Investigating Coagulation Mediators Fibrinogen and Platelets in Abdominal Aortic Aneurysm Pathophysiology

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Abstract

Proteins and mediators of the coagulation cascade have been long found in association with abdominal aortic aneurysm (AAA). Specifically, the coagulant protein fibrinogen, and platelets have been found to be linked to aneurysmal disease. They have also been associated with multiple inflammatory processes and diseases, and AAA is primarily an inflammatory disease. However, their exact impact and role in the pathogenesis of AAA have not been studied in detail. To provide potential explanations for the mechanisms of fibrinogen and platelets in AAA, in-depth studies were required. The studies presented in this dissertation test the overall hypothesis that fibrinogen and platelets are important in early aneurysm for the formation and development of the aneurysmal aorta. We first infused AngII into *LDLR-/-* mice on a C57Bl6 background that had been treated with antisense oligonucleotide (ASO) to β-fibrinogen. We discovered significant improvements in AAA disease burden in these mice. These experiments were followed-up with mouse mutations that demonstrate this improvement can be recapitulated in models were leukocyte interactions with fibrinogen have been hampered. We also collected data that suggest that AAA improvement with fibrinogen impairment is due to decreases in IL-6 and other pro-inflammatory pathways. Separately, we found that platelet activation markers are significantly increased in AAA patients, however platelet impairment in *in vivo* mouse models in the initial aneurysm stages result in catastrophic failure of the aortic structure and rupture-induced death of the animal. Mutations in platelets that result in incomplete fibrinogen-signaling in platelets and unstable clot structure also recapitulate this finding, suggesting that the structural failure observed is due to poor clot structure, highlighting a crucial role for platelets in early aneurysm formation. Overall, these data suggest many potential impacts of fibrinogen and platelets in AAA pathogenesis.

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List of Tables and Figures

List of Abbreviations and Acronyms

AA	Arachidonic Acid
AAA	Abdominal Aortic Aneurysm
AAT	Alpha-1-antitrypsin
AAV	Adeno-associated virus
AngII	Angiotensin-II
ApoE	Apolipoprotein E
APR	Acute Phase Reactant
APP	Acute Phase Protein
ASA	Acetyl-Salicylic Acid
ASO	Antisense Oligonucleotide
BAPN	β3-aminopropionitrile fumarate salt
CAT	Calibrated Automated Thrombography
CI	Confidence Interval
CR3	Complement Receptor 3
CVD	Cardiovascular Disease
DOAC	Direct Oral Anticoagulant
DOCA	Deoxycorticosterone acetate
ECM	Extracellular Matrix
ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous Thrombin Potential
EVAR	Endovascular Aneurysm Repair
FXIII	Clotting Factor XIII
GPVI	Glycoprotein VI
HFD	High-fat Diet
IBD	Inflammatory Bowel Disease
IF	Immunofluorescent
IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor
ILT	Intraluminal Thrombus
LDLr	Low-density Lipoprotein Receptor
LNK	Lymphocyte Adaptor Protein
LP	Lectin Pathway
MMP	Matrix Metalloproteinase
P4AP	Par4 Agonist Peptide
PAR	Protease-Activated Receptor
PCSK9	Proprotein convertase subtilisn kexin 9
PDGF	Platelet-Derived Growth Factor
PMN	Polymorphonuclear
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PT	Prothrombin Time
PTT	Partial Thromboplastin Time

PAD	Peripheral Arterial Disease
PVD	Peripheral Vascular Disease
ROS	Reactive Oxygen Species
SD	Standard Deviation
sGPVI	Soluble Glycoprotein VI
siRNA	Short Interfering Ribonucleic Acid
SBP	Systolic Blood Pressure
tPA	Tissue plasminogen activator
TAT	Thrombin-Antithrombin
TF	Tissue Factor
TGF-β	Transforming Growth Factor Beta
TLR4	Toll-like Receptor 4
T_xA_2	Thromboxane A_2
T_xB_2	Thomboxane B_2
VSMC	Vascular Smooth Muscle Cell
vWF	von Willebrand Factor
WGCNA	Weighted Gene Correlation Network Analysis

Organization of Dissertation

This dissertation is composed of five chapters and includes: an introduction into the current status of abdominal aortic aneurysm (AAA) in both the pathobiology and clinical realms, followed by a review of relevant literature (Chapter 1); a description of the overall research approach, objectives, and general methods used in this dissertation (Chapter 2); a summary of the experimental results in the form of two scientific manuscripts (Chapters 3-4); and a discussion of the major conclusions, limitations, and future directions of this work (Chapter 5). Chapter 1 begins discussing the current clinical situation of AAA, as well as the basis of fibrinogen, platelets and IL-6, and their applications to our current understanding of AAA. The current literature on the topic is also discussed. Chapter 2 will explain the basis and objectives of the research outlined in this dissertation. Chapter 3 will detail experiments and results investigating the contributions of the 390-396 binding motif on the γ-chain of fibrinogen in abdominal aortic aneurysm. Chapter 4 will detail the experiments and results investigating the contributions of platelets in the early stages of AAA formation. Chapter 5 will discuss these findings and their impacts. Also in chapter 5, we will discuss the limitations and some remaining questions in fibrinogen and platelets in AAA and recommend some future directions for the project.

CHAPTER 1

Background and Review of the Literature

1.1 Introduction

Abdominal Aortic Aneurysm (AAA) is a cardiovascular disease (CVD) accounting for approximately 10,000-15,000 deaths annually in the United States [1]. The disease is characterized by a permanent dilation of the abdominal region of the descending aorta, with >50% expansion of the normal diameter, leading to a diameter of >3.0cm. Growth of the aneurysm can continue to the point of rupture, which is often fatal [2]. AAA has been cited as a leading cause of sudden death, with many aneurysms being discovered on autopsy after rupture. [3] Even in surgically-advanced hospital facilities, ruptured aneurysm leads to a 30-day mortality as a high as 60%.[4] The risk of rupture is correlated with the diameter of the aneurysm, though an aneurysm can rupture at any diameter. [5]

A feature of many AAAs is the presence of an intraluminal thrombus (ILT), a non-occlusive, laminated thrombus which occurs in the lumen of the aorta. Though this ILT is present in up to 70% of cases, [6] the contributions of this ILT are unclear, with very little of the literature in AAA investigating this feature. Investigation of the ILT is difficult, as until recently, animal models of AAA were unable to recapitulate this phenotype in a way fully comparable to the human phenotype. [7, 8] A common model of AAA is the Angiotensin-II model (AngII), which induces an internal inflammatory insult, and then subsequent aortic dissection and remodeling, resulting in an aneurysm; [7, 9-12] in this model, a thrombus is formed, however, the thrombus is not found in the intraluminal

space, but in the wall of the aorta itself. Another model of AAA is the topical elastase model [13], in which elastase is applied to the outside of the aorta, resulting in structural damage that creates an artificial aneurysm. When the topical elastase model is combined with infusion of either anti-TGFβ antibody [8], or β3-aminopropionitrile fumarate salt (BAPN) [14], an ILT will form in mouse models, though the elastase model is not a true representation of natural, physiologic aneurysm formation [15]. This makes studying the ILT *in vivo* difficult. However, the possible implications of a biologically active thrombus and the hemostatic and inflammatory impacts this has are not difficult to conceive theoretically.

Several pharmaceutical options have shown minor or moderate effects on slowing AAA growth in animal models or human explant studies. [16] However, to date, no pharmaceutical interventions have proven reliably effective in treating human AAA [17-19] The only treatment option available to AAA patients is surgical repair of the aorta, either through endovascular repair, or open repair. [20] Though significant interest exists in developing pharmacotherapeutic options, this effort is hampered by poor understanding of the disease pathobiology.

Fibrinogen is a hemostatic and pro-inflammatory protein, the plasma levels of which are associated with CVD risk. [21] Fibrinogen is also recognized as an Acute Phase Reactant

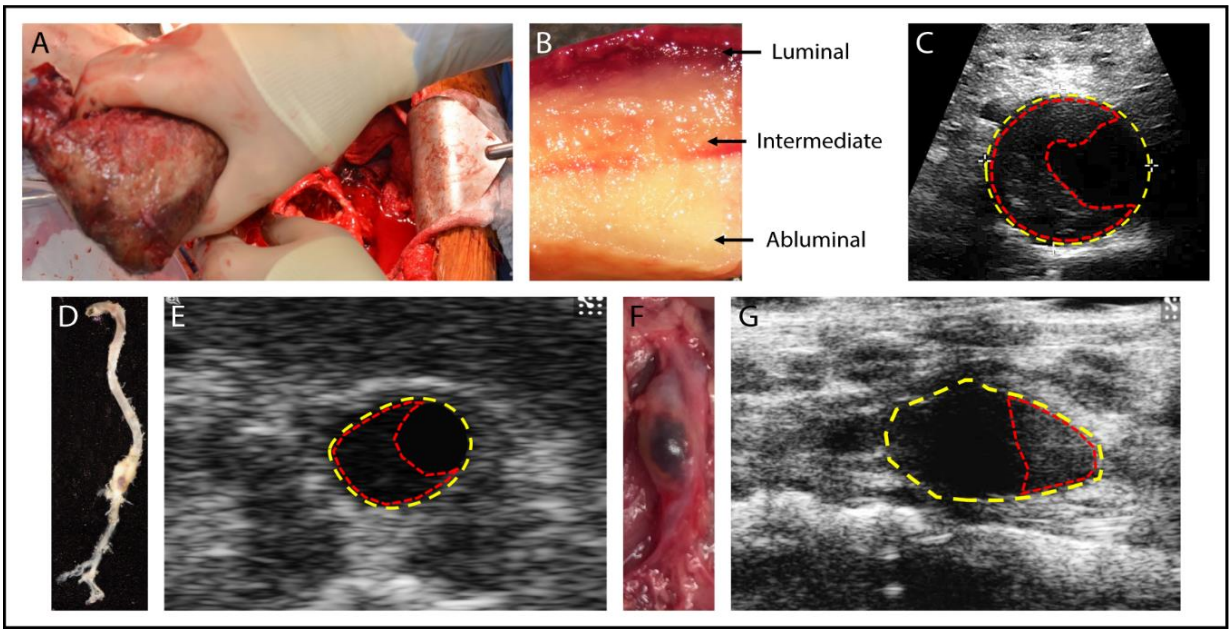

Fig 1.1 **Composition of the ILT in humans and mouse models.** (A) Representative picture of a 4.6-cm-diameter ILT (42.9 cm^2) being removed from a 6-cm diameter aneurysm (79.4 cm^2) during an open surgical procedure. (B) Macroscopic view of an ILT demonstrating the luminal, intermediate, and abluminal layers. (C) Ultrasound image of a human AAA with yellow and red dashed lines outlining the area of the aneurysm and thrombus, respectively. (D) A representative suprarenal AAA aorta from a low-density lipoprotein receptor–deficient (*Ldlr−/−*) mouse infused with AngII (1000 ng/kg per min) with an (E) in vivo ultrasound image captured at the maximum diameter on a Vevo 2100 (FUJIFILM VisualSonics Inc), where yellow dashed lines represent the area of the aneurysm and red dashed lines outline the extraluminal thrombus. (F) A representative infrarenal AAA aorta from a C57BL/6J mouse treated with topical elastase to induced aneurysm and administration of β3-aminopropionitrile fumarate salt (BAPN) ad libitum via water bottle to induce an ILT. (G) In vivo ultrasound of the topical elastase/BAPN model at the point of maximal expansion, where yellow dashed lines represent the area of the AAA and the red dashed lines outline the ILT. Adapted from *Scott J. Cameron, Hannah M. Russell, A. Phillip Owens, III, Antithrombotic therapy in abdominal aortic aneurysm: beneficial or detrimental?, Blood, 2018, Figure 1.*

(APR), and is an indicator of inflammatory processes. [22] Increased plasma fibrinogen has been correlated with AAA, when compared to age-matched, gender-matched controls in multiple cohorts. [23, 24] Nevertheless, no causal-link has been identified or investigated between fibrinogen and AAA.

