Platelet Inhibitors Reduce Rupture in a Mouse Model of Established Abdominal Aortic Aneurysm

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Objective—Rupture of abdominal aortic aneurysms causes a high morbidity and mortality in the elderly population. Platelet-rich thrombi form on the surface of aneurysms and may contribute to disease progression. In this study, we used a pharmacological approach to examine a role of platelets in established aneurysms induced by angiotensin II infusion into hypercholesterolemic mice.

Approach and Results—Administration of the platelet inhibitors aspirin or clopidogrel bisulfate to established abdominal aortic aneurysms dramatically reduced rupture. These platelet inhibitors reduced established abdominal aortic platelet and macrophage recruitment resulting in decreased active matrix metalloproteinase-2 and matrix metalloproteinase-9. Platelet inhibitors also resulted in reduced plasma concentrations of platelet factor 4, cytokines, and components of the plasminogen activation system in mice. To determine the validity of these findings in human subjects, a cohort of aneurysm patients were retrospectively analyzed using developed and validated algorithms in the electronic medical record database at Vanderbilt University. Similar to mice, administration of aspirin or P2Y₁₂ inhibitors was associated with reduced death among patients with abdominal aortic aneurysm.

Conclusions—These results suggest that platelets contribute to abdominal aortic aneurysm progression and rupture. (Arterioscler Thromb Vase Biol. 2015;35:2032-2041. DOI: 10.1161/ATVBAHA.115.305537.)

Key Words: angiotensin II ▪ aortic aneurysm, abdominal ▪ aspirin ▪ blood platelets ▪ clopidogrel ▪ mice

Abdominal aortic aneurysms (AAA) affect 5% to 10% of the male and 1% of the female population over the age of 65 and is the 13th leading cause of death (estimated from 15 000 to 30 000 people, both primary and contributing) in the United States.1,2 AAA is defined as a permanent localized dilation in the arterial wall with a diameter ≥50% of normal.3 It is an inflammatory disease that is often associated with formation of an intramural clot. Rupture of AAA frequently causes death.4 In spite of a high incidence and catastrophic consequences, there is limited information about the sequence of events that lead to initiation, progression, and the eventual rupture of AAAs. Currently, the only treatment for AAA is surgical intervention after the aneurysm has reached a diameter of ≥5.5 cm.5,6 Because AAA is categorized as a peripheral artery disease, it is currently recommended that patients with AAA start a regimen of low-dose aspirin (ASA) therapy.7,8

Infusion of angiotensin II (AngII) into hypercholesterolemic mice induces formation of AAA localized to the suprarenal aorta.9,10 This model is highly reproducible and has been used to define mechanisms of vascular pathology associated with AAA.11-13 AngII infusion promotes elastin fiber destruction, proteolytic destruction of medial connective tissue, inflammation, atherosclerosis within the aneurysm, and rupture, which are all features that occur in human AAA.4,14 Other mouse models of chemically induced AAA have been developed that include exposure of the aorta to elastase15 or calcium chloride.16 In addition, there is a xenograft rat model of AAA, which results in aortic dilatation and the presence of a mural thrombus in ≥20% of rats.17 However, AngII infusion is the only consistent mouse model of aortic dilatation and rupture.14,18 Platelets are required for hemostasis but also contribute to thrombosis and inflammation.19 Primary hemostasis results from
Platelet adherence to selected adhesive glycoproteins in subendothelial matrix. Platelet activation, spreading, degranulation, and aggregation lead to formation of a platelet-rich hemostatic plug. Platelet activation occurs through stimulation of a variety of G protein–coupled receptors with soluble agonists, such as thrombin, ADP, and thromboxane A2. Activation of coagulation and production of thrombin activate platelets by cleavage of protease-activated receptors. Activated platelets release ADP and thromboxane A2, that are required for sustained platelet activation and accumulation in a thrombus. ADP stimulates the ADP receptors P2Y1 and P2Y12, whereas thromboxane A2 activates the thromboxane receptor. The uncontrolled growth of a platelet-rich thrombus can occlude the blood vessel and result in myocardial infarction and stroke. Patients at risk for thrombosis are treated with platelet inhibitors, such as ASA, which blocks TXA (thromboxane A2) generation, and clopidogrel bisulfate, which inhibits P2Y12 activation. In addition, the protease-activated receptor 1 inhibitor, Vorapaxar, has recently been approved for treatment of patients with cardiovascular disease.

Studies in rat and mouse models demonstrate that platelet inhibitors reduce abdominal diameter (dilatation) and incidence of experimental aneurysm, suggesting that platelets may enhance AAA. Furthermore, patients with AAAs...
have an activated coagulation system and levels of thrombin generation correlate with the maximum diameter of the aorta in the patients.\textsuperscript{27–32} Importantly, platelets and platelet-specific secretions (soluble P-selectin, soluble CD40L, soluble glycoprotein V, and platelet-derived microparticles) are present in plasma of AAA patients and are specifically released from the luminal thrombus of an aneurysm.\textsuperscript{25} Despite these results, several meta-analyses and retrospective clinical trials reported no significant benefit of platelet inhibitors on aneurysm growth and incidence of rupture.\textsuperscript{33–35}

In this study, we investigated the effects of pharmacological inhibition of platelet activation on aneurysms that were established by infusion of AngII. In addition, we evaluated the progression and rupture of AAA in patients with or without ASA or platelet inhibitors.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Effect of Platelet Inhibition on Established AAAs

Most patients are treated with ASA after being diagnosed with an AAA.\textsuperscript{7,8} To determine the effect of platelet inhibition on established AAAs, we generated AAAs in mice and then administered ASA or clopidogrel bisulfate. \textit{Ldlr}^{-/-} mice were fed a high-fat diet for 1 week before, and throughout AngII infusion for 28 days. Abdominal aortic diameters were measured by in vivo ultrasound and then mice were implanted with an additional 42-day AngII pump (Figure 1A and 1D). Mice were divided into 4 groups: placebo versus ASA and placebo versus clopidogrel bisulfate. ASA significantly reduced arachidonic acid–mediated integrin activation (data not shown) and completely suppressed plasma thromboxane B\textsubscript{2} (Figure IA in the online-only Data Supplement). Clopidogrel bisulfate administration resulted in attenuation of ADP-mediated integrin activation (Figure IB in the online-only Data Supplement). All mice had similar body weights, cholesterol, lipid fractions, and systolic blood pressures (Table I in the online-only Data Supplement).

