Aging induces endothelial dysfunction while sparing arterial thrombosis

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Abstract: OBJECTIVE: To assess the effects of aging on arterial thrombus formation by comparing 2-year-old with 11-week-old C57Bl6 mice. METHODS AND RESULTS: Aging is a major risk factor for cardiovascular disease. In humans, assessing the direct effects of aging on vascular homeostasis is difficult because it occurs in the presence of other risk factors. Arterial thrombosis is the critical event in cardiovascular diseases; however, it is not known whether aging per se promotes its occurrence. Mice represent an interesting system to address this issue because they age without spontaneously developing other risk factors. Organ chamber experiments confirmed the advanced level of aging of old mice. As previously shown, old mice exhibited endothelial dysfunction; however, arterial thrombosis induced by photochemical injury was unchanged. Arterial tissue factor expression and activity; expressions of tissue factor pathway inhibitor, thrombomodulin, and plasminogen activator inhibitor 1; prothrombin time; partial thromboplastin time; thrombin-antithrombin complex; and platelet activation were comparable in both groups. CONCLUSIONS: Although these results cannot be directly extrapolated to humans, this study contributes novel important information on the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals.

DOI: https://doi.org/10.1161/ATVBAHA.110.206920

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: https://doi.org/10.5167/uzh-40002

Accepted Version

Originally published at:
Stämpfli, S F; Akhmedov, A; Gebhard, C; Lohmann, C; Holy, E W; Rozenberg, I; Spescha, R; Shi, Y; Lüscher, T F; Tanner, F C; Camici, G G (2010). Aging induces endothelial dysfunction while sparing arterial thrombosis. Arteriosclerosis, Thrombosis, and Vascular Biology, 30(10):1960-1967.
DOI: https://doi.org/10.1161/ATVBAHA.110.206920
Aging induces endothelial dysfunction while sparing arterial thrombosis

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Running title: Aging, endothelial function, and arterial thrombosis

Word count of Body: 3808
Word count of abstract: 198
Number of figures: 5

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Abstract

Objective: Aging is a major risk factor for cardiovascular disease. Assessing direct effects of aging on vascular homeostasis is difficult since in humans it occurs in the presence of other risk factors. Arterial thrombosis is the critical event in cardiovascular diseases; however, it is not known whether aging *per se* promotes its occurrence. Mice represent an interesting system to address this issue since they age without spontaneously developing other risk factors. Thus, we assessed the effects of aging on arterial thrombus formation by comparing 2 years old and 11 weeks old C57Bl6 mice.

Methods and Results: Organ chamber experiments confirmed the advanced level of aging of old mice. As previously shown, old mice exhibited endothelial dysfunction however arterial thrombosis induced by photochemical injury was unchanged. Arterial tissue factor expression and activity, expression of tissue factor pathway inhibitor, thrombomodulin, and plasminogen activator inhibitor 1 as well as prothrombin time, partial thromboplastin time, thrombin-antithrombin complex and platelet activation were comparable in both groups.

Conclusions: Although these results cannot be directly extrapolated to humans, this study contributes novel important information on the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals.

Key words: aging; endothelial function; atherosclerosis; cardiovascular risk factor; mouse
Condensed Abstract

Aging is a major risk factor for cardiovascular disease however its direct effect on acute arterial thrombosis is not known. Despite aging-dependent vascular dysfunction, thrombotic responses remain unchanged irrespective of the age. Although carried out on mice, this study sheds light of the pathogenesis of aging-induced cardiovascular disease.
Introduction

The percentage of elderly people in our society is steadily increasing and age is considered a major risk factor for the development of cardiovascular disease\(^1\). As most cardiovascular events such as myocardial infarction and ischemic stroke are caused by arterial thrombosis, it is tempting to speculate that advanced age promotes the development of arterial thrombosis. Yet, the direct effects of aging on arterial thrombosis have not been studied since aging in humans always occurs in parallel to other risk factors or atherosclerosis.

Unlike aged humans, old mice fed a regular diet do not spontaneously develop cardiovascular risk factors. Previous work showed that aged mice develop nitric oxide (NO) dependent endothelial dysfunction, but do not develop atherosclerotic lesions, diabetes or hypertension\(^2\text{-}^5\). Therefore, mice represent an interesting system to study the process of aging in isolation and to investigate its direct effects on vascular homeostasis.