Platelets are an important mediator of blood coagulation, and are also recognized as independent inflammatory modulating cellular fragments. Though more literature exists regarding platelets in AAA than does for fibrinogen, their exact role in progression and pathogenesis are not fully understood and extensive investigation is lacking.

Furthermore, their varying contributions to AAA in different stages of the aneurysm development and progression have not been extensively defined.

1.2 Epidemiology and Disease Burden of Abdominal Aortic Aneurysm

AAA is a CVD currently accounting for up to 20 million deaths per year worldwide. [25] Most affected by AAA are males, those >65 years of age, and those with past or current smoking history. [3] Analysis has revealed that up to 75% of AAA deaths can be accounted for by smoking history. AAA is rare in females, those under 65, and those with no smoking history [26]. Recent studies have identified some genetic risk, with males having first-degree relative history of AAA showing increased risk of developing AAA[27, 28]. Some large studies have suggested that a small proportion of AAAs may be accounted for by genetic defects in collagen III, but most genetic risk has not been specifically identified [29, 30]. Most general risk factors of CVD are also associated with increased risk of AAA, with the exception of diabetes mellitus. [31] These findings may be true for females, though limited data on female AAA cases pose difficulty in identifying risks. Female gender is associated with reduced risk, as are black race and diabetes. [32-34] While female gender is associated with reduced risk for developing AAA, females with AAA are at an increased risk for rupture, and show higher mortality upon rupture, compared to male patients [35, 36].

Generally, AAA is asymptomatic, or presents with non-descript symptoms such as lower backpain, mild paresthesia, and a pulsating sensation the abdomen. Screening by ultrasonography is recommended for males age 65-75 who have ever smoked. Unfortunately, many AAA cases are not discovered until autopsy, with some studies in recent years citing autopsy incidence as high as 25%. [37] Though AAA is typically

attributed to account for 10,000-15,000 deaths annually in the United States, some studies have estimated the true disease burden to be closer to 40,000 deaths per year, due to undetected AAA, and AAA contribution to other cardiovascular risks. [38] However, screening protocols and risk factor mitigation have been demonstrated to reduce the burden and rupture incidence in AAA. [39-43]

1.3 Current Treatment and Recommendations in Abdominal Aortic Aneurysm

Current recommendations suggest all males between ages 65 and 75 who have smoked at least 100 cigarettes be screened one time for AAA. In cases where aorta measures <3.0 cm on ultrasound, the aorta is not aneurysmal. In cases where the aorta measures >3.0 cm but <5.5 cm, the aneurysm should be monitored, but no surgery pursued unless other criteria are met. Patients with aneurysm measuring >5.5 cm are considered good candidates for surgery. Other surgical criteria include fast growth rate, symptomatic aneurysm (such as flank, back or abdominal pain; evidence of embolization; etc), female gender and diameter >5.0 cm, infected aneurysm, and association with other arterial disease. The aorta can be repaired either through an open surgical repair, or endovascularly (EVAR). [44] Though pharmaceutical treatment options continue to emerge in the treatment of CVD, pharmacotherapeutic options remain elusive for AAA. Despite significant scientific interest in non-surgical treatment options, surgical or endovascular intervention remains the only approved treatment for AAA. As AAA patients do meet the criteria for peripheral vascular disease (PVD), they are commonly put on a regimen of aspirin or $P2Y_{12}$ Inhibitors, however, these approaches have not shown reliable efficacy in human reviews, [17] and have shown mixed results in mouse models. [45, 46] Furthermore, though screening and surgical

interventions have improved access to care, the worldwide mortality of AAA has not decreased. [47]

1.4 Pathophysiology of Abdominal Aortic Aneurysm

The pathophysiology of AAA is characterized by structural failure of the aortic vasculature [48-50]. The vascular wall shows failure of both elastin and collagen, leading to localized proteolytic destruction of the extracellular matrix (ECM), vascular smooth muscle cell (VSMC) apoptosis, neovascularization, atherothrombosis and thinning of the tunica media[51]. This leads to a loss of resistance and elasticity, creating a physical environment for edema and inflammation, and a staging ground for platelets, macrophages, lymphocytes, and other proinflammatory cells [52, 53] .

These physiological changes result in a weakening of the aortic wall and a widening of the aorta lumen. These results contribute to the risk of rupture and subsequent death. However, the exact cellular and molecular mechanisms that trigger the initiation of AAA, as well as factors governing progression and rupture, are poorly understood. Degradation of elastin fibers is thought to be one of the first events to occur in the formation of AAA. Elastin degradation leads to exposure of the underlying media, which leads to activation of VSMCs, and leukocytes, and cytokine release. As further degradation occurs, endothelial damage and denudation leads to the activation of platelets and the coagulation cascade. As the aneurysm continues, matrix metalloproteinases (MMPs), gelatinases, collagenases and elastases are released leading to further destruction of the vascular integrity. [2] Attempting to ameliorate this loss of integrity, Type I and III collagen is deposited on the vessel wall.

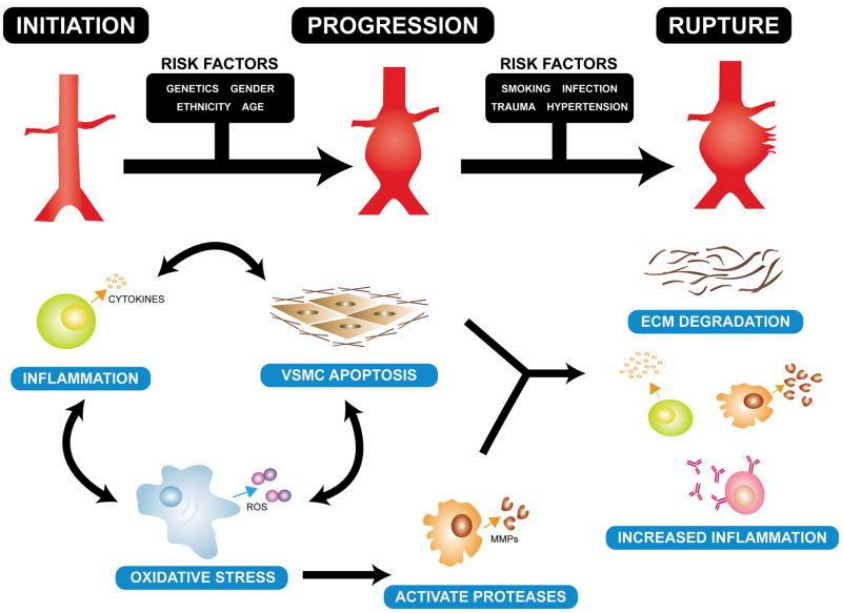

Fig 1.2 A summary of the currently understood pathobiology of AAA, both in the initiation, progression and eventual rupture of AAA. Reproduced with permission from Kuivaniemi H, Ryer EJ, Elmore JR, Tromp G. Understanding the pathogenesis of abdominal aortic aneurysms. *Expert Rev Cardiovasc Ther*. 2015

Oftentimes, this proinflammatory environment, and denuded tunica media also gives rise to an intraluminal thrombus (ILT), which is formed through activation of both platelets and coagulation cascade proteins [54]. The ILT is present in approximately 70% of AAA cases[55]. The exact formation of the ILT is not fully understood, however, it is hypothesized that the formation of the ILT is started by platelet and coagulation cascade activation due to an exposed subendothelium after elastin degradation [56]. The ILT is a non-occlusive clot which permanently interfaces blood, and may contribute to further growth of the aneurysm, specifically through the entrapment of polymorphonuclear (PMN) leukocytes, and the accompanying release of MMP-9 and MMP-2[57]. The contribution of the ILT in rupture risk of the aneurysm is controversial. Some reports have found that the presence and size of the ILT is correlated with rupture risk, imputing hypoxia and subsequent degradation of the vessel wall. [58] Other reports find that the ILT can work to reinforce the aneurysm area, fill open space in the lumen, normalize blood flow pressure and rate, and ultimately reducing wall stress and contributing to greater aneurysm stability. [59, 60]

The location of the AAA is most commonly in the infra-renal area, however, AAA can also be localized to the supra-renal, para-visceral or para-renal regions of the abdominal aorta. Some connective tissue disorders, such as Loeys-Dietz syndrome, can contribute to AAA risk. [2] However, AAA, and other aortopathies, are usually the result of atherosclerosis or inflammation [61].

1.5 Fibrinogen

Fibrinogen is a plentiful plasma protein, found in concentrations around 350 mg/dL of human plasma. It is a hexamer consisting of two alpha chains, two beta chains and two gamma chains. Fibrinogen is exclusively synthesized by the liver and is then released into bloodstream. [62] After activation of either the intrinsic or extrinsic coagulation cascade, followed by the activation of the common pathway and cleavage of prothrombin to thrombin, thrombin acts to bring about the culmination of the coagulation cascade. The culminating event of the coagulation cascade is the cleavage of insoluble fibrinogen into soluble fibrin, enabling the formation of a fibrin clot. [63] After cleavage of thrombin, thrombin will cleave fibrinogen at the fibrinopeptide cleavage side in the E region of fibrinogen from the N-termini of the Aα and Bβ chains. Following this cleavage, Factor XIII (FXIII) will generate covalent bonds on the fibrin clot, which will protect the fibrin both from fibrinolysis, and from mechanical

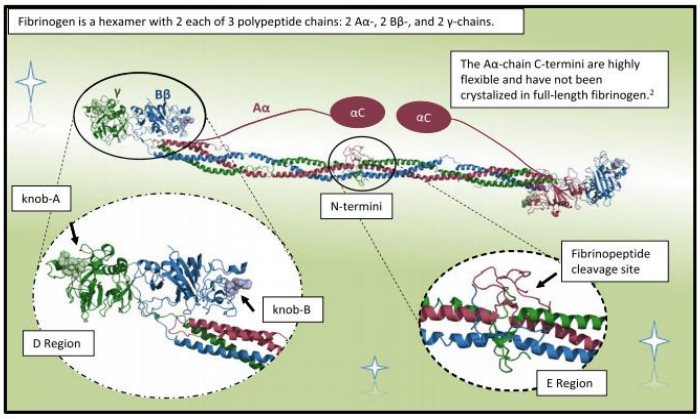

Fig 1.3 A graphic representation of the molecular structure of fibrinogen. Reproduced, with permission, from Pieters M, Wolberg AS. Fibrinogen and fibrin: An illustrated review. *Res Pract Thromb Haemost*. 2019

stress. Fibrin, in addition to being the structural protein in a clot, is involved in further amplification of the coagulation cascade, and in initiation of fibrinolysis. There are plasminogen binding sites within the fibrinogen structure that, upon fibrin polymerization become exposed, helping to facilitate the initiation of fibrinolysis [64]. Furthermore, fibrin also has the capability to bind tissue plasminogen activator (tPA), which will further enhance fibrinolysis.