Figure 2. Platelet inhibition decreased platelet and macrophage accumulation and matrix metalloproteinase (MMP) activity in mouse abdominal aortas. \textit{Ldlr}^{-/-} mice underwent interventional therapy with placebo or ASA and placebo or clopidogrel bisulfate. Platelets (5 days before euthanasia) and macrophages (24 hours before euthanasia) were labeled with anti-GPIX (glycoprotein IX) conjugated 700-nm fluorophore (red) or MMP 680-sense fluorophore (red) and dextran-coated nanoparticles conjugated to DyLight 800 fluorophore (green), respectively (treatments), or IgG placebo controls (controls). A, Representative platelet, macrophage, merged, and grayscale images. B, Representative MMP, macrophage, merged, and grayscale images. C, Subsequent quantification. Histobars represent means±SEM of 4 to 8 mice. The abdominal aorta within the dotted yellow lines were analyzed for total fluorescent signal in C. *\textsubscript{P}<0.001 placebo vs treatment groups. Data were analyzed with a 1-way ANOVA on ranks with Dunn’s post hoc. ASA indicates aspirin.
Supplement). We observed a decrease in abdominal aortic diameters with clopidogrel bisulfate or ASA versus placebo controls, but this was not significant (Figure 1A, 1B, 1D, and 1E). Similarly, mice treated with ASA or clopidogrel bisulfate had a nonsignificant reduction in aortic arch area and diameter of the thoracic aorta (data not shown). Importantly, both platelet inhibitors protected mice with established AAA from rupture-induced death versus placebo controls (Figure 1C and 1F; ASA 0% versus placebo 50%; clopidogrel bisulfate 0% versus placebo 47%; P < 0.01). Furthermore, all deaths were because of rupture of the suprarenal abdominal region of the aorta. Interestingly, both platelet inhibitors also reduced the visible thrombi in mice with aortic arch or thoracic aneurysms (data not shown; P < 0.048).

Platelet Inhibitors Decreased Platelet and Macrophage Accumulation and Matrix Metalloproteinase-2 and -9 Activity in Abdominal Aortas
Matrix metalloproteinase (MMP)-2 and MMP-9 have been shown to contribute to the initiation and progression of AAAs.15,16,36 Macrophages are a primary source of MMP-9 and platelets contain both MMP-2 and MMP-9. 37–39 Notably, we found that platelet and macrophage accumulation was decreased significantly in mice treated with platelet inhibitors versus placebo controls (Figure 2A and 2C). Therefore, we examined whether platelet inhibitors reduced levels of MMP activity in the aorta. Importantly, MMP activity in the aorta was significantly decreased with platelet inhibitors (Figure 2B and 2C).

To further characterize the effect of platelet inhibitors on MMP activity in the aorta, abdominal aortas were removed and levels of pro and active MMP-2 and MMP-9 were quantified by ELISA and visualized with gelatin zymography. Platelet inhibitors significantly decreased levels of active MMP-2 and MMP-9 versus placebo controls (Figure 3A–3D). Interestingly, platelet inhibitors also decreased abdominal aortic tissue concentrations of total MMP-2, with a nonsignificant decrease in total MMP-9, as measured by ELISA (Figure 3A and 3B). Importantly, the decrease in both MMP-2 and MMP-9 were significantly correlated with decreased macrophage (\( r^2=0.835 \)) and platelet (\( r^2=0.913 \)) counts in the AAAs of ASA or clopidogrel-treated mice (data not shown; P < 0.001 for all correlations).

Platelet Inhibition Decreased Plasminogen Activators and Plasma Cytokines in AngII-Infused Mice
Plasmin generation by the plasminogen activators, urokinase plasminogen activator (uPA), and tissue-type plasminogen activator (tPA) is associated with increased levels of active MMP-2 and MMP-9.40 We determined that total and active uPA and tPA in both plasma and abdominal aorta were decreased by platelet inhibitors. Protein was harvested from pooled aortas obtained from the intervention study (3 aortas pooled for n=1; total different pools analyzed n=4). Pooled lysates (1-µg total protein/well) were analyzed by MMP-2 (A) and 9 (B) Biotrak Activity Assay ELISAs. Protein (20 µg/ lane) from clopidogrel bisulfate and ASA total MMP-2, active MMP-2, and active MMP-9 vs placebo controls. Data were analyzed with a 1-way ANOVA with a Holm–Sidak post hoc.
significantly decreased by platelet inhibitors (Figure 4C–4F). Furthermore, the endogenous inhibitor of uPA and tPA, plasminogen activator inhibitor 1, was significantly increased by platelet inhibitors (Figure 4A and 4B).

Platelets contain and secrete a variety of inflammatory and thrombotic molecules upon activation. We ascertained if antiplatelet therapy decreased secretion and circulation of these molecules. Circulating and abdominal aortic platelet factor 4 was significantly decreased by antiplatelet therapy (Figure 5A). In addition, antiplatelet therapy significantly attenuated several cytokines that are expressed by platelets, such as granulocyte colony stimulating factor, interferon-γ, regulated on activation normal T cell expressed and secreted (RANTES), interleukin-1α and β (IL-1α, β; Figure 5B–5F). Several other plasma inflammatory cytokines were also significantly decreased by antiplatelet therapy, including: IL-4, IL-5, IL-6, IL-7, IL-12(p70), IL-13, IL-17, KC, MCP-1 (monocyte chemotactic protein 1), MIP-1α (macrophage inflammatory protein) and β, MIP-2, and tumor necrosis factor-α.

Treatment With P2Y₁₂ Inhibitors and ASA Significantly Reduce Rupture and Dissection in Aneurysm Patients

A total of 1578 eligible patients (nonmissing data for all covariates) with aortic aneurysms (AAAs; defined as either thoracic, abdominal, or thoracoabdominal) were identified totaling 5592 years of person time with an average follow-up of 2.28 years per individual. In total, 351 AA dissections (227) or ruptures (124) were recorded. Summaries of drug categories, demographic, and vital characteristics are presented in Table 1.