In light of the above, we compared 2 C57Bl6 male mice groups, bred and housed in same conditions, aged 2 years (old) and 11 weeks (young) and investigated age-induced changes in arterial thrombosis in the carotid artery. To this end, we employed an established photochemical injury model which principally affects the intimal layer as demonstrated by transmission electron microscopy (TEM) sections (data not shown) and mimics endothelial injury as occurring in atherothrombotic human complications\(^6\text{-}^7\). Endothelial function was assessed to document the stage of vascular aging. It was however not our intention to establish a causal link between NO-mediated endothelial dysfunction and age-related changes in thrombosis. In fact, this hypothesis had already been addressed by a previous study showing that mice lacking endothelial NO synthase (eNOS) exhibit no changes
in arterial thrombosis\textsuperscript{8}. In light of the previous work linking inflammation and thrombosis\textsuperscript{9} as well as inflammation and aging\textsuperscript{10}, the inflammatory state of young and old mice was assessed by immunohistochemistry and real time PCR.
Materials and Methods

Mice

Male C57BL/6 mice of the following age groups were examined: a) young group: 11 weeks, b) intermediate group: 62 weeks, and c) old group: 105 weeks. All mice were bred and housed in a specific pathogen free (SPF) animal facility with conventional light cycle at the Institute of Laboratory Animal Science, University of Zurich. All experiments were approved by the local authorities.

Organ chamber

Thoracic aorta was dissected free, excised, and immediately placed into ice-cold Krebs-Ringer bicarbonate solution. Vessels were cut into 3 mm rings and suspended in organ chambers for isometric tension recording (System 700 MO, Danish Myo Technology A/S, Aarhus, Denmark) containing 6 mL of Krebs solution (37°C, pH 7.4) aerated with 95% O₂/5% CO₂. After a 30 min equilibration period, rings were progressively stretched to the optimal point of their length-tension curve, and the presence of a functional endothelium was tested in norepinephrine precontracted rings by adding 10⁻⁷ mol/L acetylcholine. Endothelial function was assessed in norepinephrine precontracted rings by studying the relaxation to acetylcholine (10⁻⁹ to 10⁻⁴ mol/L; Sigma–Aldrich) in the presence or absence of L-NAME (3×10⁻⁴ mol/L; 30 min incubation, Sigma–Aldrich). The NO donor sodium nitroprusside (10⁻⁹ to 10⁻⁴ mol/L; Sigma–Aldrich) was applied to test endothelium-independent relaxations. Concentration-dependent contractions were established by norepinephrine (10⁻⁹ to 10⁻⁴ mol/L; Sigma–Aldrich).
Arterial thrombosis

Mice were anesthetized with pentobarbital (87 mg/kg i.p.). Rose bengal (Fisher Scientific, Fair Lawn, NJ) was injected into the tail vein in a volume of 0.12 mL at a concentration of 50 mg/kg body weight. Mice were placed under a dissecting microscope and the right common carotid artery was exposed following a midline cervical incision. A Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) was applied and connected to a flowmeter (Transonic, Model T106) supplying a data acquisition system (PowerLab 4/30, AD Instruments). A 1.5-mW green light laser (540 nm) (Melles Griot, Carlsbad, CA) was directed at the desired site of injury at a distance of 6 cm for 60 minutes or until complete occlusion. Flow was monitored for 120 minutes from the onset of injury. Occlusion was defined as flow ≤0.1 mL/min for at least 1 minute. Data were analyzed with ChartPro Software (AD Instruments). The photochemical injury protocol leads to an endothelial-specific injury largely confined to the diameter of the laser beam which reacts with the laser-sensitive rose bengal. As a result of the photochemical injury, endothelial and subendothelial procoagulant factors come in contact with circulating coagulation factors resulting in the formation of an arterial thrombus. Kinetics of and time to occlusion are monitored in real-time with a Doppler probe.

