beneficial in experimental thrombotic and inflammatory conditions in mice,[30,34,64] and the recent use of DNase 1 in patients with Alzheimer disease,[65] cystic fibrosis,[66] or in an ongoing trial to treat patients with SARS-CoV-2,[67] our findings underscore the clinical potential of this treatment to target thrombotic consequences in aging.

ARTICLE INFORMATION

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Author contributions: Kumar designed and conducted the experiments, interpreted the results, and co-wrote the manuscript. Sonkar assisted with DNase 1 infusion and conducted in vivo thrombosis studies. Swamy also conducted experiments. Sharathkumar aided with thrombin generation assay. Pierce collected, processed, and stored the human samples. Dayal directed the project, designed the experiments, interpreted the results, and wrote the manuscript. All authors assisted with the preparation and editing of the manuscript.

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Disclosures

None.

Supplemental Material

Data S1
Tables S1–S3
Figures S1–S10
References 68–70

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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 °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

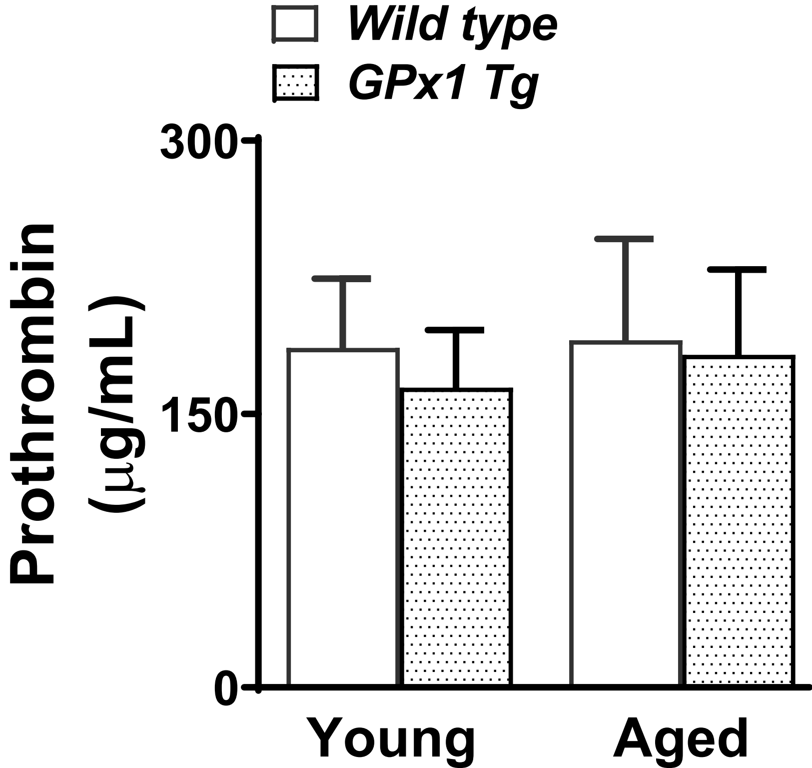

Figure S1: Plasma prothrombin levels in mice. Prothrombin level was measured in plasma from young (4 month) and aged (20 month) wild type mice and their littermates overexpressing Gpx1. N = 7-8 mice in each group. Data expressed as mean ± SD. Two-way ANOVA.