After a diagnosis of AAs, P2Y₁₂ inhibitors (hazard ratio, 0.49; 95% confidence interval, 0.32–0.74; P <0.05) were significantly associated with decreased dissection or rupture after adjustment for vital (blood pressure and body mass index),

**Figure 4.** The plasminogen activation system was decreased in plasma and abdominal aorta of mice receiving platelet inhibitors. Protein was harvested from pooled aortas obtained from the intervention study (3 aortas pooled for n=1; total n=4). Plasma (n=14–16 each treatment group) was analyzed on total or active (A) plasminogen activator inhibitor 1 (PAI-1), (C) tissue-type plasminogen activator (tPA), or (E) urokinase plasminogen activator (uPA) ELISA plates. Protein (1–µg protein/well) was run on total or active (B) PAI-1, (D) tPA, or (F) uPA ELISA plates. Histobars represent means±SEM of n=14 to 16 plasma samples (A, C, and E) or n=4 pooled aortas (B, D, and F). *P<0.001 clopidogrel and aspirin (ASA) total and active PAI-1, uPA, and tPA vs placebo controls. Data were analyzed with a 2-way ANOVA with a Holm–Sidak post hoc.
demographic (age, sex, and race), and comorbid factors (diabetes mellitus, atrial fibrillation, heart failure, and chronic kidney disease; Table 1). This effect seems to be modified by whether the location of the aneurysm is thoracic versus abdominal, and the effect estimate in thoracic aneurysms is less protective and not statistically significant. ASA also protected against dissection or rupture (hazard ratio, 0.50; 95% confidence interval, 0.35, 0.72; \( P=1\times10^{-4} \)) in adjusted analyses in both the thoracic and abdominal aorta (Table 2). Kaplan–Meier plots of survival for each drug exposure are presented in Figure 6A and 6B. Participants with at least 30 days of follow-up underwent sensitivity analyses, which did not substantively change event ratios (data not shown).

**Discussion**

A better understanding of the underlying pathophysiology in aneurysm disease is essential to develop nonsurgical therapeutics to reduce the burden of this condition in our aging population. A prominent feature of human AAAs is the accumulation of a laminated mural platelet-rich thrombus that develops along the luminal surface.\(^{22,41} \) Interestingly, a thrombus is a dynamic biological entity that is balanced between luminal renewal and abluminal fibrinolysis.\(^{24,25,31,44} \) Importantly, clinical studies suggest that thrombus volume or blood displacement caused by presence of mural thrombus may be a predictor of both AAA expansion and rupture.\(^{43,45} \)

Therefore, it is surprising that the role of platelets in the formation of mural thrombi and their potential contribution to both the progression of AAAs has not been investigated systematically in a mouse model. We found that inhibition of platelets reduced rupture of established AAAs. Furthermore, we found ASA or P2Y\(_{12} \) inhibitors administration may protect human AAA patients from rupture. In summary, our results suggest that inhibiting platelet activation slows AAA progression and reduces AAA rupture, which may support the clinical use of ASA and P2Y\(_{12} \) inhibitors in AAA patients.

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Platelet inhibition reduced plasma and tissue cytokines in established abdominal aortic aneurysms in mice. Protein was harvested from pooled aortas obtained from the intervention study (3 aortas pooled for n=1; total n=4). Plasma (n=14–16 each treatment group) or protein (1-µg protein/well) was run on a platelet factor 4 (PF4) ELISA (A). Plasma samples (n=10 each treatment group) were analyzed on a chemokine/cytokine luminex array and (B) granulocyte colony stimulating factor (G-CSF), (C) interferon-γ (IFN-γ), (D) regulated on activation normal T cell expressed and secreted (RANTES), (E) interleukin-1α (IL-1α), or (F) IL-1β were quantitated. Histobars represent means±SEM of n=10 plasma samples (A–F) or n=4 pooled aortas (A). *\( P<0.001 \) clopidogrel and aspirin (ASA) vs placebo controls. Data were analyzed with a 1-way ANOVA on ranks with Dunn’s post hoc (B–F) or 2-way ANOVA with a Holm–Sidak post hoc (A).
ASA therapy is recommended for patients with AAA from the time of diagnosis until the perioperative period. It is currently hypothesized that the benefit of ASA in reducing cardiovascular morbidity and mortality and potentially AAA progression outweigh the risks of bleeding and AAA rupture. However, clinical studies examining the effects of platelet inhibitors (clopidogrel bisulfate and ASA) on non-genetically categorized AAA ruptures are extremely limited. Among a large meta-analysis of 6 studies, only 1 reported data with regard to AAA rupture. The UKSAT (United Kingdom Small Aneurysm Trial) study reported that antiplatelet administration resulted in a rupture rate hazard ratio of 0.83, which was not significant. The mechanism by which ASA reduces aneurysm expansion is hypothesized to be via a decrease in thrombus formation, a reduction in aortic wall inflammation, and stabilization of the aortic wall. A small retrospective study reported patients with medium-sized AAAs had significantly reduced AAA expansion and time to aneurysm repair on low-dose ASA. Other studies have demonstrated a reduction in progression of small AAAs, although a definitive association between platelet inhibitors and aneurysm reduction was not established. However, a large meta-analysis demonstrated that antiplatelet therapy resulted in a nonsignificant decrease in AAA growth compared with untreated aneurysm patients (P=0.241) after adjusting for confounding variables. In our study, we observed protective associations between platelet inhibitors and AA rupture or dissection in 351 patients. We also found an inverse relationship between adverse AA events and non-ASA platelet inhibitor use, as well as an independent effect of ASA use. This finding indicates that addition of ASA or P2Y12 inhibitors to standard therapy may be beneficial to AAA patients in addition to effects on other presumed cardiovascular diseases. In support of our findings, a phase 2 clinical trial is examining the efficacy of ticagrelor on patients with small AAAs (government clinical trial identifier: NCT02070653).

To better mimic this clinical situation, we administered platelet inhibitors to mice with established AAAs. We found that prolonged AngII infusion increased rate of aortic rupture, which was significantly reduced with clopidogrel bisulfate or ASA. These inhibitors also had no effect on abdominal aortic diameter (clopidogrel bisulfate, P=0.19 and ASA, P=0.08). Other studies have shown that a GP IIa/IIIb platelet inhibitor (abciximab) and a P2Y12 receptor antagonist (AZD6140) prevented aneurysm growth in the rat xenograft model of aneurysm. While this model does not exhibit rupture, it does exhibit an intraluminal thrombus similar to human aneurysms in a certain percentage of rats. We speculate that our results may be different because of the large amount of ruptures in our placebo groups, resulting in a lack of subsequent measurements of aortic diameters. Alternatively, a previous publication demonstrated a distinct difference between the incidence or maximal diameter of AngII-induced AAAs and increased mortality because of rupture.

The continued accumulation of platelets and macrophages may result in proteolytic destruction of the aortic architecture via release of MMPs. Platelets and macrophages are a robust source of MMPs. Furthermore, platelet-derived chemo- kines can regulate the expression of MMPs from VSMCs and macrophages. In addition, plasmin production by uPA or tPA is a critical step in fibrinolysis and MMP activation. Here, we demonstrate that clopidogrel bisulfate and ASA intervention reduce platelet and macrophage infiltration into the vessel wall, circulating platelet-derived cytokines.