Tissue factor activity

Carotid arteries were homogenized in 50 µl of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton-X 100, pH 7.4) by manual grinding on ice. Samples were then centrifuged at 14000 rpm for 15 min at 4°C and the supernatant was transferred to a fresh tube. Tissue factor (TF) activity was assessed with a colorimetric assay (Actichrome TF, American Diagnostica); incubation at 37°C allowed TF to form a complex with FVII and this complex to cleave FX to FXa. Absorbance was
determined at 405 nm and compared to a standard curve generated using known amounts of lyophilized TF.

Coagulation pathways

Plasma from citrated blood (3.2% citrate 1/10) was extracted by 15 minutes centrifugation (2500g, 4°C) and stored immediately at -80°C until analysis. Prothrombin time (PT) and partial thromboplastin time (aPTT) were assessed by the Start4 analyzer (Diagnostica Stago, France), using the according reagents (Roche Diagnostics, Switzerland). Thrombin-antithrombin (TAT) complex was measured by sandwich ELISA (Enzyme Research Laboratories, USA).

Shear stress-dependent platelet function

Shear stress-dependent platelet function was assessed with a cone and platelet analyzer. Citrated whole blood (200 µL) was circulated in polystyrene wells at a shear rate of 1875 s⁻¹ for 2 minutes with a rotating polytetrafluoroethylene cone as described¹²⁻¹⁴. Wells were washed, stained with May-Grünwald dye, and analyzed with a microscope connected to an image analysis system (ImageJ). Results are expressed as the percentage of surface covered by platelets.

Real-time PCR

Aortic arches were snap frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated using TRizol reagent (Invitrogen). cDNA was generated using Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscience) and first-strand cDNA primer pd(N)₆. Real-time PCR was performed using SybrGreen Jump start kit (Sigma). Murine primers were as follows: tissue factor (TF): forward (5'-3') CAATGAATTCTCGATTGATGTGG, reverse: (5'-3')
GGAGGATGATAAAGATGGTGGC; tissue factor pathway inhibitor (TFPI): forward (5'-3') ACTGTGTGTCTGTTGCTTAGCC, reverse: (5'-3') GTTCTCGTCCCTTCACATCCC; plasminogen activator inhibitor (PAI-1): forward (5'-3') ATCAATGACTGGGTGGAAGGC, reverse: (5'-3') GTTGAACCTGGCTCTGAGCCThrombomodulin (TM): forward (5'-3') TTTCTTTCTGCTGGGTGCTGGC, reverse: (5'-3') TGCTCTGGCTCAGCAAGG; matrix metalloproteinase 9 (MMP-9): forward (5'-3') CCTGGAACTCACAGCATCTTC, reverse: (5'-3') TGGAAACTCACACGACATCTTC, GGAAGCTGCCAAAGCCTTAGA, reverse (5'-3') AACTGCAACCAAAACCCTTC.

The amplification program consisted of 1 cycle at 95˚C for 10 min, followed by 40 cycles with a denaturing phase at 95˚C for 30 s, an annealing phase of 1 min at 60˚C and an elongation phase at 72˚C for 1 min. For verification of the correct amplification, PCR products were analyzed on an ethidium bromide stained 1,5% agarose gel. Quantification was performed using 2^ΔΔCt method.

**Blood glucose, total cholesterol, plasma triglycerides**

All measurements were performed in the morning after a 14 hours fasting period. Glucose was measured in whole blood with ACCU-CHEK aviva system (Roche). Cholesterol and triglycerides were determined in heparinized plasma (15 minutes centrifugation, 2500g, 4˚C) by the enzymatic CHOD-PAP and GPO-PAP method (both from Roche), respectively.

**Immunohistochemistry**

Aortic roots and carotid arteries were mounted in O.C.T. (Tissue-Tek).
Specimens were cut into 8 mm sections, fixed with 4% paraformaldehyde. Sections were stained with Oil Red-O (ORO) for 1 hour at room temperature. For the assessment of myeloperoxidase (MPO; Labvision), CD68 (Serotec), and tumor necrosis factor α (TNF-α; Abcam) sections were fixed in acetone, blocked with 1% FCS, and incubated with specific primary antibody. Primary antibodies were revealed by goat anti-species specific immunoglobulin antibodies, followed by alkaline phosphatase-labelled donkey anti-goat antibodies. Alkaline phosphatase was visualized using naphtol AS-BI phosphate and new fuchsin as substrate. Sections were counterstained with hemalaun and coverslipped.