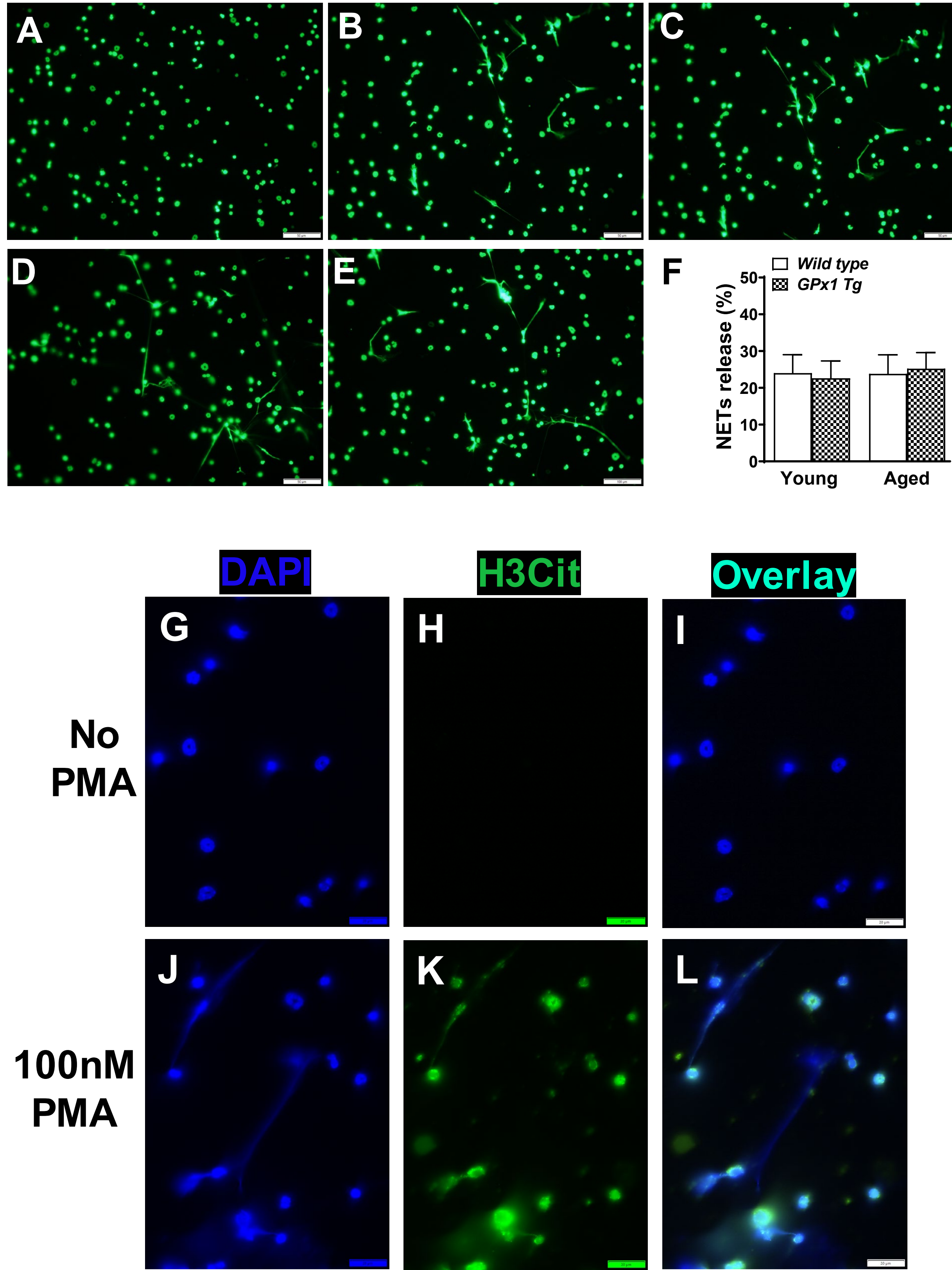
A
B
C
D
E
F
Wild type
GPx1 Tg
NETs release (%)
50
40
30
20
10
0
Young
Aged
DAPI
H3Cit
Overlay
G
H
I
No PMA
J
K
L
100nM PMA

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.