### Table 1. Demographic, Vital, and Medication Characteristics for AA Individuals Stratified by Outcome

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Events</th>
<th>Nonevents</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristic</strong></td>
<td>351</td>
<td>1524</td>
<td></td>
</tr>
<tr>
<td>European ancestry, %</td>
<td>82.1</td>
<td>75.5</td>
<td>0.009</td>
</tr>
<tr>
<td>Women, %</td>
<td>68.7</td>
<td>69.9</td>
<td>0.635</td>
</tr>
<tr>
<td>Age, mean (SD)</td>
<td>66.5 (11.1)</td>
<td>67.6 (10.9)</td>
<td>0.125</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>28.0 (5.81)</td>
<td>27.9 (5.8)</td>
<td>0.667</td>
</tr>
<tr>
<td>Type II diabetes mellitus, %</td>
<td>2.85</td>
<td>4.72</td>
<td>0.121</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>89.8</td>
<td>85.5</td>
<td>0.045</td>
</tr>
<tr>
<td>Atrial fibrillation, %</td>
<td>10.5</td>
<td>15.8</td>
<td>0.012</td>
</tr>
<tr>
<td>Heart failure, %</td>
<td>12.3</td>
<td>21.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>8.83</td>
<td>13.5</td>
<td>0.017</td>
</tr>
<tr>
<td>Dialysis</td>
<td>3.42</td>
<td>2.10</td>
<td>0.141</td>
</tr>
<tr>
<td>AA dissection, %</td>
<td>64.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AA rupture, %</td>
<td>35.4</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Abdominal AA, %</td>
<td>22.7</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Thoracic AA, %</td>
<td>17.7</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Thoracoabdominal AA, %</td>
<td>59.5</td>
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<td>NA</td>
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<tr>
<td>Antiplatelet drugs, %</td>
<td>11.7</td>
<td>25.9</td>
<td>&lt;0.001</td>
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<tr>
<td>P2Y12 inhibitors</td>
<td>49.9</td>
<td>74.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Categorical variables assessed with exact tests, continuous variables with Student t test. AA indicates aortic aneurysm; ASA, aspirin; BMI, body mass index; and NA, not applicable.

### Table 2. Cox Proportional Hazards Regression Analysis of Drugs by Class

<table>
<thead>
<tr>
<th>Term</th>
<th>HR</th>
<th>95% CI</th>
<th>HR</th>
<th>95% CI</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y12 inhibitors (crude)</td>
<td>0.36</td>
<td>0.24–0.53</td>
<td>0.21</td>
<td>0.10–0.42</td>
<td>0.42</td>
<td>0.15–1.15</td>
</tr>
<tr>
<td>ASA (crude)</td>
<td>0.35</td>
<td>0.26–0.45</td>
<td>0.28</td>
<td>0.18–0.43</td>
<td>0.27</td>
<td>0.17–0.45</td>
</tr>
<tr>
<td>P2Y12 inhibitors (adjusted)</td>
<td>0.49</td>
<td>0.32–0.74</td>
<td>0.24</td>
<td>0.12–0.51</td>
<td>0.81</td>
<td>0.27–2.41</td>
</tr>
<tr>
<td>ASA (adjusted)</td>
<td>0.50</td>
<td>0.35–0.72</td>
<td>0.47</td>
<td>0.22–1.00</td>
<td>0.30</td>
<td>0.15–0.60</td>
</tr>
</tbody>
</table>

Adjusted models are fit with terms for age, sex, race, body mass index, smoking, diabetes mellitus, heart failure, atrial fibrillation, chronic kidney disease, and dialysis. Abd. indicates abdominal; ASA, aspirin; CI, confidence interval; HR, hazard ratio; and Thor., thoracic.
plasminogen activators, and ultimately the amount of active MMP-2 and MMP-9 in the abdominal aortas. Importantly, MMP-2 and MMP-9 are correlated with increased aneurysmal disease and rupture.\(^{16,55}\) Furthermore, several attenuated cytokines and chemokines play a significant role in AAA progression.\(^ {52-55}\) In addition, there is a role for the uPA, uPAR, tPA, and plasminogen activator inhibitor 1 plasminogen axis in both the progression and the rupture of experimental aneurysm.\(^ {48,56-58}\) Although it is uncertain whether these MMPs, cytokines, and plasminogen activators/inhibitors are primarily derived from platelets or macrophages, there are significant correlations between platelets and macrophages among all of these inflammatory mediators. However, there are several contradictions in the literature about the role of Th1/Th2 chemokines and cytokines and MMPs with regard to the outcome of AAA pathogenesis.\(^ {59,60}\) Indeed, the differences in cytokine and MMP profile only reflect a single stage of AAA development/regeneration, thus complicating a proper analyses and interpretation of these correlations.

In conclusion, we show that platelet accumulation and activation is detrimental in a mouse model of established AAAs. The pathological role seems to involve macrophage recruitment and the production of MMPs resulting in vessel instability and rupture. We further show a positive association with platelet inhibitors and ASA in the prevention of human AA rupture or dissection. The results indicate that platelet inhibitors are beneficial in pre-existing aneurysms. Future studies will be directed at dissecting the role of platelet signaling in AAAs.

**Acknowledgments**

We acknowledge helpful suggestions provided by the VTRACC (Vanderbilt Center for Translational and Clinical Cardiovascular Research) working group that contributed to this work, as well as helpful advice from Dr Digna Velez Edwards.

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**Disclosures**

None.

**References**

Cardiovasc Med J


Platelet Inhibitors Reduce AAA Rupture

Significance

Abdominal aortic aneurysm is a progressive expansion of the aorta which may result in catastrophic rupture and death. This cardiovascular disease is estimated to affect almost 10% of people over the age of 50 years with an estimated 1 of every 250 people affected. Despite decades of research, there are no clinically approved drug regimens for this disease with surgical intervention as the only approved therapy. Here, we demonstrate commonly used antiplatelet drugs that prevent rupture of advanced abdominal aortic aneurysms in a mouse model. Antiplatelet therapy dramatically reduces the amount of destructive enzymes and tissue/circulating inflammatory proteins. Finally, we verify this effect in a retrospective analysis of human aneurysm patients. These results identify platelets as a critical component of aneurysm rupture and suggest using antiplatelet therapy may be beneficial in patients with abdominal aortic aneurysm.
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The cover image indicators in the issue have been corrected.
## Supplemental Table I: Mice weights, systolic blood pressures, and lipid profiles