Though the most well-known function of fibrinogen is in hemostasis, fibrinogen has been identified to have myriad roles in several physiological processes. Fibroblasts and leukocytes are recruited by fibrinogen, giving fibrinogen both a crucial role in wound healing and pro-inflammatory activity[65-67], and fibrinogen interacts with red blood cells to help govern both blood viscosity and erythrocyte sedimentation. Residues of the fibrinogen γ-chain interact with the β_3 integrin of platelets, enabling platelet aggregation and clot retraction and contraction [68]. Furthermore, fibrinogen has proteomic associations with several lipoproteins, coagulation factors, and other proteins of relevance in CVD [69-72].

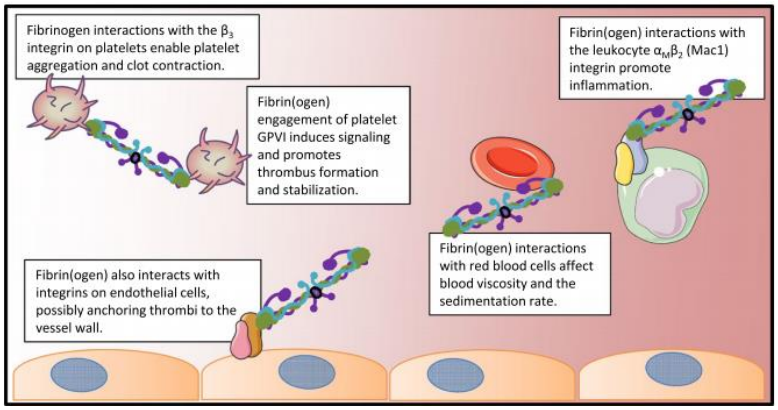

Fig 1.4 The most prominent roles of fibrinogen in inflammatory processes. Reproduced, with permission, from Pieters M, Wolberg AS. Fibrinogen and fibrin: An illustrated review. *Res Pract Thromb Haemost*. 2019

Importantly, fibrinogen is a pro-inflammatory molecule, being implicated in many inflammatory diseases including inflammatory bowel disease (IBD) [73, 74] sepsis, [75] cerebral amyloid angiopathy [76], *Staph aureus* infection [77], liver disease [78], and arthritis [79].

Recently, fibrinogen has been extensively investigated as a factor in serious and fatal COVID-19 disease, separate from its known action in sepsis [80]. Fibrin(ogen) may also mediate obesity-driven inflammation [81] and is known to be a risk factor in CVD [82, 83].

1.6 Fibrinogen in Abdominal Aortic Aneurysm

Fibrinogen has been identified to be a crucial risk factor for CVD, as well as mortality from CVD. [82-84] In the early 2000s, population studies emerged which showed that elevated fibrinogen is also found in AAA patients, when compared to age-matched, gender-matched controls. [23, 24] However, little has been done to investigate this finding. The current understanding of fibrinogen and its actions—particularly its known actions in inflammation—and the current understanding of abdominal aortic aneurysm, coupled with the presence of an ILT in many AAA patients, and the finding of elevated fibrinogen in AAA patients provide strong motivation to further investigate fibrinogen in AAA patients.

1.7 Interleukin-6

IL-6 is a proinflammatory cytokine which is secreted by macrophages after activation by microbial PAMPs or other inflammatory instigators. IL-6 is the primary mediator of fever response, and of APR production. Many APR proteins, also known as Acute Phase Proteins (APPs) are directly responsive to IL-6's actions. APPs are categorized as being Type I or Type II, delineated by its response to either IL-1 and TNF-α (Type I) or response to IL-6 and IL-6-like molecules (Type II). After synthesis in response to a pro-inflammatory event, IL-6 travels to the liver where it acts upon hepatocytes to signal release of APR proteins such as CRP, serum amyloid A, fibrinogen, haptoglobin and α-1-chymotrypsin. In the liver, IL-6 also works to downregulate other proteins, such as albumin, transferrin, and fibronectin [85] By increasing hepcidin levels and reducing

transferrin levels, IL-6 also acts to create a net decrease in iron levels throughout the body, thus providing a major component of the mechanism for anemia of chronic inflammation. [86] Similarly, IL-6 induces hepatocyte uptake of zinc, accounting for the hypozincemia demonstrated in chronic inflammatory disease.

As well as signaling APR and fever responses, IL-6 contributes to further immune action by influencing the maturation of neutrophils and their release from the bone marrow. IL-6 also promotes maturation of lymphocytes, through signaling the maturation of naïve CD4+ T-cells, which helps to couple the innate immune response carried out by macrophages, to the acquired immune response. IL-6 will also contribute to heightened maturation of megakaryocytes, increasing platelet release from the bone marrow.

Overall, IL-6 is a well-studied potent mediator of immune response and accounts for many hallmarks of both immunity and inflammation.

1.8 IL-6 in AAA

The relationship between IL-6 and AAA has been well-documented in the literature. IL-6 has been noted to be elevated in AAA patients, both in the circulating plasma, and in the aneurysmal tissue itself [87], and has been proposed as a potential biomarker for AAA. Furthermore, Asp358Ala, a well-studied polymorphism of the IL-6 receptor (IL-6R) has been correlated not only with AAA, but also with AAA growth rate[88]. The IL-6 relationship has also been well-documented in animal models. Recently, treatment with IL-6 inhibitory antibody, tocilizumab, has been observed to blunt AAA progression in mouse models. [89] This same result was observed when using a neutralizing monoclonal antibody to the IL-6 receptor [90].

1.9 IL-6 and Fibrinogen

Fibrinogen is a known class II acute phase reactant (APR), increasing with levels of inflammation, and specifically under the influence of IL-6. The synthesis of fibrinogen is closely related to the cytokine interleukin-6 (IL-6), and fibrinogen is released from hepatocytes under the influence of IL-6, with sites on the fibrinogen γ gene promoter responding to IL-6.[91] Transcription factors for the fibrinogen α chain have also been demonstrated to be IL-6 responsive [92]. However, the intersection of fibrinogen and IL-6 in the context of AAA has never been fully investigated.

1.10 Platelets

Platelets, also called thrombocytes, are cellular fragments formed after release from a parent megakaryocyte. [93] The exact mechanism of platelet formation is debated and may be a result of either an explosive rupture of the megakaryocyte after megakaryocytes grow to a large size, expanding their cytoplasm without cellular replication, or from a more mediated replication resulting from controlled release from pseudopodia. [94] The production of platelets is controlled by the actions of thrombopoietin.

After release from the bone marrow, the platelets are in a resting state and patrol the vasculature for endothelial breaks. Platelets can then be activated through several mechanisms. In one mechanism, upon encountering collagen exposed under a broken endothelium, either glycoprotein VI (GPVI) or integrin α2β1, both membrane-bound receptors on the platelet will bind to collagen, with the assistance of von Willebrand factor (vWF). After the initial activation by endothelial collagen, further internal cascades contribute to granule release which amplifies the response, activating more platelets, and inducing platelet aggregation. Upon activation, platelets also undergo

substantial morphological change, increasing surface area and spreading. [95] This shape change induces the release of platelet granules, which contain more components that help to amplify platelet activation. After activation, platelet membrane receptor GPIIb/IIIa is expressed which receives fibrinogen to stabilize the platelet plug [96].

Platelet activation is also crucial for secondary hemostasis. In the morphological changes mentioned above, the surface of the platelet becomes negatively charged and becomes important for proper assembly and action of the tenase and prothrombinase complexes. [97] Also critical for secondary hemostasis is the release of calcium from activated platelets. [98]

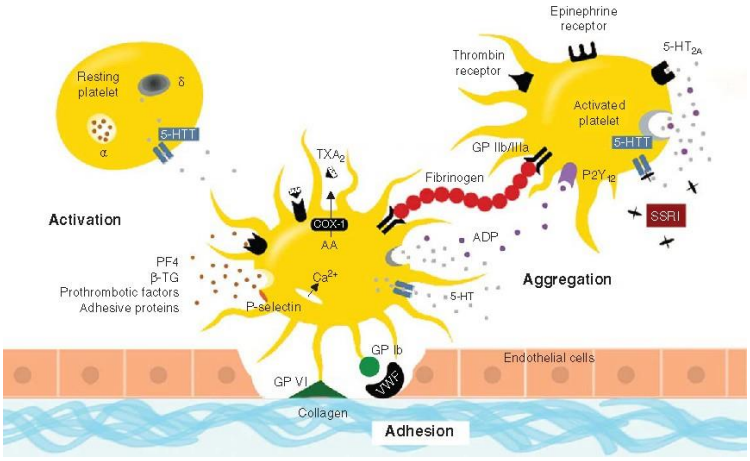

Fig 1.5 A summary of the steps of platelet activation, adhesion and aggregation. Reproduced, with permission, from de Abajo FJ. Effects of selective serotonin reuptake inhibitors on platelet function: mechanisms, clinical outcomes and implications for use in elderly patients. Drugs Aging. 2011

A growing body of literature demonstrates that platelets are not only hemostatic in nature, however. In the simplest sense, platelets are known to have the ability to directly bind to and inactivate foreign pathogens. [99] However, beyond this, platelets are known to secrete cytokines and chemokines [98-103], as well as platelet-derived growth factor (PDGF) [104], a potent immunostimulant, and can increase immune cell sensitivity and general inflammatory environment.

1.11 Platelets and Fibrinogen

Though platelets are traditionally considered to be the driving actor in primary hemostasis, and fibrinogen to be the functional unit in secondary hemostasis, the common perception of primary and secondary hemostasis being separate and distinct pathways is oversimplified. In fact, primary and secondary hemostasis are two pathways that are occurring often simultaneously and both contribute to the progression of the other. Though platelets can activate and aggregate without the assistance of fibrinogen, fibrinogen signaling and action is vital to forming a robust and strong platelet plug, and ultimately, blood clot. Poor fibrinogen signaling results in poor clot retraction and stability, and an unstable platelet plug. [105]

The growing understanding of platelets acting not only as hemostatic cellular fragments, but independent inflammatory instigators further enriches this new understanding of fibrinogen and its actions.

1.12 Platelets and AAA

The data surrounding platelets in AAA is mixed. Not much investigation has been done directly on platelet effects on aneurysm, either in human or animal models. Analysis of the ILT organization demonstrates that there is platelet deposition and accumulation contributing to ILT growth[54, 106], particularly in the biologically active component of the ILT, the luminal layer[57, 107, 108]. Some animal studies have demonstrated that antiplatelet therapy can be beneficial in slowing aneurysm growth or in decreasing rupture rates [46, 107, 109], however, human data has not successfully recapitulated these findings. While many patients are put on low-dose aspirin, as is recommended for any patient with any peripheral arterial disease (PAD) [110, 111], there is not much data to support any impact from this treatment. Where some small trials of platelet therapy favored use of

antiplatelet drugs or showed non-significant positive results [19], metaanalysis has not shown any improvement in AAA outcome when patients are using antiplatelet drugs for other comorbid conditions [17, 19] . However, a recent database analysis has shown improvement in both aneurysm outcomes and aortic dissections [112]. However, some data may even suggest that antiplatelet drugs would be detrimental, as antiplatelet therapy can result in poor outcome after aneurysmal repair [45]. Overall, early data and scientific theory implicate platelets as a promising area of AAA research.