**Statistics**

Data are represented as mean±SEM. Statistical analysis was performed by 2-tailed unpaired Student t test or ANOVA as appropriate. A probability value of <0.05 was considered significant.
Results

Vascular tone

Endothelium-dependent relaxation to acetylcholine was impaired in old as compared to young mice (maximal relaxation: -58.9±9.6% vs. -88.0±4.4%, p<0.01; -log ED50: 6.52±0.27 vs. 7.05±0.09, p=n.s.; n=5; Figure 1A). The nitric oxide synthase inhibitor L-NAME abrogated acetylcholine induced relaxation in both groups (maximal response: 8.6±5.2% vs. 0.08±3.5%; p=n.s.; n=5; Figure 1A). In contrast, endothelium-independent relaxation to sodium nitroprusside was similar in old and young mice (maximal relaxation: -93.3±6.8% vs. -111.9±7.4%, p=n.s.; -log ED50: 7.89±0.21 vs. 7.69±0.17, p=n.s.; n=5; Figure 1B). No difference was observed in the contraction to KCl (0.79±0.07 g vs. 0.71±0.07 g; p=n.s.; n=10; Figure 1C), L-NAME (11.1±10.9% vs. 10.4±13.2%; p=n.s.; n=5; Figure 1D), or norepinephrine (118.7±16.3% vs. 138.9±13.8%; p=n.s.; n=5; Figure 1E). Levels of eNOS mRNA expression were comparable in young and old mice (p=0.0739; n=5; data not shown).

Arterial thrombosis

Age-related changes in arterial thrombosis were examined by a carotid artery photochemical injury model and assessed by real time blood flow measurement. Both time to thrombotic occlusion and the kinetics of thrombus formation were unaltered in old (45.5±3.7 min; n=5) as compared to middle aged (53.0±5.8 min; p=n.s.; n=6;) or young mice (45.6±5.9 min; p=n.s.; n=5; Figures 2A and 2B). Carotid artery blood flow before initiation of photochemical injury was similar in old and young mice (0.81±0.07 vs. 0.77±0.07 mL/min; p=n.s.; n=5).

To improve our understanding of the laser-induced damage to the arterial wall, we
examined sections of non-injured and injured carotid arteries by electron microscopy. Both the internal elastic membrane and the vascular media remained morphologically intact after the laser injury under our experimental conditions indicating that the injury is largely confined to the endothelial layer (data not shown).

Analysis of coagulatory and fibrinolytic factors and platelets

TF activity in carotid artery did not differ between old and young mice (OD405: 0.68±0.01 vs. 0.5±0.05; p=n.s.; n=5; Figure 3A). In line with this, TF mRNA levels (ΔΔCt: 3.7±1.1 vs. 4.8±1.5; p=n.s.; n=5; Figure 3A) as well as levels of its antagonist TFPI (ΔΔCt: 0.0134±0.0028 vs. 0.0120±0.0017; p=n.s.; n=5; Figure 3A) were comparable in old and young mice. PAI-1 (ΔΔCt: 0.210±0.049 vs. 0.408±0.088; p=0.0862; n=5; Figure 3B) as well as TM expression (ΔΔCt: 0.342±0.087 vs. 0.434±0.153; p=n.s.; n=5; Figure 3B) were not different between the two groups. Extrinsic and intrinsic coagulation pathways were examined by measuring PT and aPTT, respectively. No changes were observed between old and young mice neither for PT (10.7±0.23 vs. 10.9±0.25 s; p=n.s.; n=5; Figure 3C) nor aPTT (26.7±2.8 vs. 26.2±1.5 s; p=n.s.; n=5; Figure 3C). TAT complex was assayed in plasma and no difference was found between young and old mice (4.79±0.16 vs. 4.47±0.093 ng/mL; p=0.1236; N=5; data not shown). Platelet function was assessed by determining shear stress-dependent platelet adhesion. Old and young mice exhibited similar values (5.7±2.0% vs. 4.1±1.5%; p=n.s.; n=5; Figure 3D).