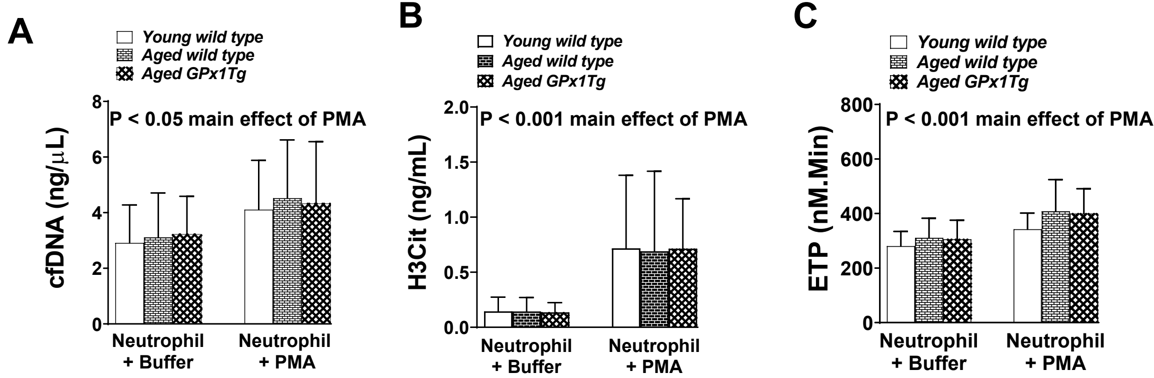

Figure S3: Neutrophils from young or aged mice display similar potential to release NETs and increase thrombin generation potential. Neutrophils were isolated from young and aged wild type mice or aged Gpx1 Tg littermates. A total of 1 x 10^5 cells was added to pooled mouse plasma and incubated with 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.

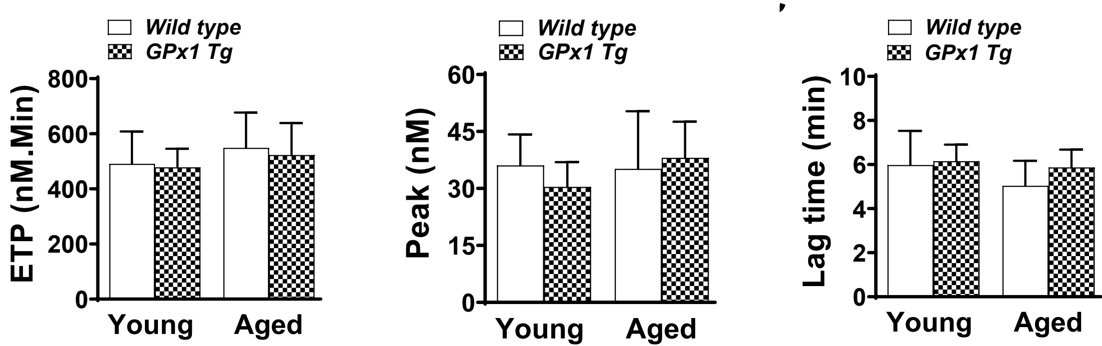

Figure S4: Murine aging does not increase platelet-dependent thrombin generation. (A) endogenous thrombotic potential, i.e. ETP, **(B**) thrombin peak and **(C)** lag time in PRP of young and aged wild type mice as well as littermates overexpressing Gpx1 (Gpx1 Tg). Data expressed as mean ± SD. N = 5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

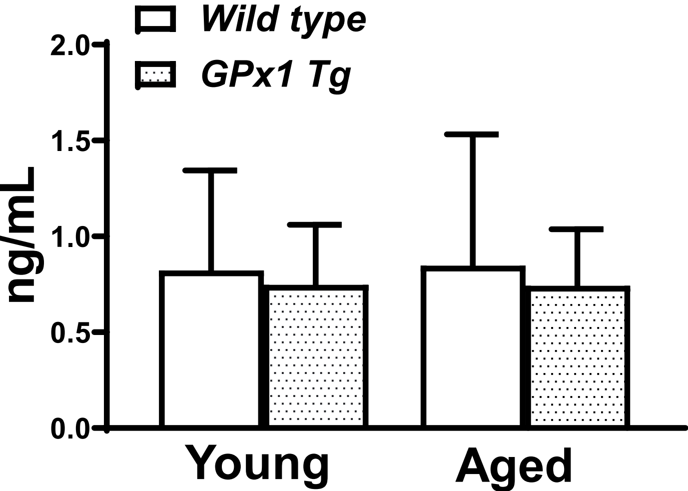

Figure S5: Plasma DNase 1 levels in mice. Antigen levels of DNase 1 were measured in plasma from young (4 month) and aged (20 month) wild type mice and their littermates overexpressing Gpx1. N = 6-8 mice in each group. Data expressed as mean ± SD. Two-way ANOVA.