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Gender</th>
<th>Mouse number</th>
<th>Treatment</th>
<th>Weight (grams)</th>
<th>SBP (mmHg)</th>
<th>TPC (mg/dL)</th>
<th>Total VLDL (mg/dL)</th>
<th>Total LDL (mg/dL)</th>
<th>Total HDL (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
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<td>Ldlr&lt;sup&gt;-/-&lt;/sup&gt; Intervention</td>
<td>Male</td>
<td>14</td>
<td>Placebo</td>
<td>35.8 ± 2.8</td>
<td>159 ± 7.8</td>
<td>1455.9 ± 122.1</td>
<td>117.2 ± 11.9</td>
<td>1268.9 ± 99.1</td>
<td>69.8 ± 3.2</td>
<td>732.8 ± 111.7</td>
</tr>
<tr>
<td>Ldlr&lt;sup&gt;-/-&lt;/sup&gt; Intervention</td>
<td>Male</td>
<td>16</td>
<td>ASA</td>
<td>34.9 ± 3.1</td>
<td>165 ± 11.8</td>
<td>1511.9 ± 117.1</td>
<td>120.8 ± 10.1</td>
<td>1319.7 ± 55.2</td>
<td>71.4 ± 7.7</td>
<td>754.9 ± 27.7</td>
</tr>
<tr>
<td>Ldlr&lt;sup&gt;-/-&lt;/sup&gt; Intervention</td>
<td>Male</td>
<td>15</td>
<td>Placebo</td>
<td>36.2 ± 2.7</td>
<td>167 ± 14.8</td>
<td>1588.9 ± 45.7</td>
<td>115.7 ± 9.7</td>
<td>1398.9 ± 88.1</td>
<td>74.3 ± 9.9</td>
<td>722.9 ± 89.2</td>
</tr>
<tr>
<td>Ldlr&lt;sup&gt;-/-&lt;/sup&gt; Intervention</td>
<td>Male</td>
<td>15</td>
<td>Clopidogrel</td>
<td>35.8 ± 2.5</td>
<td>164 ± 12.6</td>
<td>1553.2 ± 104.4</td>
<td>122.4 ± 10.8</td>
<td>1349.7 ± 77.9</td>
<td>81.1 ± 11.8</td>
<td>764.8 ± 77.9</td>
</tr>
</tbody>
</table>

Abbreviations: TPC—total plasma cholesterol; VLDL—very low density lipoproteins; LDL—low density lipoproteins; HDL—high density lipoproteins; BM: bone marrow; Ldlr: low density lipoprotein receptor.
Supplemental Figure I: In vivo administration of ASA and clopidogrel bisulfate inhibit platelet activation. Ldlr<sup>-/-</sup> mice were fed a HFD and infused with AngII for 28 days. Mice were then stratified based on in vivo suprarenal abdominal aortic diamters into 4 equal-sized groups and then placed on placebo (n = 14) or ASA (n = 16) and placebo (n = 15) or clopidogrel bisulfate (n = 15) and infused for an additional 42 days. (A) To verify effective in vivo delivery of ASA, plasma was analyzed for inhibition of T<sub>XB2</sub> via ELISA. (B) To verify effective in vivo delivery of clopidogrel bisulfate, blood was collected retro-orbitally, prior to sacrifice, treated with ADP, and αIIβ3 integration activation was measured. Circles represent individual mice, diamonds represent means ± SEM. *P < 0.001 treatment groups versus controls. Data was analyzed with an unpaired two-tailed Student's t-test.
Supplemental Data and Methods

Platelet Inhibitors Reduce Rupture in a Mouse Model of Established Abdominal Aortic Aneurysm

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Methods

Mice and diet:

Male \( \text{Ldlr}^-/^- \) mice (8-12 weeks of age) were originally obtained from The Jackson Laboratory (stock number 002207) and bred in-house at UNC-CH. To induce hypercholesterolemia, \( \text{Ldlr}^-/^- \) mice were fed a diet enriched with saturated milk fat (21\% wt/wt) and cholesterol (0.15\% wt/wt, Harlan Teklad diet TD.88137 produced by Purina) for 1 week prior to AngII infusion and throughout the duration of infusion.

Platelet inhibition:

For P2Y\(_{12}\) inhibition, \( \text{Ldlr}^-/^- \) mice were fed a custom-made Western diet containing peanut butter flavoring with or without clopidogrel bisulfate (50 mg/kg, purchased from UNC pharmacy: 75 mg Bristol-Meyers Squibb/Sanofi Pharmaceutical tablets) after diameter stratification and 42 day AngII pump implantation (Dyets Inc., based on Harlan Teklad TD.88137). Pills consisted of 28.6\% active compound and 71.4\% filler. Mice eat an average of 4 g food/day, therefore 1048 mg of pills were added per kg food (0.1048\%). The placebo diet consisted of 0.1048\% cellulose as a substitute for the filler. Bacon flavoring was added to both clopidogrel bisulfate and placebo diets for palatability. ASA (30 mg/L, Sigma Aldrich) was administered to \( \text{Ldlr}^-/^- \) mice via water after diameter stratification and 42 day AngII pump implantation, as described (1). ASA was dissolved in 200 proof sterile molecular grade ethanol (~200\( \mu l \), Sigma Aldrich) before addition to the water (0.1\% final concentration). Placebo groups received 200\( \mu l \) of ethanol alone (0.1\% final concentration). Strawberry MiO Liquid
Water Enhancer (Kraft Foods) was added to the water for palatability (500µl or 0.5%/water bottle).

**Verification of drug delivery:**

Clopidogrel bisulfate inhibition of $P2Y_{12}$ was determined by measurement of $\alpha_{IIb}\beta_3$ activation (2, 3). Briefly, blood was drawn from the retro-orbital plexus with heparinized capillaries (FisherBrand; ~50 µl). Whole blood was then diluted with apyrase (0.03U/ml; Sigma Aldrich, catalog A6535) and modified Tyrode’s buffer (137 mM NaCl, 0.3 mM Na$_2$HPO$_4$, 2mM KCl, 12 mM NaHCO$_3$, 5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 5 mM glucose, and 1 mM CaCl$_2$ at pH 7.3) were activated with ADP (10uM; Sigma Aldrich catalog A2754) in the presence of JON/A-PE (2 µg/ml). Following 10 minutes of incubation, samples were diluted with PBS and analyzed immediately with a BD Accuri C6 Flow Cytometer (BD Biosciences). Effective ASA absorption was determined by plasma concentration of thromboxane B2.