Arterial morphology

Aortic roots, descending aortae, and carotid arteries were stained by HE and Oil-Red-O. Histological features of the arteries were comparable in old and young mice (Figure 4). No lipid deposits or fatty streaks were detected in either group. In the
aortic root, sporadic single subendothelial cells stained positive for Oil-Red-O in old, but not in young mice (Figure 4B).

**Blood glucose, plasma cholesterol, and plasma triglycerides**

Fasting blood glucose (81.1±8.3 vs. 60.8±1.3 mg/dL; p=n.s) and total plasma cholesterol (81.2±2.2 vs. 59.3±20.3 mg/dL; p=n.s) did not differ in old and young mice. Plasma triglyceride levels after 14 hours of fasting were significantly lower in old mice as compared to young controls (35.6±7.9 vs. 99.8±4.2 mg/dL; p<0.001).

**MMP-9 expression and immunohistochemical analysis of inflammation**

Levels of MMP-9 mRNA expression were comparable in both groups. (ΔΔCt: 0.00023±0.00004 vs. 0.00026±0.00006; p=n.s.; n=5, Figure 5). Immunohistochemical analysis of carotid arteries revealed comparable expression levels of the inflammatory markers MPO, CD68, and TNF-α in young and old mice indicating a similar and very low level of inflammation in both groups (Figure 5).
Discussion

Increasing age is considered a major risk factor for the development of cardiovascular disease\textsuperscript{1}. Thus, studying its molecular mechanisms is an important scientific goal especially in view of the growing percentage of elderly people in our society. This study demonstrates that aging \textit{per se} does not promote arterial thrombosis. Endothelial function was assessed in order to document the advanced stage of vascular aging of old mice but not to establish a direct link between NO-mediated endothelial function and thrombosis. This link had indeed been investigated in a previous study were deletion of eNOS gene was shown not to affect arterial thrombus formation\textsuperscript{8}. Old mice indeed exhibited impaired endothelium-dependent relaxation, while maintaining an unaltered thrombotic potential. Moreover, the kinetics of thrombus formation, arterial TF activity, plasma coagulation times, and platelet adhesion as well as modulators of coagulation (TFPI, PAI-1, TM) remained unchanged as compared to young controls. Although levels of PAI-1 and eNOS were not statistically different, a trend was visible and we cannot fully exclude that a higher number of animals might have yielded a different result. Mice have a life expectancy of roughly two years; and the aged mice used in this study were over one hundred and four weeks old; thus, their age corresponded roughly to seventy/eighty years of age for a human.

Mice fed a regular diet do not exhibit elevated blood pressure, glucose, or cholesterol with age\textsuperscript{15}. Indeed, not only remained fasting blood glucose and plasma cholesterol levels unaltered in old mice, fasting triglyceride levels were even decreased in these animals; hence, in line with previous studies\textsuperscript{3}, old mice fed their entire lifespan a normocaloric diet did not exhibit glucose, cholesterol, or triglyceride levels known to be associated with vascular disease. Consistent with these observations, histological analysis did not reveal atherosclerotic lesions such as fatty
streaks in aortic roots, carotid arteries, or descending aortas of old mice. Sporadic single cells stained positive for Oil Red-O in the aortic root, one of the typical sites of atherogenesis in mice, most likely representing intracellular lipids; nevertheless, accumulation of inflammatory cells or extracellular lipids were not noted. Vascular inflammation, assessed by immuohistochemical staining for the inflammatory marker MPO, macrophage marker CD68, and the inflammatory cytokine TNF-α as well as expression of MMP-9 was very low in both groups.

Rupture of atherosclerotic plaques and consequent thrombus formation is the main cause of acute cardiovascular events in humans. A very important question in this context is whether the increasing incidence of thrombosis with aging solely depends on more severe atherosclerosis or whether aging promotes thrombosis by itself. In light of these considerations, the absence of atherosclerosis in our mouse model is a prerequisite for this study. Hence, we were especially interested in studying aging-related changes in the absence of atherosclerosis in order to dissect these two parameters. In light of the above and despite the obvious differences to humans, mice represent an interesting model for the independent assessment of aging on vascular function or, like in the present study, for investigating the direct effect of aging on arterial thrombus formation.