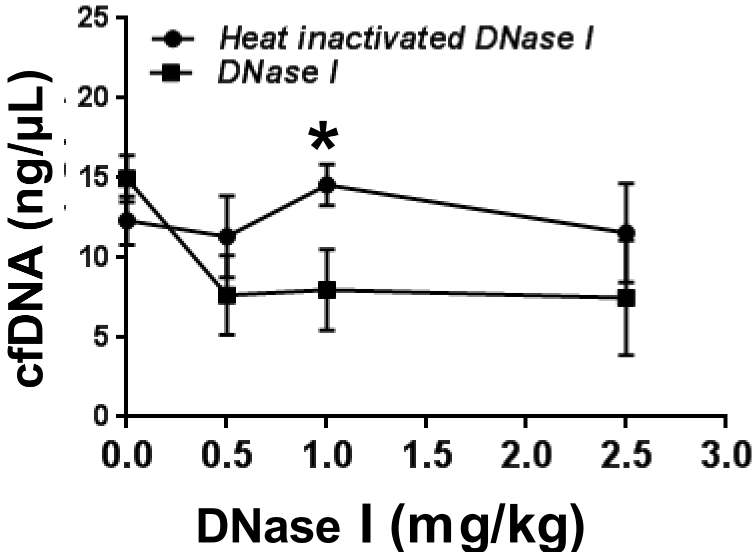

Figure S6: Dose dependence of DNase 1 infusion on plasma cfDNA. Wild type mice were treated retro-orbitally with either DNase 1 or heat-inactivated DNase 1, blood was collected 60 minutes post-infusion and cfDNA was measured. Data expressed as mean ± SEM. N = 4 mice in each group. *P < 0.05 vs. DNase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

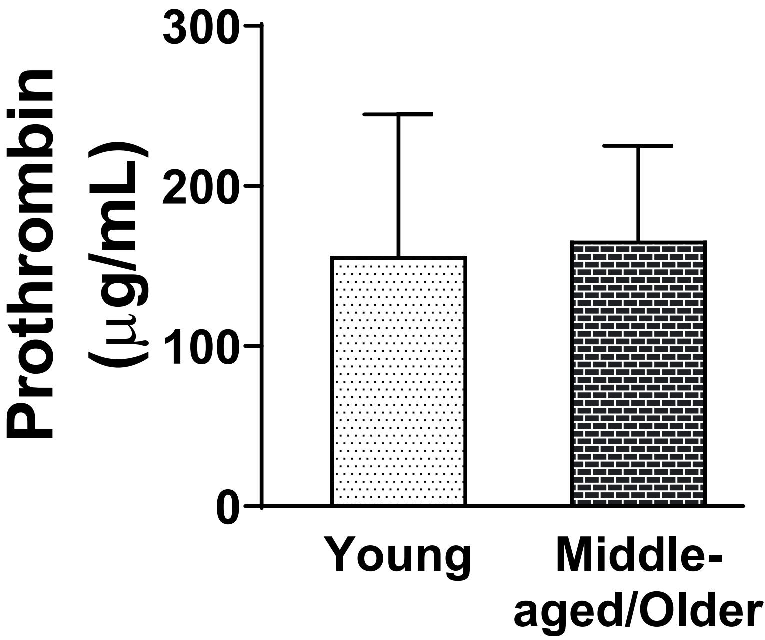

Figure S7: Plasma prothrombin levels in humans. Prothrombin level was measured in plasma from healthy young (18-39 years) and Middle-aged/older (50-71 years) subjects. N = 17-18 subjects in each group. Data expressed as mean ± SD. Unpaired t-test.

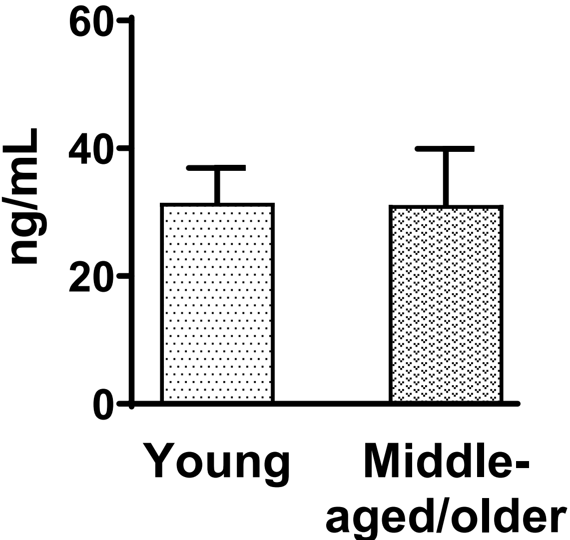

Figure S8: Plasma DNase 1 levels in humans. Antigen levels of DNase 1 were measured in plasma from healthy young (22-38 years) and Middle-aged/older (51-71 years) subjects. N = 12-15 subjects in each group. Data expressed as mean ± SD. Unpaired t-test.