**Preparation of platelet antibody:**

A rat anti-mouse GPIX antibody (Emfret Analytics, catalog M051-0) was labeled with 680 nm anti-rat Alexa Fluor dye (Life Technologies, catalog A-21096) for in vivo platelet labeling. Briefly, several vials of the antibody were concentrated 10 fold to 5 mg/100 µl. Fresh 1M sodium bicarbonate (pH 9.0, 11 µl) was added to the antibody followed by 2 µl of Alexa Fluor 680 (stock 10 mg/mL in DMSO). This solution was incubated in the dark for 1.5 hours. The antibody/dye solution was then centrifuged at
2500 rpm for 3 minutes in a Bio-Spin 30 (BioRad), washed in sterile 1 x hanks balanced salt solution (HBSS), recentrifuged, and the flow-through resuspended at a concentration of 0.025 µg/µl in 1 x HBSS. Mice were given an initial loading dose of 5 µg/mouse followed by a maintenance dose of 2.5 µg/mouse/day. This process was repeated for the Alexa Fluor 680 IgG control using a Rat IgG1 (BD Biosciences, catalog 553921).

**Preparation of macrophage nanoparticles:**

Dextran-coated magnetic iron oxide (Fe$_3$O$_4$) nanocrystals were utilized in the in vivo labeling of macrophages, as described previously with modifications (4-6). Aminated (-NH$_2$) dextran-coated magnetic iron oxide 20 nm nanocrystals in water were purchased from M K Impex Corp (MKnano, Ontario Canada; product number MKN-IOW-DX-020NH$_2$). These nanoparticles were labeled with a DyLight 800 NHS Ester kit (Thermo Scientific, catalog 46421), per the instruction manual. Extraneous dye (270,000 MW) was removed using Vivaspin 6 centrifugal concentrators (Sartorius, 300,000 MWCO). Nanoparticles were further purified by a SuperMag Separator (Ocean NanoTech, incubated overnight per instructions) and resuspended with sterile 1 x Hanks Balanced Salt Solution for in vivo use. Control, unlabeled, nanoparticles underwent the same procedures without addition of DyLight 800 NHS Ester. Labeling was verified by injection into the peritoneum of mice, extraction of peritoneal macrophages 24 hours later, and quantification on an Odyssey LiCor imager with an 800 nm filter.
Intervention study:

*Ldlr⁻/⁻* mice (n = 100) were fed a Western diet for 1 week prior to and throughout a 28 day AngII infusion. Of those, 60 mice had AAAs as measured by 50% diameter increase from day 0 to day 28. Fifteen mice did not develop aneurysms, and 25 mice died of ruptures. Of those 25 mice, it was ascertained that 12 mice died of arch rupture, 3 mice died of thoracic rupture, and 10 mice died of abdominal aortic rupture based on necropsy. Surviving mice were stratified by aneurysm size, via ultrasonography, into 4 groups with equivalent aortic diameter: Placebo for clopidogrel bisulfate (50 mg/kg cellulose filling in diet), clopidogrel bisulfate (50 mg/kg clopidogrel in diet), placebo ASA (ethanol + Strawberry MiO), and ASA (30 mg/L and Strawberry Mio). Immediately before treatment, mice were implanted with an additional 42 day pump (Durect Model 2006) for a total of 70 days of AngII infusion.

One week prior to sacrifice, select mice were retroorbitally injected (daily) with an anti-GPIX mouse antibody (emission: 700 nm; 5.0 µg initial loading dose; 2.5 µg total antibody per mouse/day afterward). One day prior to sacrifice, all mice were injected retroorbitally with a solution of dextran-coated nanoparticles (emission: 800 nm; 10 mg final labeled iron oxide/kg body weight) (6). For MMP analysis, select mice were injected retroorbitally with a MMP-sense solution (680 nm, 150 nmol/kg body weight, Perkin Elmer), 1 day prior to sacrifice (6).

Aortic imaging and quantification:
After sacrifice, aortas were perfused, removed, and immediately cleaned free of all adventitia. Aortas were then placed on an Odyssey Infrared Bioimaging System (LI-COR Biosciences) and positioned appropriately. Aortas were then scanned with both the 700 and 800 nm filters on to detect platelets/MMPs or macrophages, respectively. All aortic preparations and scanning parameters remained constant throughout the procedure (Resolution 42 µm; Quality high; Focus offset 1.0 mm; 700 nm channel intensity 5.0; 800 channel intensity 2.0). Aortas were analyzed with the Odyssey software (on-site) or Li-Cor Image Studio Lite version 3.1.4 (remote computer).

For quantification of raw platelet and macrophage cells, exact numbers of cells were labeled and analyzed and tissue samples extrapolated. Platelets: non-study Ldlr-/- mice were injected retro-orbitally with Alexa Fluor 680-labeled GPIX, similar to study animals, for 1 week. Mice were sacrificed and blood was isolated. Platelets were then enumerated using a BD Accuri C6 Flow Cytometer (BD Biosciences). Specific numbers of platelets were then seeded into a 96 well plate (50 µl/well) using a dose curve from $5 \times 10^8$ platelets to 0 (Resolution 86 µm; Quality high; Focus offset 3.0 mm; 700 nm channel intensity 7.0). This was performed 5 times and a standard curve was created. Macrophages: non-study Ldlr-/- mice were injected intra-peritoneally with DyLight 800-labeled dextran-coated nanoparticles. Twenty-four hours later, macrophages were obtained via peritoneal lavage and enumerated using a hemocytometer. Specific numbers of macrophages were then seeded into a 96 well plate (50 µl/well) using a dose curve from $1.0 \times 10^7$ macrophages to 0 (Resolution 86 µm; Quality high; Focus offset 3.0 mm; 800 nm channel intensity 6.0). This was performed 5 times and a standard curve was created. For extrapolation to tissues, abdominal aortas, from the
celiac artery to the right renal artery were excised and cleaned from of adventitia. Aortic segments were then placed in separate solutions of type II porcine pancreatic elastase (250 µg/mL, Sigma) and type I collagenase (1 µg/mL, Worthington) for 2 hours at 37°C. All contents were then passed through a 40 µ nylon cell strainer (BD Biosciences) and quantified.

**ELISAs and cytokine array:**

The following commercially available kits were used: platelet factor 4 (PF4) (R&D Systems), thromboxane B2 (Cayman Chemical), matrix metalloproteinase (MMP)-2 and MMP-9 Biotrak Activity Assay System (GE Healthcare), mouse plasminogen activator inhibitor 1 (PAI-1) activity and PAI-1 total antigen ELISA (Molecular Innovations), mouse urokinase plasminogen activator (uPA) activity and uPA total antigen ELISA (Molecular Innovations), and mouse tissue plasminogen activator (tPA) activity and tPA total antigen ELISA (Molecular Innovations). The MMP-2 and MMP-9 Biotrak Activity Assay System ELISAs specifically detects epitopes from ‘active’ MMPs. To determine the amount of endogenously active MMPs, untreated plasma/tissue are placed into each well. To detect the amount of ‘total active' MMPs, 4-Aminophenylmercuric acetate (APMA)-treated plasma/tissue were applied to each well, as suggested by the GE Healthcare manual. APMA treatment activates latent (pro-MMP) to active MMPs by dissociation of cysteine residues in the propeptide from the zinc active site. To obtain the level of pro-MMPs, the amount of endogenously ‘active’ is subtracted from the ‘total active’.
Cytokine/chemokine panel I (EMD Millipore Company) was analyzed by the Luminex MAGPIX system by the Animal Clinical Chemistry and Gene Expression Laboratory Core at UNC-CH.