1.13 Summary

Abdominal Aortic Aneurysm is a deadly, inflammatory, cardiovascular disease with abysmal treatment options. Efforts to develop a non-surgical treatment have not been successful and are hampered by unclear understandings of its pathobiology. Though surgical options are available, they are costly and present their own set of challenges and risks. There is a critical need to further understand the disease process and investigate treatment options. The procoagulant, pro-inflammatory protein fibrinogen is known to be elevated in AAA patients and offers a possible of avenue of research which is, as of yet, uninvestigated. This dissertation will detail our efforts to identify a pathological relationship between fibrinogen and AAA development or progression, and to investigate possible roles for AAA in the pathobiology of AAA development, progression, or rupture.

CHAPTER 2

Research Approach and Objectives

2.1 Research Scope and Approach

AAA remains a leading cause of morbidity and mortality in elderly Americans. Though treatment protocols are developed for this disease, no pharmaceutical treatment has been recognized to demonstrate consistent, positive effect in AAA patients. Surgical intervention remains the only option for AAA treatment. Therefore, there is critical need to develop more effective treatment for this disease. However, poor understanding of AAA pathogenesis hampers identification of pharmaceutical targets. The long-term goal of this dissertation is to understand the molecular mechanisms which drive AAA pathogenesis, and identify possible pharmacotherapeutic targets for AAA treatment.

Acknowledging the demonstrated statistical link between AAA and elevated fibrinogen, this dissertation seeks to understand the cause of this correlation. Through this investigation, this work hopes to identify a role for fibrinogen in AAA pathogenesis, and elucidate a mechanism by which fibrinogen drives AAA initiation and progression. The objective of this thesis was to identify a role for the plasma protein fibrinogen and associated proteins in AAA pathogenesis.

A secondary objective was also identified throughout the progression of this work, in investigating the contribution of platelets in AAA pathogenesis. Acknowledging both the importance of the ILT in AAA, the immunomodulatory actions of platelets, the significant crosstalk between fibrinogen and platelets, and the growing literature on platelets in AAA, the actions of fibrinogen and platelets were both considered in this thesis.

2.2 Specific Aims and Hypotheses

The central hypothesis of this thesis is that platelet response to the site of elastin injury, followed closely by the response of fibrinogen, work together to build a pro-inflammatory environment that drives aneurysmal growth in AAA.

This hypothesis was investigated in two aims.

Aim 1 will elucidate the contribution of leukocyte binding to fibrinogen on aneurysm pathogenesis.

Scientific premise: Fibrinogen is an important member of clot formation, however, it is also an acute phase reactant which is closely linked with inflammatory cytokines and macrophage activation. Furthermore, the ILT is an active site of leukocyte activity and recruitment, promoting proteolytic degradation of the aortic wall. Preliminary data demonstrate that fibrinogen depletion in mice attenuates AAA, and decreases proinflammatory cytokines, suggesting that fibrinogen induces AAA through inflammatory processes. Our working hypothesis is that fibrinogen interacts directly with proinflammatory factors to induce AAA progression. This hypothesis will be tested using genetic mouse models with deletions in the macrophage-binding motif of fibrinogen (Fibγ390-396), and mice bearing deletions in the Mac-1 integrin on bone-marrow derived cells. Mouse models will be tested to determine whether the effects of fibrinogen depletion can be replicated in mice with deletions of only the macrophage-binding motif or with deletions in the Mac-1 integrin, but with fibrinogen which shows no hemostatic defect.

Aim 2 will investigate the importance of platelets in early aneurysm development.

Scientific premise: Preliminary data suggested that platelets are essential to prevent early rupture in animal aneurysmal models, and that platelet transcripts are richly upregulated in human ILT tissue. Though antiplatelet therapy has not been seen to have any impact on aneurysmal disease when analyzed in indirect retrospective analysis, some animal data suggests that in a controlled trial, antiplatelet therapy may be beneficial in blunting the growth rate of aneurysm. Therefore, it is important to understand the temporal importance of platelets in the AAA disease process, as platelets appear to show different impacts at different points in the disease. This will be tested using different animal models of AAA and different methods of platelet depletion or impairment.

2.3 Significance and Impact

Abdominal Aortic Aneurysm (AAA) rupture is a significant cause of morbidity and mortality in the United States today, and is a leading cause of sudden death in the United States. [20, 113] This number is expected to rise with an increasing elderly population, and poor-lifestyle choices leading to cardiovascular disease. Currently, only surgical repair is recognized as being effective in treating AAA. [20] Pharmaceutical interventions against atherosclerosis and thrombotic conditions have been tested in AAA patients (i.e. platelet inhibitors, ACE inhibitors, statins). However, these interventions have shown little success, [20] and surgical repair is associated with high morbidity and mortality. [20, 114-117] Developing pharmaceutical interventions in AAA needs to be the focus of the scientific and medical community in this ailment. However, this development is difficult while the pathogenesis of AAA remains elusive. Fibrinogen is known to be an independent risk factor in CVD, and an increase in plasma fibrinogen levels has been repeatedly demonstrated in AAA patients. [21, 23, 24, 118] Furthermore, d-

dimer levels [119] and fibrin degradation product levels [120] can be successfully correlated to the size of the intraluminal thrombus found in AAA. Our proposal marks novel research into fibrinogen, an understudied, yet recognized associate of AAA; into platelets, a poorly understood, complex contributor to AAA; and into the ILT aspect of AAA, which could reveal scientific premise for further studies and treatment.

2.4 Methods

2.4.1. Animal Models of AAA

Several animal models of AAA have been developed to study this disease. As with all animal models, each presents its own set of advantages, disadvantages and applications. This section will summarize the basis, advantages and disadvantages of each major animal model of AAA used in this dissertation. Though models for other animals than mice exist, this section will focus on the models developed for mice. Generally, aneurysm models can be delineated into non-dissecting models and dissecting models. In this paper, among those discussed, the Angiotensin-II model and the DOCA salt model are dissecting models, where Calcium Chloride and Elastase models are both non-dissecting models.

Angiotensin-II Model

The majority of this dissertation will use the Angiotensin-II model. This model was developed in 2000 [12], and has been cited to be the most common model of AAA [121]. In this model, *LDLR*$^{-/-}$ or *ApoE*$^{-/-}$ mice at 8-12 weeks of age are fed a high fat diet and then implanted with a subcutaneous minipump, delivering a continuous infusion of AngII over a period of 4 weeks. Angiotensin results in microtears to the intima, resulting in

dissection formation. The subsequent remodeling of this injury results in the improper inflammatory response that ultimately causes aneurysm formation in the suprarenal aortic region. The localization of the response is due to endothelial cell heterogeneity [122]. The main inflammatory mediators of the AngII model are macrophages, [123] though B- and T-lymphocytes have also been reported to respond [11]. The AngII model does result in clot formation, though the clot is intramural, rather than intraluminal.

The advantages of the AngII model include its relative technical simplicity, compared to several other models that require involved surgical skill. It is also a model that shows the importance of atherothrombosis in human disease and allows investigation of this feature. As the AngII model is an inside-out model, rather than outside-in like many models, it allows for analysis of leukocyte extravasation, which is important to human phenotypes. The AngII model is cited as being markedly less artificial in its alterations of structural integrity.

Unfortunately, unlike direct injury models, the AngII model does not show aneurysm formation in all mice, which increases the need for higher experimental numbers, and also lends to some experimental variability. Also, the aneurysm in the AngII model is found in the suprarenal region, where human aortas form in the infrarenal regions. Explanations for this finding include that there is genetic variability in the endothelial cells comparing mice to humans, or that the turbulent flow of blood is altered between bipedal and quadrupedal animals. Lastly, the AngII model does require impairment of lipid homeostasis, whether by removal of the *LDLR* or *ApoE,* either genetically or with gene editing. This can make experimental preparation costly and time-consuming when mouse lines need to be crossed.

Elastase Models

The earliest elastase models were developed in the 1990s, originally in rats [124]; however, the model is now used routinely in mice. In an elastase model, porcine pancreatic elastase is introduced to the aorta. In original experiments, the animal was abdomen was opened with a midline incision, the aorta was isolated and elastase was infused into the aorta by catheter. [125] As time has progressed, the topical elastase model has become common, in which porcine pancreatic elastase, rather than being infused, it topically applied to the outside of the aorta, with similar results, [126] reducing the surgical intricacy of the procedure. In this model, dilation of the aorta is induced as elastin fibers degrade upon penetration of porcine elastase into the wall. Further infiltration of neutrophils results in MMP-9, urokinase and leukocyte elastase driving additional aneurysm development [121].

The elastase model is mainly driven by neutrophils and the destruction of vessel structure. This makes the elastase model good for studying the effects of neutrophils in aneurysm. Also, the expansion of the aneurysm is, comparably to humans, non-linear and occurs in separate phases. This allows a time window to study early aneurysms that some other models do not. The elastase model, unlike many aneurysm models, does not depend on hypercholesterolemia, which allows for experiments that would be confounded by the altered immune functions of *ApoE* deficiency, or the off-target effects of adipose tissue seen in hypercholesteremic mouse models. This also eliminates the need to cross genetic lines to either an *ApoE* or *LDLr* deficient background [127].

The elastase model can induce extremely large aneurysm size, even to 300-400% of the initial aortic diameter. However, it is somewhat inconsistent in the size of the aneurysm. Furthermore, because all mice should develop an aneurysm of some kind, the elastase model does not allow for an analysis of incidence rates. Another drawback of the elastase model is the complete destruction of the extracellular matrix (ECM). Though human aneurysms do show some endothelial disorganization and ECM degradation, the elastase model shows a complete lack of subendothelial organization. Oftentimes, the elastic line is completely destroyed, and no true intima, media or adventitia can be discerned in sections. The elastase model in mice also does not natively form an ILT, nor does it result in a human-like rupture phenotype.

Some of the drawbacks of the elastase model can be overcome by combining it with other treatments. For example, treatment with anti-TGFβ antibody [8] or BAPN in addition to elastase application will result in an ILT, and will show a rupture.

Calcium Chloride Model

Originally developed in a rabbit model in 1988, [128] and then applied to mice in 2001, [129] the Calcium Chloride ($CaCl_2$) model involves applying calcium chloride directly to the animal's adventitial space of the aorta. Though the exact physiology of this model is unknown, it is thought that calcium ions penetrate the vasculature and disrupt elastin fibers, resulting in an injury from $CaPO_4$ crystal formation and subsequent immune response [128]. The immune response of crystal phagocytosis and MMP production results in an aneurysm, rich in neutrophils, as well as some T-lymphocytes, VSMC loss, elastin fragmentation and calcification.

As advantages of this model, the phenotype is comparable to human disease, particularly in the high amount of MMPs [130], M1 macrophages, [131] elastin damage and VSMC apoptosis. [128] Most notably, this model is used to study the interplay of M1 and M2 macrophages. However, unfortunately, the $CaCl_2$ lacks both an ILT, and a rupture phenotype, as well as the human atherothrombotic phenotype in human AAA.

DOCA Salt Model

The DOCA Salt Model is a recent, chemically-inducible model that does not require any surgical intervention, developed in 2013. [132] In this model, aged mice of 10 months are administered a high salt diet and deoxycorticosterone acetate (DOCA) over a period of 3 weeks.