Following the photochemical injury protocol, old mice displayed unaltered time to thrombotic occlusion compared to young ones. Furthermore, the kinetics of arterial thrombus formation, as assessed by measuring real time blood flow following photochemical injury, was comparable. Both the internal elastic membrane and the vascular media remained morphologically intact after the laser injury under our experimental conditions, as confirmed by TEM (data not shown). Altogether, the model has many similarities with an endothelial erosion, which is a well known entity triggering an occlusive arterial thrombosis in humans. Hence, these data provide
convincing evidence that aging per se does not affect arterial thrombosis in the mouse in the absence of additional cardiovascular risk factors or atherosclerosis.

Age-related endothelial dysfunction occurs in both human\textsuperscript{17} and murine\textsuperscript{3} arteries and is characterized by a decreased bioavailability of NO\textsuperscript{2,18,19}. Since aging and other risk factors\textsuperscript{20} occur together in humans, their independent assessment is extremely difficult. The old mice examined in this study, exhibited a markedly dysfunctional endothelium but no additional risk factor or atherosclerosis; moreover, the decreased endothelium-dependent relaxation in old mice underscores a relevant impairment of vascular function, providing appropriate conditions to study the impact of age-related vascular alterations on arterial thrombosis. These alterations are restricted to the endothelium, since endothelium-independent relaxations to sodium nitroprusside as well as contractions to both receptor-dependent and -independent agonists remained unaltered. Moreover, the observed alterations are solely functional, since histological analysis of the arteries did not reveal any structural difference between old and young mice. Furthermore, levels of eNOS mRNA expression were comparable as also shown in a previous study\textsuperscript{3}. Furthermore, genetic deletion of eNOS does not influence arterial thrombus formation\textsuperscript{8}. In line with this, despite endothelial dysfunction in the old mice, arterial thrombosis in these animals remained unaltered as compared to young controls, indicating that aging \textit{per se} does not directly affect arterial thrombosis.

In both humans and mice, age-related changes of coagulation factors have been reported. Factor VIII, factor VII, and fibrinogen plasma levels increase with age in humans\textsuperscript{21-23} and mice\textsuperscript{23}. In the former, some of these alterations are associated with coronary artery disease or overall mortality, although no causal relationship has been established\textsuperscript{24}. Such prothrombotic alterations may well be counterbalanced in humans by increased plasma levels of anticoagulant proteins like TFPI or protein C,
which have been reported as well\textsuperscript{25}. Moreover, such changes may occur secondary to activation of coagulation by endothelial damage or endothelial erosion\textsuperscript{26} rather than playing a direct role in thrombosis. In addition, many of these parameters are acute phase reactants and may simply represent an enhanced inflammatory burden\textsuperscript{27}. Hence, the clinical relevance of such changes in plasma coagulation factors with aging is questionable. Consistent with this interpretation, studies in centenarians revealed that these individuals exhibit higher levels of procoagulant factors than old controls, suggesting that such changes correlate with health and longevity and do not necessarily lead to a higher risk for arterial thrombosis. In addition, mRNA expression and protein activity of TF, the main trigger of coagulation\textsuperscript{28}, and expression of its antagonist TFPI as well as PAI-1 and TM were not altered in these animals. PT, aPTT and TAT complex are globally accepted parameters for assessing changes in coagulation\textsuperscript{29}; the integral character of these tests renders them well suited for assessing the activity of coagulation pathways. Age-related changes of these parameters have not been described\textsuperscript{30}, and clinically used reference ranges are not age-dependent\textsuperscript{31}. In line with these observations and with the reported similar kinetics of arterial thrombosis in vivo, PT, aPTT and TAT complex remained unchanged in old mice. Therefore, no functionally relevant alteration of the coagulation system was apparent. Additionally, given the unchanged thrombotic potential and unchanged PAI-1 levels observed in young and old mice it is conceivable to expect that also activation of the fibrinolytic system did not vary with age.

Platelets are crucially involved in arterial thrombus formation. In aged humans – not controlled for classical risk factors – elevated levels of $\beta$-thromboglobulin and platelet factor 4\textsuperscript{32} as well as a lower aggregation threshold to adenosine diphosphate and collagen\textsuperscript{33} have been reported. In addition, von Willebrand factor expression is
known to increase with age and correlate with age related risk factors\textsuperscript{34} and may in these subjects enhance platelet activation in vivo. Furthermore, phospholipid composition of platelets differs with age in healthy subjects\textsuperscript{35}, suggesting an age related alteration in transmembrane signaling. In the present study, platelet function was assessed by shear stress induced adherence. No age-dependent alteration in platelet function was recorded in these experiments suggesting comparable platelet function among young and old mice, implying that the age-related changes described may not be directly influenced by aging but rather by other risk factors.