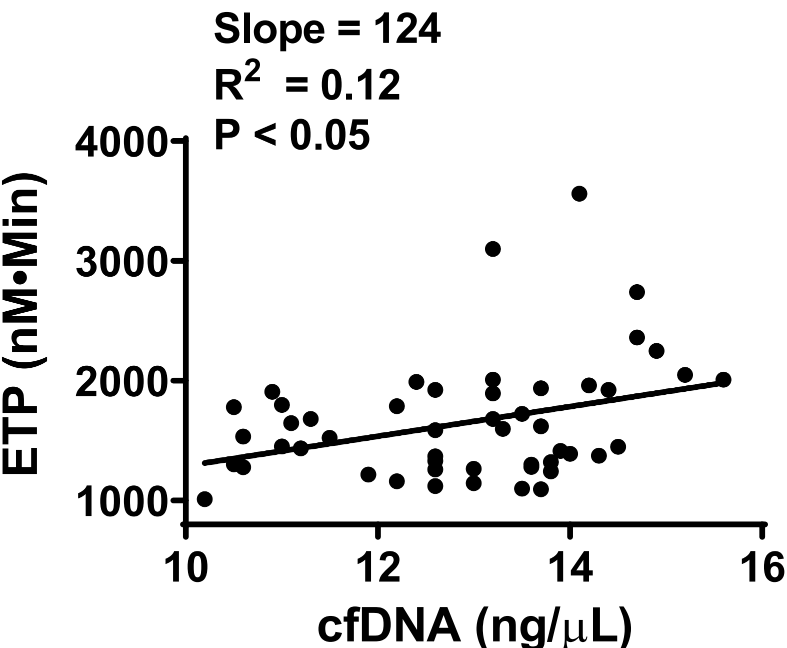

Figure S9: Elevated plasma cfDNA level in humans is associated with increased thrombin generation. Plasma cfDNA and endogenous thrombin potential (ETP) was measured using platelet poor plasma from young (18-39 years) and middle-aged/older subjects (50-72 years). N = 55. Data set was analyzed using simple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

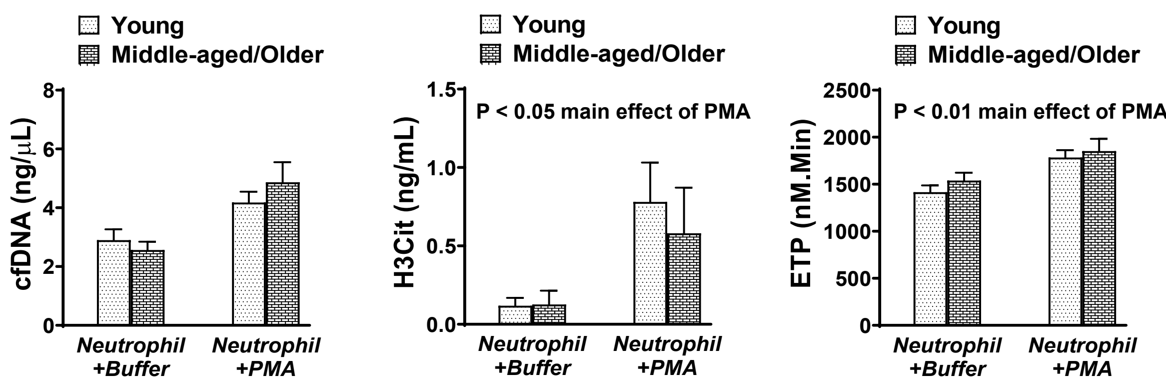

Figure S10: Activation of neutrophils from either young or middle-aged/older humans with PMA produces similar increases in plasma cfDNA, H3Cit and thrombin generation. Neutrophils were isolated from healthy young and middle-aged/older humans. A total of 1 x 10^5 cells was added to the pooled human plasma 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.