In this model, DOCA alters aldosterone homeostasis as a ligand of the mineralocorticoid receptor (MCR), and with the addition of advanced-age and high-salt diet, suprarenal dissection and aneurysm formation occurs in ~60% of mice. It has been proposed that primary hyperaldosteronism is an independent risk factor for aortic dissection in humans, [133] which creates significant human parallelism in this model. It is thought that MCR stimulating results in oxidative stress, leading to lymphocyte and macrophage accumulation. ECM degradation, VSMC apoptosis, inflammatory infiltrate, increased MMP activity and oxidative stress are all seen in this model, in common with human dissecting aneurysms.

Advantages of the DOCA salt model include its lack of surgical instigation, and its ability to be used in genetically unaltered animals. However, the significant investment of waiting for mice to come to age is a drawback in the overall cost of the model. The

model highlights internal changes in humans that lead to aneurysm and is valuable in investigating early aneurysm development because of its spontaneity.

2.4.2 Summary of methods used in this dissertation.

Mice and diet: For aneurysm experiments, *Fibγ$^{390-396A}$*, *Fib$^{-/-}$*, and *FXIII$^{-/-}$* mice were obtained from Matthew Flick (University of North Carolina). For AngII experiments, mice were then crossed to *Ldlr$^{-/-}$* background to increase susceptibility to cardiovascular disease. *Par4$^{-/-}$* mice (Sambrano et al. 2001) were crossed into the *Ldlr$^{-/-}$* background to create *Ldlr$^{-/-}$/Par4$^{+/+}$* and *Ldlr$^{-/-}$/Par4$^{-/-}$* mice. *P2Y$_{12}$$^{+/+}$* and $^{-/-}$ mice, originally from Portola Pharmaceuticals [134], were used as donors in platelet bone marrow transplant experiments. For *Fibγ$^{390-396A}$* mice, mice were crossed until homozygous for both alleles. For *FXIII$^{-/-}$* mice, mice were crossed until homozygous for *Ldlr$^{-/-}$*, and heterozygous for *FXIII$^{-/-}$,* as female mice with a homozygous mutation proved unable to reliably carry pregnancy to term, or to safely deliver pups. Male offspring homozygous for both mutations were used for experiments. For fibrinogen bone marrow experiments, Male *Ldlr$^{-/-}$* recipient mice and Female *Itgam1$^{-/-}$* or *Itgam$^{+/+}$* donor mice were obtained from Jackson laboratories. For *Fib$^{-/-}$* experiments, mice were not crossed to *Ldlr$^{-/-}$* background, but were instead treated with AAV-Ldlr vector. The virus used was AAV8-U6-SA-WTmLdlrEx14-gRNA2-N22-CB-SACas9-HA-OLLAS-spA. This is a AAV8 adenoviral associated vector, with Ldlr Crispr/Cas, as outlined in previous publications. [135] This virus was obtained from Elizabeth Tarling and Thomas Vallim (UCLA). For ASO-treated experiments and siRNA-treated experiments, *Ldlr$^{-/-}$* mice were obtained from Jackson laboratories. To induce hypercholesterolemia, *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. For elastase experiments, mice were fed a normal laboratory diet throughout experimentation. Due to low penetrance of aneurysm models in female mice,[136] only male mice were utilized in experiments. All mice were maintained under barrier conditions. Water and normal laboratory diet were available ad libitum. All procedures involving animals were approved by the Animal Care and Use Committee at the University of Cincinnati.

Platelet and thrombin inhibition:

Platelets were depleted in *apoE*$^{-/-}$ mice by injecting a rat anti-mouse GP1bα (5μg/g bodyweight, R300, Emfret Analytics, n = 5), via tail vein, twice weekly for 1 week prior to and throughout AngII infusion. Rat IgG (5μg/g bodyweight, C301, Emfret Analytics, n = 5) was similarly administered for negative control.

For inhibition of thrombin, *Ldlr*$^{-/-}$ mice were fed a custom-made HFD containing peanut butter (10 g/kg diet) with or without dabigatran etexilate (10 g/kg diet, purchased from UNC pharmacy: 150 mg capsules from Boehringer Ingelheim) 1 week prior to and throughout AngII infusion (Dyets Inc., based on Harlan Teklad TD.88137). Dabigatran-induced thrombin inhibition in the plasma was measured with a HEMOCLOT Thrombin Inhibitors kit (Aniara).

For $P2Y_{12}$ platelet inhibition, *Ldlr*$^{-/-}$ mice were fed either 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) or intraperitoneally injected with 30 mg/kg clopidogrel bisulfate 1 week prior to

and throughout AngII infusion (Dyets Inc., based on Harlan Teklad TD.88137). Clopidogrel bisulfate inhibition of *P2Y*$_{12}$ was determined by platelet agonism by ADP or tail bleeding time. ASA (30 mg/L) was administered to *Ldlr*$^{-/-}$ mice via water 1 week prior to and throughout AngII infusion, as described previously [137]. Effective ASA absorption was determined by plasma levels of thromboxane B2.

Platelet Isolation and Immunoblotting: Healthy patients were volunteers without any known medical history, aneurysmal disease, or on antiplatelet agents were recruited in studies approved by the Institutional Review Board at the Cleveland Clinic. All AAA patient samples were collected by venipuncture from pre-operative patients under serial imaging surveillance. For each subject, blood was drawn by a medical professional into citrate plasma tubes, then centrifuged in a tabletop centrifuge at 1100 rpm for 15 mins. Platelet rich plasma (PRP) well above the buffy coat was decanted and the final platelet centrifugation step at 2600 rpm for 5 mins was conducted with a final concentration of 10 μM PGI_2 (in Tris Buffer, pH 9.0). The final washed platelet pellet from one human plasma citrate tube was resuspended in 1000 μL of fresh Tyrode's solution which was diluted 1:10 in fresh Tyrode's solution. This was aliquoted into 100 μL platelet samples before reducing SDS-PAGE and Immoblotting as described by us previously [138, 139]. Blocking buffer was 3% bovine serum albumin/Tris-buffered saline–Tween 20 for 60 minutes at room temperature with agitation. TBST-T Primary anti-GPVI antibody (ABCAM, # ab129019) was used in a 1:4000 dilution in 3% bovine serum albumin/Tris-buffered saline–Tween 20 for 12 hours at 4 degrees C with agitation. GAPDH antibody (Cell Signaling Technology #5174S) was used in a 1:000 dilution in 3% bovine serum albumin/Tris-buffered saline–Tween 20 for 12 hours at 4 degrees C with agitation.

Secondary antibody (GE Healthcare, Buckinghamshire, UK) was used in a 1:2000 titer in 5% milk/Tris-buffered saline–Tween 20 for 1 hour at room temperature with agitation. Final autoradiographic films (Bioblot BXR, Laboratory Product Sales, Rochester NY) were quantified by densitometry using ImageJ software (National Institutes of Health).

Platelet surface GPVI Quantification by Flow Cytometry: Isolated 100 µL platelet samples were incubated with 1 µL of labeled GPVI antibody (BD Biosciences, Clone HY101**,** #565241 in the dark for 30 minutes.) This reaction was then stopped by adding 100 µL of 2% formalin to each reaction, and then quantification of platelet surface GPVI was made possible using an Accuri C6 Plus Flow Cytometer (BD Biosciences) at 10K events per sample. Data was then processed through FloJo (Ashland, Oregon). Platelet surface GPVI was quantified using the geometric mean.

Time-course study: *Ldlr*$^{-/-}$ mice were fed a Western diet for 1 week prior to and throughout AngII infusion for 2, 4, 7, and 28 days (n = 20 each time point). In vivo platelet and macrophage labelling and ex vivo examination are detailed in the online supplement.

Intervention study:

Ldlr$^{-/-}$ mice (n = 60) were fed a Western diet for 1 week prior to and throughout a 28 day AngII infusion. Mice were stratified by aneurysm size, via ultrasonography, into 4 groups with equivalent aortic diameter: placebo Clopidogrel bisulfate (50 mg/kg cellulose filling in diet), Clopidogrel bisulfate (50 mg/kg Clopidogrel in diet), placebo ASA (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, certain mice were retro-orbitally injected (daily) with an anti-GPIX mouse antibody (700 nm). One day prior to sacrifice, all mice were retro-orbitally injected with a solution of dextran-coated nanoparticles (800 nm). For MMP analysis, mice were retro-orbitally injected with a MMP-sense solution (680 nm, 150 nmol/kg body weight, Perkin Elmer), one day prior to sacrifice, as described previously [140]. Aortas were imaged and quantified as described in the online supplement.

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 [141].

Antisense Oligonucleotides: For fibrinogen suppression experiments, antisense oligonucleotide to β-Fibrinogen and scrambled control were obtained from IONIS Pharmaceuticals. Mice were injected intraperitoneally 30 mg/kg twice per week, 3 weeks prior to and throughout experimentation.

siRNAs: For FXIII suppression experiments, siRNA to FXIII-A obtained from Christian Kastrup (University of British Columbia) was injected retro-orbitally 1 week prior to, and 2 weeks into elastase experiments, as described [142].

Topical Elastase Surgery: At at least 8 weeks of age, male mice were anesthesized with isoflurane inhaled to effect and the abdominal cavity was opened. The abdominal region of the aorta was treated, topically, with porcine elastase and wounds were closed by suture.

Human Cohort: Human samples of AAA patients and age-matched, gender-matched controls were obtained from the University of Uppsala (Uppsala, Sweden) biobank. For AAA patients, inclusion criteria in the present study included: aortic diameter > 30 mm, and follow-up of > 6 months. Patients with ruptured AAA, co-existing malignant disease, dialysisdependence, recent thrombo-occlusive disease, or on anticoagulation therapy were excluded. In the present study, plasma samples from participants with AAAs (n = 166) and normal (non-aneurysmal; n = 129) aortas were analyzed.

ELISAs and plate assays: The following commercially-available kits were used: total cholesterol colorimetric assay (Wako Diagnostics, Cat No. 439-17501), mouse fibrinogen ELISA (ICL Cat No E-90FIB), TAT ELISA (Enzygnost TAT micro kit from Dade Behring), mouse PF4 (R&D systems), thromboxane B2 (Cayman Chemical), MMP-2 and MMP-9 Biotrak Activity Assay System (GE Healthcare), triglycerides (L-Type TG M), and HDL-C (L-Type HDL-C) from Wako Chemicals (Richmond, VA). Manufacturer instructions were followed. For lipid calculations, LDL-C was calculated using the Friedewald equation. VLDL-C was then calculated by subtracting HDL-C and LDL-C from total plasma cholesterol.

Cytokine/chemokine panel: 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.

Osmotic minipump implantation: At at least 8 weeks of age, male mice were implanted with Alzet osmotic minipumps (Model 1004 or 2004, Durect corporation) subcutaneously. Infusion of AngII (1,000 ng/kg/min; Bachem) continued for 28 days, as previously described. [12]

Bone marrow transplantation: *Ldlr-/-* mice were irradiated using gamma irradiation with a Cs137 irradiator (J.L. Shepherd) with a total dose of 1200 rads of radiation, in two doses across a period of 4 hours. Irradiated mice were re-populated with bone marrow harvested from *Mac1*$^{+/+}$ (n = 15), *Mac1*$^{-/-}$ (n = 15), *Ldl*$^{+/+}$ donor mice via retro-orbital injection. Mice were allowed to recover for 4 weeks with prophylactic Sulfatrim (Patterson Veterinary) available in 0.5 mg/mL concentration in water, *ad libitum.* After 4 weeks, mice were fed hypercholesterolemic diet as described and infused with AngII.