In summary, this study shows that aging \textit{per se} does not affect arterial thrombosis in the mouse and that age-dependent vascular dysfunction in the absence of additional risk factors does not alter arterial thrombus formation. Although limited by the use of mice, this study contributes novel important information concerning the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals. Studying the effect of additional risk factors and/or atherosclerosis in concert with aging on arterial thrombosis could certainly represent an interesting future development of this study.
Sources of Funding

The study was supported by Swiss National Science Foundation (to TFL, FCT and GGC), Velux Foundation, Wolfermann Nägeli Foundation, MERCATOR foundation, Hartmann Müller-Stiftung and Olga Mayenfish fountadion (to GGC) and the Swiss Heart Foundation.

Disclosures

None.
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Figure legends

Figure 1
Isometric tension studies in aortic rings of old (filled symbols) and young (open symbols) mice

A: Endothelium-dependent relaxations to acetylcholine are impaired in old as compared to young mice (*p<0.01; **p<0.001; n=5). Pretreatment with L-NAME abrogates relaxations in both groups (p=n.s. for old vs. young mice; p<0.001 for control vs. L-NAME; n=5).

B: Endothelium-independent relaxations to sodium nitroprusside do not differ between old and young mice (p=n.s.; n=5).

C: Contractions to KCl (80 mM) are similar in old and young mice (p=n.s.; w=weeks; n=5).

D: Contractions to L-NAME (3x10^-4 mol/L) are similar in old and young mice (p=n.s.; w=weeks; n=5).

E: Contractions to norepinephrine are similar in old and young mice (p=n.s.; n=5).

Figure 2
Aging does not affect arterial thrombus formation

A: Old and young mice exhibit similar time to thrombotic occlusion (p=n.s.; n=5).

B: Real time blood flow plot analysis reveals no difference in the flow pattern over time (representative records).

Figure 3
TF expression and activity, expression of TFPI, PAI-1, and TM, coagulation times, and platelet adhesion in old and young mice

A: Old and young mice exhibit comparable arterial TF activity and TF mRNA (p=n.s.; n=5).

B: No significant difference in mRNA expression of TFPI, PAI-1 and TM was observed.

C: Prothrombin time (PT) and partial thromboplastin time (aPTT) do not differ in old and young mice (p=n.s.; n=5).

D: Shear-stress dependent platelet adhesion is similar in old and young mice (p=n.s.; n=5).
Figure 4

Similar arterial histology in old and young mice A: No structural difference is evident in aortic roots of old and young mice (HE stain). B: No lipid deposits are detected in aortic roots of old and young mice, while sporadic single subendothelial Oil-red-O positive cells are detectable in old mice. Descending aorta (C) and carotid arteries (D) exhibit similar histology in old and young mice.

Figure 5

Expression of inflammatory markers MPO, CD-68, and TNF-α in the arterial wall A: Expression of the neutrophil marker MPO was comparable in old and young mice. B: Macrophage marker CD68 was expressed to a similar extent in both groups. C: The pro-inflammatory cytokine TNF-α exhibited a similar expression pattern in old and young mice. D: mRNA levels of MMP-9, assessed by real time PCR analysis, were comparable in both groups.
Figure 1

A

Acetylcholine (-logM)

B

Sodium nitroprusside (-logM)

C

Contraction to KCl (g)

D

Contraction to L-NAME (% KCl)

E

Contraction (% KCl)

Norepinephrine (-logM)
Figure 3

A. \( \Delta C_i \) (TFPI/S12, AU)

B. \( \Delta C_i \) (PAI-1/S12, AU)

C. Partial thromboplastin time (s)

D. Area covered (%)
Figure 4

11 weeks vs. >104 weeks

A

B

C

D
Figure 5

11 weeks vs. >104 weeks

A

B

C

D

ΔΔCt (MMP-9/β-actin AU)