Protein assay:

Where applicable, protein was quantified in cell or tissue samples utilizing the DC protein assay (BioRad Inc.) in the 96 well plate assay according to the product manual.

Gelatin zymography:

MMP-2 and MMP-9 activities were analyzed in the homogenates of aortic tissues (70 day infused mice) by gelatin zymography. Briefly, abdominal aortas (from the celiac artery to the right renal artery) were extracted, cleaned of all adventitia, and homogenized in ice cold 50 mM Tris-HCL pH 7.5 with 0.25% triton X-100 with a Fischer Scientific PowerGen Homogenizer and immediately flash frozen in liquid nitrogen. All gelatin gels (catalog 161-1167), loading buffer (zymogram sample buffer cat 161-0764) and processing buffers (10 x zymogram development buffer cat 161-0766; 10 x zymogram renaturation buffer cat 161-0765) were obtained from Bio-Rad Laboratories. Three aortas from each group were pooled 4 times for a total n = 4. Protein was measured and 25 µg was placed into loading buffer and run under non-reducing conditions. Gel electrophoresis was performed at 125 V for 90 minutes. SDS was removed using the renaturation buffer with slight agitation for 6 hours at room
temperature. Gels were placed in water overnight at 4°C. The next day, gels were incubated in development buffer for 6 hours at 37°C, washed 3 x in 200 mL ddH₂O, and stained with Gel Code Blue for 1 hour at room temperature. Gel Code Blue was destained using several washes with ddH₂O and gels captured on an illuminated platform with a Sony NEX-5. For loading control, two gels (the gelatin gel and a separate 10% Tris-HCL gel) were loaded with similar amounts of loaded protein made at the same time. The Tris-HCL gel was run, transferred, and GAPDH quantified using a Odyssey LiCor machine, as previously described (7).

Osmotic minipump implantation:

At 8 to 12 weeks of age, male mice were implanted with Alzet osmotic minipumps (Model 1004 or 2004, Durect Corporation) subcutaneously into the right flank. Infusion of AngII (1,000 ng/kg/min; Bachem) continued for 28 days, as described previously (8). Pump implantation for the intervention study is described in an earlier section.

Blood pressure measurements:

Systolic blood pressure (SBP) was measured on conscious mice using a Coda 8 (Kent Scientific Corporation) tail-cuff system, as described previously (9). SBP was measured for 5 consecutive days prior to pump implantation, and during the last week of AngII administration.
**Plasma collection and aortic dissection/processing:**

Seventy days after initial pump implantation, mice were sedated with 3% isoflurane and blood was collected from the inferior vena cava into a 25 gauge x 1' needle pre-coated with 3.8% sodium citrate. The mice were then humanely euthanized. An aliquot of blood was analyzed for complete blood count utilizing a Hemavet 950 LV veterinary multi-species hematology system (Drew Scientific). Blood was centrifuged at 4,000 x g for 15 minutes to prepare platelet poor plasma and then stored at -80°C until use. Aortas were perfused with saline, extracted, and either immediately processed for imaging and dye quantification or placed into formalin (10% wt/vol) until dissection and processing.

**Measurements of abdominal aortic diameters:**

Abdominal aortas were visualized with high-frequency ultrasound (Vevo 2100, VisualSonics, Toronto, ON, Canada) on day 0, 27, and 69 as described previously (10). Luminal diameters were measured on images with the maximal dilation. No difference was found between ultrasound measurements and ex-vivo diameter measurements (P = 0.97). Represented diameters in publication are from in vivo ultrasound measurements.

**Measurements of aortic arch area, thoracic diameters, and visible thrombi:**
Aortic arch area and thoracic diameters were determined as described previously (11, 12). Briefly, aortas were pinned and photographed. Maximal ex vivo diameters of the thoracic aortas were measured with NIH Image J software (http://imagej.nih.gov/ij/download.html). Aortas were cut longitudinally and pinned. Aortic intimas were then photographed and the extent of dilatation and area was quantified in aortic arches via Image J. Diameters were calculated from aortic arch circumferences. Intimal areas were quantified from regions of ascending arches protruding from the ventricle to 3mm proximal from the subclavian artery.

Thrombi were assessed by visual examination of the aortas by two blinded investigators. Thrombi were deemed similar to the type III aneurysm in Figure 2 of the publication by Manning and colleagues (13). Briefly, thrombi were determined to be any portion of the aneurysm which had a visible clot structure, which were dark red to black in color.

**Plasma lipid analyses:**

Mouse plasma lipid concentrations were analyzed with the following commercially kits: total plasma cholesterol (Total Cholesterol E), triglycerides (L-Type TG M), LDL-C (L-Type LDL-C), and HDL-C (L-Type HDL-C) from Wako Chemicals (Richmond, VA). VLDL-C was then calculated by subtracting HDL-C and LDL-C from total plasma cholesterol.

**Research statistics and data representation:**
All bar and line graphs were created with Sigma Plot v.11 (SPSS, Chicago, IL). All statistical analysis was performed using SigmaStat, now incorporated into Sigma Plot v.11. Data are represented as mean ± SEM. For two group comparison of parametric data, a Student’s t-test was performed, while non-parametric data was analyzed with a Mann-Whitney Rank Sum. Statistical significance between multiple groups was assessed by One Way analysis of variance (ANOVA) on Ranks with a Dunn’s post hoc, One Way ANOVA with Holm Sidak Post Hoc, or Two Way ANOVA with Holm Sidak Post Hoc, when appropriate. Statistical significance among groups performed temporally was assessed by either a One Way Repeated Measures ANOVA (parametric) or Repeated Measures ANOVA on Ranks (non-parametric), where appropriate. Percentage incidence of aneurysms were analyzed by a Fisher’s exact test. Linear dependencies between macrophages or platelets to active MMPs, cytokines, chemokines, or plasminogen activators were calculated utilizing the Pearson linear correlation coefficient (r) and strengthened by analyzing the coefficient of determination (r²). Values of $P < 0.05$ were considered statistically significant.