Aortic tissue and plasma collection: Twenty-eight days after pump implantation, or twenty-one days after elastase surgery, mice were terminated, and blood was drawn from the inferior vena cava in sodium citrate and plasma processed by centrifugation. Aortas were perfused with saline, extracted, and placed into formalin (10% wt/vol) for 24 hours and then transferred to a standard PBS solution.

Measurements of abdominal aortic diameters: Mouse aortas were measured *ex vivo.* Aortas were dissected under dissecting scope and measured at widest suprarenal point (AngII) or infrarenal point (elastase). In FibASO experiments, abdominal aortas were visualized with high-frequency ultrasound (Vevo 2100, VisualSonics, Toronto, ON, Canada) on day 0 and 27, as described previously [9, 10]. Luminal diameters were measured on images with the maximal dilation. *Ex vivo* measurements were also taken in these animals. Reported values represent *ex vivo* measurements, though no significant difference was observed.

Measurement of inflammatory markers: Aortas were perfused with saline, extracted and placed into RNAlater solution (ThermoFisher scientific). After dissection, RNA was isolated from aortas. First, aortas were processed by Beadbug homogenization

(Millipore Sigma) in QiaZol (Qiagen), and product was isolated in chloroform and isopropanol. RNA was then isolated and purified using a Macherey-Nagel purification kit, according to manufacturer instructions (Macherey-Nagel). The formation of cDNA was done using iScript Reverse Transcription Supermix (BioRad) according to manufacturer instructions. Inflammatory markers were measured by qPCR using iTaq Universal SYBR Green Supermix and CFX-96 qPCR machine (BioRad).

Human Fibrinogen Measurement: Human fibrinogen was quantified by the Clauss Method, using a Stago STart 4 (Stago Hemostasis Systems). The measurement was done according to the procedure outlined by the manufacturer.

RNAseq: Aortas were perfused with saline, the abdominal region was extracted and placed in RNAlater (ThermoFisher scientific). Prior to processing, all surfaces were treated with RNAseZAP (MilliporeSigma). Aortas were then removed and washed with RNAse-free PBS and flash frozen in liquid nitrogen. The aortas were pulverized using a Spectrum Bessman tissue pulverizer (Fisher Scientific) and then processed in a using BeadBug microtube homogenization system (Millipore Sigma) in QiaZol Lysis reagent (Qiagen), and isolated using chloroform and isopropanol. RNA was then purified using a Direct-zol RNA Miniprep Plus kit (Zymo Research), according to manufacturer and instructions. RNA yield was measured by a Nanodrop 2000 (ThermoFisher scientific). RNAseq was performed by Cincinnati Children's Hospital and Medical Center Gene Expression Core using the Tecan Genomics and Illumina Protocols on an Ovation RNA-seq System v.2.

Clottable Assay Measurement: Mouse coagulation was assessed by prothrombin time (PT), activated partial prothrombin time (aPTT) and bleeding time. PT and aPTT were

measured using a Stago Start4 (Stago Hemostasis Systems) according to manufacturer instructions. Reagents were obtained from Pacific Hemostasis. Bleeding time was measured by tail transection.

Histology: For frozen sections, dissected aortas were embedded in TissuePlus OCT compound (Fisher scientific) and section in 10 um slices using a HM525NX cryostat (ThermoFisher). For paraffin-embedded sections, dissected aortas were fixed in formalin, and then processed and embedded by the University of Cincinnati Histology Core. Blocks were sectioned in 6um slices using a HM 315 Microm microtome (Thermo Scientific).

Histological Staining: For picrosirius staining, paraffin sections were rehydrated, stained with picrosirius red (Abcam), dehydrated, mounted in Permount (Fisher Scientific) and visualized under polarized light using a LMC-4000 (Laxco). Images were analyzed using ImageJ Fiji (National Institutes of Health).

Immunofluorescent Staining: For fibrinogen staining, in mouse, frozen sections were stained with Dako Antibody to Human Fibrinogen (polyclonal rabbit anti-human; clone A0080) and labeled with fluorescent secondary Alexa488 (goat anti-rabbit, Invitrogen). In human fibrinogen stains, paraffin sections were stained with the same antibodies mentioned above. For CD68 staining, frozen sections were stained with CD68 (Bio-rad MCA1957; rat anti-mouse) and labeled with fluorescent secondary Alexa488 (goat anti-rat, Invitrogen). Sections were visualized with a LSM 710 LIVE Duo Confocal Microscope (Zeiss). Stained area was quantified by ImageJ Fiji (National Institutes of Health). We acknowledge the use of the University of Cincinnati Live Microscopy Core.

Research statistics and data representation: All bar and line graphs were created with Sigma Plot v.14 (SPSS, Chicago, IL). All statistical analysis was performed using SigmaStat, now incorporated into Sigma Plot v.14. 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. Values of $P < 0.05$ were considered statistically significant.

Calibrated Automated Thrombography: Calibrated Automated Thrombography (CAT) uses a fluorogenic substrate of thrombin to measure, in real-time, thrombin generation of Platelet-poor plasma (PPP) or platelet-rich plasma (PRP). CAT was run by our collaborators at the University of North Carolina, Chapel Hill. Briefly, in a 96-well plate, a trigger solution containing tissue factor (TF) is added to a well, and calibrator to another. Citrated plasma is then added to the wells. At time zero, calcium is added to the samples. As thrombin generation continues, the fluorescent signal is measured in both the triggered and the calibrated wells.

Animal Blood Component Analysis: Mouse blood was analyzed by either on a HemaVet 950 (Drew Scientific), or Heska HT5 (Heska Corporation).

CHAPTER 3

Fibrinogen-γ augments Abdominal Aortic Aneurysm Disease Severity via a Mac-1 binding motif dependent mechanism

Abstract

Background: Abdominal aortic aneurysm is a cardiovascular disease accounting for 10,000-15,000 deaths each year in the United States. Despite this, surgical intervention remains the only treatment option for these patients. Fibrinogen levels have been noted to be a potent CVD risk factor, and to be elevated in AAA patients compared to healthy individuals in multiple populations. Here, we investigate a causal link between fibrinogen and AAA.

Methods and results: We noted a significant increase in procoagulant activity in both humans with AAA and in mice with an induced AAA, as measured by ETP, TAT and fibrinogen levels. When fibrinogen is suppressed or deleted in mice, an AngII model of AAA shows a decrease in abdominal aortic size, aneurysm incidence and rupture rate. Furthermore, mutations in a Mac-1 binding site on the γ-chain of fibrinogen suppress abdominal aortic size, aneurysm incidence and rupture rate. Bone marrow transplantation of Mac-1 deficient bone marrow into mice shows similar suppression of diameter, incidence and rupture rate. RNA-sequencing demonstrated decreased activity in inflammatory pathways, including in IL-6, as well as significant reductions in SERPIN proteins, correlated with MMPs.

Conclusions: Fibrinogen is a crucial factor in the development of AAA in AngII mouse models. The Mac-1 binding site on the γ-chain of fibrinogen is necessary for proper formation of AngII-induced AAA in mice.

Keywords:

Introduction

Abdominal aortic aneurysm (AAA) is a cardiovascular disease (CVD) that affects approximately 2 million Americans, and accounts for approximately 10,000-15,000 deaths in the United States annually [1]. Risk factors for AAA include male sex, age >65, and smoking history [2, 28, 31]. AAA is an inflammatory disease resulting in permanent, localized dilation of the abdominal region of the descending aorta. AAA commonly occurs with an intraluminal thrombus (~70% of cases) [55] Rupture of the aneurysm can occur, which is fatal in >90% of cases, accounting for most of its fatalities. [2] Unfortunately, to date, no pharmaceutical interventions have shown success in decreasing the aneurysm size or slowing the rate of growth [143]. The current standard of care suggests monitoring the aneurysm and pursuing surgical intervention when dilation has reached a diameter >5cm, or other ancillary circumstances are met. [44] However, the surgical procedure is not without risk, and can lead to further complications [20, 44, 114-117]. There is clear need for the development of pharmaceutical interventions. However, development is inhibited by poor understanding of the pathogenesis of AAA.

Fibrinogen is a hexameric plasma protein, most known for its procoagulant effects. However, it is also recognized as a crucial pro-inflammatory molecule and has effects in both wound healing and leukocyte recruitment [62, 63, 82, 84]. Fibrinogen is also recognized as a potent risk factor for CVD, and is known to be elevated in AAA patients as compared to age-matched, gender-matched controls [23, 24]. However, the exact role of fibrinogen in AAA pathogenesis has not been studied.

Macrophages are an integral part of the AAA disease process [52]. M2 macrophages are highly enriched in all sections of the aneurysmal aorta wall, and are key regulators in the pathogenic remodeling of the vascular wall in AAA. M1 Macrophages are also enriched in segments of the aneurysm and drive inflammatory progression of AAA [52]. Macrophages are characterized into several classes, but all classes unified by the presence of the Mac-1 integrin. [144] Mac-1 is a complement activator that mediates recognition and phagocytosis of invading bacteria, and contributes to opsonization. [145] Importantly, fibrinogen contains a binding site for this integrin on its gamma chain, crucial to macrophage recruitment. [146]

Together, this suggests a possible relationship between fibrinogen and macrophages in AAA pathogenesis. This paper seeks to understand the contribution of fibrinogen-mediated macrophage recruitment to AAA pathogenesis.

Results

To confirm the association of fibrinogen and other procoagulants with AAA, we measured endogenous thrombin potential (ETP) by calibrated automated thrombography (CAT), and thrombin-antithrombin complexes (TAT) by ELISA, in both human patients, and a mouse model of AAA (Fig 1a&b). Both measures were found to

be elevated in mouse and human specimens. Fibrinogen was also measured in humans with AAA and in age-matched, gender-matched controls, and showed elevation consistent with that seen in the literature (Fig 1c). To evaluate the deposition of fibrinogen localized to the abdominal aorta, human aortic sections from AAA patients and control cadaver were stained for fibrinogen fluorescently. Significant deposition was observed in the AAA patient sections (Fig 1d). Large analysis of patients from Uppsala Sweden, (n=264) fibrinogen could be related to both disease, and is further elevated in patients with fast-growing aneurysms, compared to those with slow-growing aneurysms (Fig 1f, Tables 1 and 2). Similarly, increased fibrinogen was correlated with increased aortic size (Fig 1g).

Though previous literature has demonstrated elevated fibrinogen in AAA patients, this pathogenesis has not, to date, been investigated. In an AngII *in vivo* model of AAA, mice with antisense oligonucleotide (ASO) suppression of β-fibrinogen, as compared to a scrambled ASO control, showed decreased levels of plasma fibrinogen (Fig 2a). Mice with decreased fibrinogen levels demonstrated increased survival, decreased aneurysm incidence and decreased abdominal aortic diameter, as compared to controls (Fig 2b&c). Furthermore, mice with decreased plasma fibrinogen showed decreased mRNA signal for inflammatory markers IL-1, IL-6, CD68 and Gr-1 (Fig 2d). Mice did not show any difference in fibrinogen deposition by percent stained area when stained with immunofluorescent staining (Fig 2e). Similar survival, incidence and abdominal aortic diameter results were observed in mice possessing a genetic deficiency of all fibrinogen (Fig 2f). Though plasma fibrinogen levels were low, fibrinogen suppressed mice

retained normal prothrombin times (Fig 2g) with slightly prolonged activated partial thromboplastin time (Fig 2h).

Fibrinogen is known to be a multi-faceted protein, with many physiological functions. To identify the most likely pathway for fibrinogen's effects in AAA, a mutant mouse, Fibγ$^{390-396A}$ was utilized. This mouse possesses a mutant form of fibrinogen, with non-binding alanine mutation at the 390-396 location of the γ-chain, a known site of Mac-1 binding. Mice were crossbred to an *Ldlr*$^{-/-}$ line of mice. In an AngII *in vivo* model of AAA, mice possessing this mutant fibrinogen form showed increased survival, decreased aneurysm incidence and decreased abdominal aortic diameter compared to wild-type controls (Fig 3a-c). When immunofluorescent staining was performed, mice with aneurysm showed significantly more fibrinogen deposition than mice without aneurysm, though no difference could be correlated to genotype (Fig 3d). Similar to mice with blockade of fibrinogen synthesis, mutant mice showed decreased mRNA signal for inflammatory markers IL-1, IL-6, CD68 and Gr-1 (Fig 3e). Similar aneurysm results were seen using an elastase-driven *in vivo* model of AAA (Fig 3f).

Many ligands have been identified for the region of the gamma chain of fibrinogen which is mutated in *Fibγ*$^{390-396A}$ *LDLr*$^{-/-}$ mice. Established literature suggests the Mac-1 integrin as a possible ligand to account for our findings. Therefore, to investigate, *LDLr-/-* mice were irradiated of their bone marrow. Mice were then repopulated with bone marrow from *Mac1*$^{+/+}$ or *Mac1*$^{-/-}$ donors. Mice with *Mac1*$^{-/-}$ bone marrow showed significantly decreased diameter, aneurysm incidence and death as compared to those with *Mac*$^{+/+}$ bone marrow (Fig 4a &b). No differences were observed in the fibrinogen levels of these mice (Fig 4c).

To investigate the mechanism of these findings, *Fibγ$^{390-396A}$ LDLr$^{-/-}$* mice and *LDLr$^{-/-}$* mice had aneurysms induced by AngII. After 3days, RNA sequencing was done to evaluate pathway changes in these animals. There were significant downregulation in multiple pathways related to inflammation, hemostasis and lipid homeostasis. One important pathway that was highly downregulated was acute phase reaction (Fig 5c), including IL-6.

To further analyze the findings of our RNAseq analysis, we performed weighted gene correlation network analysis (GCNA). In our analysis, we compared the precursor of fibrinogen activation, thrombin, to genes in the exosome (Table 3). In this analysis, we were able to determine that serpin A1 (also known as alpha-1-antitrypsin) and serpin A3 (also known as alpha-1-antichymotrypsin) were strongly associated with thrombin expression.

Discussion

An association of fibrinogen with AAA has been noted in the literature [23, 24], and the involvement of macrophages in AAA pathogenesis has been well-documented. [52] Here we demonstrate for the first time that fibrinogen and AAA pathology are dependently linked. First, we demonstrated that patients with AAA show increases in not only fibrinogen, but also upregulation of procoagulant activity upstream of fibrinogen. Furthermore, we were able to demonstrate that in a large cohort of human AAA patients, fibrinogen levels can be correlated with growth rate. In mice with depletions or deletions of fibrinogen, a significant reduction of AAA pathology is observed, suggesting fibrinogen is a driving factor in AAA pathogenesis. Furthermore, our data suggest that

fibrinogen drives AAA pathogenesis by macrophage activation. When animals lack either the binding site on the gamma chain, or the Mac-1 ligand on hematopoietic cells for this binding site, aneurysm pathogenesis is significantly impaired.

Accepted literature suggests that AAA forms after an unknown insult to the elastin fiber of the abdominal region of the aorta. Improper inflammatory response to this insult results in further degradation of the aortic wall, widening of the aortic lumen and structural damage. It has been well-established that Mac-1 engagement is crucial for proper activation of macrophages, and well-established that macrophage activation drives aneurysm progression. Fibrinogen control of macrophage activation, particularly to a site of tissue injury, has also been strongly demonstrated previously. It has also been hypothesized that the gamma chain of fibrinogen, after conversion to a fibrin network, helps to stabilize macrophage localization to the site of injury.

Our data suggests that fibrinogen may mediate the macrophage response to the theorized insult that is the nidus for AAA formation. It is recognized through many previous studies that macrophages are early responders to the site of AAA formation and are some of the first mediators of this process. Furthermore, studies from our lab suggest that even prior to macrophages, platelets arrive at the site of AAA formation. Substantial data continues to emerge demonstrating the powerful contribution of platelets to inflammatory processes, and crosstalk between platelets and the coagulation cascade, including fibrinogen, have been well-demonstrated. We also showed with this data that human aorta tissue collected from open repair demonstrate substantial fibrinogen deposition in advanced AAA. We also demonstrate early changes in inflammation response by RNA expression when macrophage recruitment

by fibrinogen is impaired, particularly with decreases in the acute phase reaction and IL-6. Therefore, a reasonable mechanism for our findings is that fibrinogen deposition in response to the initial elastin insult is partially responsible for recruitment and migration of macrophages to the aneurysm site, and subsequent progression of the aneurysm.

In further analysis of RNAseq data, we found that there was significant decrease in alpha-1-antitrypsin (AAT), which is thought to regulate levels of MMP-9. [147] The gelatinase effects of MMP9 are well-documented to be involved in the proteolytic destruction of the vasculature in AAA. [148] This correlation may suggest that in *Fibγ$^{390-396A}$* mice, there is no compensatory increase in AAT, because of the lack of increased MMP9 activity from white blood cells accumulating at the site of injury.

Possible other mechanisms exist. *Fibγ$^{390-396A}$* mice show biologically important differences in FXIII cross-linking in red clots [71], and the majority of FXIII in human circulation is bound to fibrinogen by the 390-396 binding motif. [149] Importantly, FXIII polymorphism His95Arg has been correlated with multiple thrombotic diseases, as well as with increased rates of AAA. Other FXIII polymorphisms have been noted as well to be in negative disequilibrium in AAA patients.[150] However, recent studies have demonstrated that FXIII cross-linking is not absolutely necessary for proper activation of macrophages *in vitro*, [151] and though *Fibγ$^{390-396A}$* mice show decreases in FXIII activity, they are not fully FXIII deficient. [71] Yet, contributions of FXIII to AAA pathogenesis should not be ignored, and the connection of FXIII and the *Fibγ$^{390-396}$* region will need to be further investigated.

The translational potential of these findings is evident. Pharmaceutical treatment of AAA remains elusive and our work demonstrates a strong potential target for drug

development. However, further studies would be necessary to demonstrate the interventional power of fibrinogen inhibition, at what point in the aneurysmal development intervention becomes most potent, and the level to which fibrinogen must be suppressed or impaired to lead to a therapeutic potential. We have demonstrated that pre-emptive inhibition, impairment, suppression or removal of various parts of the fibrinogen-macrophage communication axis can be beneficial in preventing AAA formation and progression. However, it remains to be seen if inhibition of fibrinogen after the formation of AAA could slow progression of the disease. Also, the risks of fibrinogen targeting are clear, so studies to identify a therapeutic window for fibrinogen inhibition would be crucial.

In summary, our work demonstrates that fibrinogen is a genetically alterable driver of AAA pathogenesis, particularly through the fibrin(ogen)-$\gamma^{390-396}$ motif and Mac-1 interaction, and further studies to investigate the susceptibility of this axis to drug modification and mid-aneurysmal intervention are necessary.

Methods

Mice and diet: For aneurysm experiments, *Fibγ$^{390-396A}$* or *$^{Fib-/-}$* mice were obtained from Matthew Flick. To increase susceptibility to cardiovascular disease, mice were then crossed to *Ldlr$^{-/-}$* background (*Fibγ$^{390-396A}$*) or treated with AAV-Ldlr vector (*Fib$^{-/-}$).* For viral vector treated mice, the virus used was AAV8-U6-SA-WTmLdlrEx14-gRNA2-N22-CB-SACas9-HA-OLLAS-spA. This is a AAV8 adenoviral associated vector, with Ldlr Crispr/Cas, as outlined in previous publications. [135] For bone marrow experiments, male *Ldlr$^{-/-}$* mice and female *Itgam1$^{-/-}$* or *Itgam1$^{+/+}$* mice were obtained from Jackson

laboratories. For ASO-treated experiments, *Ldlr*$^{-/-}$ mice were obtained from Jackson laboratories. To induce hypercholesterolemia, *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. Due to low penetrance of aneurysm models in female mice,[136] only male mice were utilized in experiments. All mice were maintained under barrier conditions. Water and normal laboratory diet were available ad libitum. All procedures involving animals were approved by the Animal Care and Use Committee at the University of Cincinnati.

Antisense Oligonucleotides: For fibrinogen suppression experiments, antisense oligonucleotide to β-Fibrinogen and a scrambled control were obtained from IONIS Pharmaceuticals. Mice were injected intraperitoneally 30 mg/kg twice per week, 3 weeks prior to and throughout experimentation.

Human Cohort: Human samples of AAA patients and age-matched, gender-matched controls were obtained from the University of Uppsala (Uppsala, Sweden) biobank. For AAA patients, inclusion criteria in the present study included: aortic diameter > 30 mm, and follow-up of > 6 months. Patients with ruptured AAA, co-existing malignant disease, dialysisdependence, recent thrombo-occlusive disease, or on anticoagulation therapy were excluded. In the present study, plasma samples from participants with AAAs (n = 166) and normal (non-aneurysmal; n = 129) aortas were analyzed.

ELISAs and plate assays: The following commercially-available kits were used: total cholesterol colorimetric assay (Wako Diagnostics, Cat No. 439-17501), mouse

fibrinogen ELISA (ICL Cat No E-90FIB), TAT ELISA (Enzygnost TAT micro kit from Dade Behring).

Osmotic minipump implantation:

At at least 8 weeks of age, male mice were fed a high-fat, high-cholesterol diet, and implanted with Alzet osmotic minipumps (Model 1004 or 2004, Durect corporation) subcutaneously. Infusion of AngII (1,000 ng/kg/min; Bachem) continued for 28 days, as previously described. Mice were sedated with either isoflurane inhaled to effect or ketamine/xylazine injection.

Elastase surgery:

At at least 8 weeks of age, male mice were anesthesized with isoflurane inhaled to effect and the abdominal cavity was opened. The abdominal region of the aorta was treated, topically, with porcine elastase and wounds were closed by suture.

Bone marrow transplantation:

Ldlr-/- mice were irradiated using a gamma irradiation Cs137 irradiator (J.L. Shepherd) with a total dose of 1200 rads of radiation, in two doses across a period of 4 hours. Irradiated mice were re-populated with bone marrow harvested from $Mac1^{+/+}$ (n = 15), $Mac1^{-/-}$ (n = 15), $Ldl^{+/+}$ donor mice via retro-orbital injection. Mice were allowed to recover for 4 weeks with prophylactic Sulfatrim (Patterson Veterinary) available in 0.5 mg/mL concentration in water, *ad libitum.* After 4 weeks, mice were fed hypercholesterolemic diet as described and infused with AngII, as referenced in the previous section.