Human study information:

Study population

We utilized the clinical data available from the Synthetic Derivative electronic medical record (EMR) database (14), which consists of de-identified clinical data obtained from patients at Vanderbilt University Medical Center Hospital in Nashville, TN. De-identified data from multiple sources are available within the Synthetic Derivative
database, including diagnostic and procedure codes, basic demographics, clinical documentation (e.g., discharge summaries, clinic notes, and consultation notes), nursing documentation, multi-disciplinary assessments, laboratory values, echocardiogram diagnoses, imaging reports, electronically derived data, and medication orders from both inpatient and outpatient settings. The Institutional Review Board of Vanderbilt University Medical Center approved this study.

**Data Collection**

We developed and validated an algorithm to identify within the Synthetic Derivative all eligible individuals with AA, and cases defined as those who progressed to rupture or dissection, and excluding patients who underwent surgical AA repair. A cardiologist (E.J.), blinded to the algorithm calls, reviewed full EMRs for 50 individuals who were classified as cases by the algorithm and 50 algorithm controls who did not progress to rupture, dissection or surgical AA repair. We then calculated the positive predictive value (PPV) for the algorithm classifier versus expert review for cases and the negative predictive value (NPV) for controls. Both PPV and NPV were 100% for the final algorithm for cases and controls, respectively. We also abstracted body mass index (BMI, kg/m2) on the date of AA diagnosis, race (European ancestry versus other), sex, and type 2 diabetes (T2D) status. Medication information was extracted from both structured (e.g., computerized physician order entry) and unstructured (e.g., clinic visit notes) sources using MedEx, a high performance medication information extraction system developed at Vanderbilt (15, 16). We classified smoking as ever/never or unknown on individuals using an existing EMR algorithm that employs machine learning and natural language
processing. Prior evaluation of the algorithm noted that it identified smokers with a PPV of 95% and sensitivity of 92% on a set of Vanderbilt records (17).

Using International Classification of Disease, ninth revision, (ICD 9) diagnostic codes, eligible individuals were defined as those who had at least three codes from ICD 9 code group 441 (AA and dissection), 38.04 (incision vessel, aorta), 38.44 (resection of vessel with replacement, abdominal aorta), 39.52 (other repair of aneurysm), 39.71 (endovascular implantation of graft in abdominal aorta), or 39.73 (endovascular implantation of graft in thoracic aorta), on at least two distinct dates. We excluded individuals with diagnoses of Marfan Syndrome (ICD 9: 759.82) or Ehlers-Danlos Syndrome (ICD 9: 756.83), as these inherited defects of the integument are strong risk factors for both development of AA and risk of AA progression to rupture or dissection. Also excluded were individuals younger than 40 years at AA diagnosis.

Cases with AA adverse events were defined as those having one or more codes indicating dissection or rupture. We further classified cases as rupture or dissection events based on the first such code in the record for association analysis of drugs. When dissection or rupture co-occurred with repair on the same date, we scored the case as dissection or rupture, assuming that the dissection or rupture preceded the repair. Similarly, we scored cases as dissections when dissections and ruptures occurred on the same date. We also classified AAs as abdominal, thoracic, or thoracoabdominal depending on whether ICD-9 codes indicated abdominal, thoracic, thoracoabdominal or both thoracic and abdominal, respectively.

We classified individuals as having chronic kidney disease (CKD), dialysis, heart failure or atrial fibrillation, to adjust for potential confounding by indication for drugs often
prescribed in the context of these conditions. CKD was defined using ICD-9 code group 585, evidence of renal transplant or replacement therapy using ICD-9 codes 55.6 and V42.0, or evidence of estimated glomerular flow rate of less than 60 ml/min/1.73m2 in 2 or more measures of serum creatinine on separate dates. Dialysis was defined using one or more instances of ICD-9 codes V45.1, group V56, 996.73, or 38.95 prior to the final clinical encounter for non-events or event. Atrial fibrillation was defined using four or more instances of ICD-9 code 427.31, where one or more of those codes occurred while the individual was at risk of AA progression. We validated this definition of atrial fibrillation using expert chart review, yielding a PPV of 86%. Heart failure was defined as two or more instances of codes from the ICD-9 group 428, again where one or more codes occurred while the individual was at risk of AA progression. This definition has been evaluated in the literature and has an estimated PPV of 80%-100% (18).

Time at risk began on the date of first recorded diagnosis of AA and ended on either the date of the first recorded rupture or dissection, or the date of final clinical follow up in the EMR for non-events (censoring date). Prevalent cases who presented initially with rupture, dissection, or who required immediate repair were censored. We evaluated the effects of anti-platelet, and anti-coagulant medications.

Clinical statistics and data representation:

Analyses were conducted with STATA statistical software version 12.0 (StataCorp LP, College Station, TX, USA). We used Cox proportional hazards regression models, using time at risk as the time scale to characterize the incidence of adverse AA events in relation to medication treatments (number of medications of each
class during time at risk), adjusted for putative confounders with robust estimates of standard error for estimated parameters. Effect sizes are presented as hazard ratios (HR) with 95% confidence intervals (CI) for risk of AA adverse events. Candidate confounders included factors known and suspected to influence risk of AA progression, including age at AA diagnosis and BMI modeled as continuous variables, T2D status at AA diagnosis (yes/no), and race (European (referent), other). We also included variables for chronic kidney disease (CKD), dialysis, heart failure, and atrial fibrillation to adjust for putative confounding by indication for drugs by conditions that might influence risk of adverse events.

Medication exposures were grouped by type, and each type was fit as a dichotomous variable. Proportional hazards assumptions were evaluated with the PHtest procedure in STATA and were found to be satisfied. All statistical tests assumed two-tailed distributions.

**Human medication classifications:**

Patients were classified based on anti-platelet therapy. Anti-platelet therapy was separated into ASA or P2Y$_{12}$ medication classes. Patients on P2Y$_{12}$ therapy included the following medications: clopidogrel bisulfate (Pradaxa), ticagrelor (Brilinta), or prasugrel (Effient).

**Study approvals:**

All mouse studies were performed with the approval of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. All analysis of
human data was approved by the Institutional Review Board of Vanderbilt University Medical Center.

**Clinical limitations**

Limitations of this study result from the use of EMR data, including possible missed clinical encounters (e.g., visiting a non-Vanderbilt hospital) and some missed medication exposures. We argue that we have no loss to follow-up or competing risks in this study because the censoring date is the last clinical encounter in the individual’s EMR, thereby we know that the individual was alive and evaluated clinically at the end of the time at risk. Furthermore, the occurrence of AA rupture, repair, or dissection is a clinically significant event likely to be noted at a follow up visit if the event occurred at an outside hospital. We were unable to assess medication adherence.
References