Aortic tissue, blood and plasma collection:

Twenty-eight days after pump implantation, mice were anesthetized, and blood was drawn from the inferior vena cava in sodium citrate and plasma processed by centrifugation. Mice where then terminated by bilateral pneumothorax, and aortas were perfused with saline, extracted, and placed into formalin (10% wt/vol) until dissection. For mid-study blood collections, mice were anesthetized with isoflurane and blood was collected retro orbitally into heparinized capillary tubes.

Measurement of blood composition: Platelet, red blood cell and white blood cell counts and parameters was measured in heparinized blood from retro orbital collection using a HemaVet system (Drew Scientific).

Measurements of abdominal aortic diameters: Mouse aortas were measured *ex vivo.* Aortas were dissected under dissecting microscope and measured at suprarenal area at widest point.

Measurement of inflammatory markers: Aortas were perfused with saline, extracted and placed into RNAlater solution (ThermoFisher scientific). After dissection, RNA was isolated from aortas. First, aortas were processed by Beadbug homogenization (Millipore Sigma) in QiaZol (Qiagen), and product was isolated in chloroform and isopropanol. RNA was then isolated and purified using a Macherey-Nagel purification kit, according to manufacturer instructions (Macherey-Nagel). The formation of cDNA was done using iScript Reverse Transcription Supermix (BioRad) according to manufacturer instructions. Inflammatory markers were measured by qPCR using iTaq Universal SYBR Green Supermix and CFX-96 qPCR machine (BioRad).

Human Fibrinogen Measurement: Human fibrinogen was quantified by the Clauss Method, using a Stago STart 4 (Stago Hemostasis Systems). The measurement was done according to the procedure outlined by the manufacturer.

RNAseq:

Aortas were perfused with saline, the abdominal region was extracted and placed in RNAlater (ThermoFisher scientific). Prior to processing, all surfaces were treated with RNAseZAP (MilliporeSigma). Aortas were then removed and washed with RNAse-free PBS and flash frozen in liquid nitrogen. The aortas were pulverized using a Spectrum Bessman tissue pulverizer (Fisher Scientific) and then processed in a using BeadBug microtube homogenization system (Millipore Sigma) in QiaZol Lysis reagent (Qiagen), and isolated using chloroform and isopropanol. RNA was then purified using a Direct-zol RNA Miniprep Plus kit (Zymo Research), according to manufacturer and instructions. RNA yield was measured by a Nanodrop 2000 (ThermoFisher scientific). RNAseq was performed by Cincinnati Children's Hospital and Medical Center Gene Expression Core using the Tecan Genomics and Illumina Protocols on an Ovation RNA-seq System v.2.

Histology and staining: For frozen sections, dissected aortas were embedded in TissuePlus OCT compound (Fisher scientific) and section in 10 um slices using a HM525NX cryostat (ThermoFisher). For paraffin-embedded sections, dissected aortas were fixed in formalin, and then processed and embedded by the University of Cincinnati Histology Core. Blocks were sectioned in 6um slices using a HM 315 Microm microtome (Thermo Scientific). For fibrinogen staining, sections were stained with Sections were stained with Dako Antibody to Human Fibrinogen (polyclonal rabbit anti-

human; clone A0080) and labeled with fluorescent secondary Alexa488 (goat anti-rabbit, Invitrogen). For CD68 staining, sections were stained with CD68 (Bio-rad MCA1957; rat anti-mouse) and labeled with fluorescent secondary Alexa488 (goat anti-rat, Invitrogen). Sections were visualized with a LSM 710 LIVE Duo Confocal Microscope (Zeiss). We acknowledge the use of the University of Cincinnati Live Microscopy Core.

Research statistics and data representation: All bar and line graphs were created with Sigma Plot v.14 (SPSS, Chicago, IL). All statistical analysis was performed using SigmaStat, now incorporated into Sigma Plot v.14. 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. Values of $P < 0.05$ were considered statistically significant.

Calibrated Automated Thrombography: CAT was performed on human plasma samples as previously described. [152]

RNAseq and WGCNA: Analysis was performed as previously described. [153, 154]

Study approval: All mouse studies were performed with the approval of the University of Cincinnati Institutional Animal Care and Use Committee. All analysis of human data was approved by the Institutional Review Board of Vanderbilt University Medical Center.

Acknowledgements

Disclosures

None

Figures

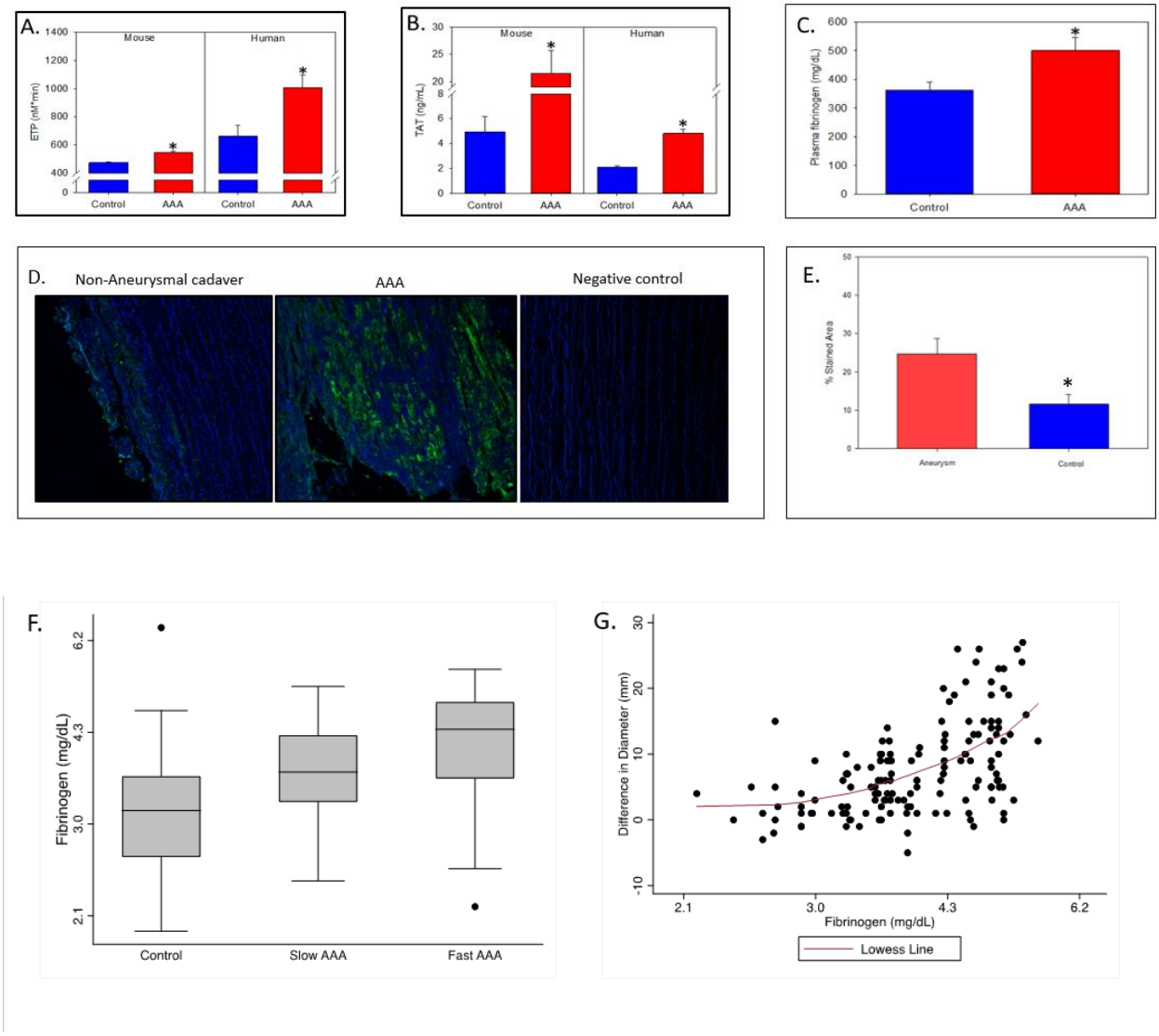

Fig 1: Procoagulant Activity is elevated in human AAA. A) Mean endogenous thrombin potential (ETP) in human AAA patients and controls (right) and AngII induced mouse aneurysm vs unchallenged mice (left) B) Mean thrombin-antithrombin complex levels (TAT) in human AAA patients and controls (right) and AngII-induced mouse aneurysm vs unchallenged mice (left). C) mean plasma fibrinogen levels in human AAA patients and controls. D) human AAA aorta sections vs human cadaver sections and negative control, stained immunofluorescently for fibrinogen deposition. E) quantification of % stained area in human sections. F) fibrinogen levels in human patients correlated to case status (n=264). G) fibrinogen levels in human patients correlated to aortic size

Table 1. Participant characteristics by case definition (n=264)

Characteristic	Control (n=115)	Slow Growing (n=75)	Fast Growing (n=74)
Age (years)	65 (65, 70)	71 (67, 77)	72 (67, 78)
Fibrinogen (mg/dL)	3.15 (2.62, 3.61)	3.67 (3.26, 4.25)	4.35 (3.58, 4.85)
Height (cm)	178 (174, 183)	181 (178, 185)	180 (177, 187)
Weight (kg)	83 (75, 90)	89 (80, 99)	87 (78, 98)
CHD	10 (8.7)	27 (36.0)	25 (33.8)
Hypertension	56 (48.7)	51 (68.0)	44 (59.5)
CVD	5 (4.4)	18 (24.0)	7 (9.5)
Claudication	1 (0.9)	8 (10.7)	1 (1.4)
COPD	5 (4.4)	12 (16.0)	11 (14.9)
Renal Insufficiency	2 (1.7)	4 (5.3)	4 (5.4)
Diabetes	17 (14.8)	17 (22.7)	4 (5.4)
Aspirin Use	17 (14.8)	37 (49.3)	33 (44.6)
Statin Use	30 (26.1)	35 (46.7)	27 (36.5)
Current Smoker	6 (5.2)	12 (16.0)	25 (33.8)
Smoking Years	23.4 (13.7)	34.2 (13.0)	39.4 (14.3)

Note: Values are median (25^{th}, 75^{th} percentile) or N (%)
Abbreviations: S.D. standard deviation; cm, centimeters; kg, kilograms; mg, milligrams; dL, deciliters; CHD, coronary heart disease; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease,

Table 2. Unadjusted and adjusted odds ratio for the relationship between fibrinogen and case status

	Control (N)	Slow AAA (N)	Fast AAA (N)	OR (95% CI)	Adjusted OR (95% CI)
Continuous					
Fibrinogen, 20% increase	-	-	-	2.69 (2.13, 3.39)	2.06 (1.59, 2.68)
Dichotomous					
<3.58 mg/dL	81	30	16	1.0 (ref)	1.0 (ref)
≥3.58 mg/dL	34	45	58	5.25 (3.22, 8.57)	2.73 (1.56, 4.77)

Note: Odds ratios obtained from ordinal logistic regression; continuous fibrinogen is log transformed; adjusted for age, smoking years, CHD, CVD, diabetes, hypertension, and COPD
Abbreviations: C.I. confidence interval; O.R. odds ratio; C.H.D. coronary heart disease; C.V.D. cardiovascular disease

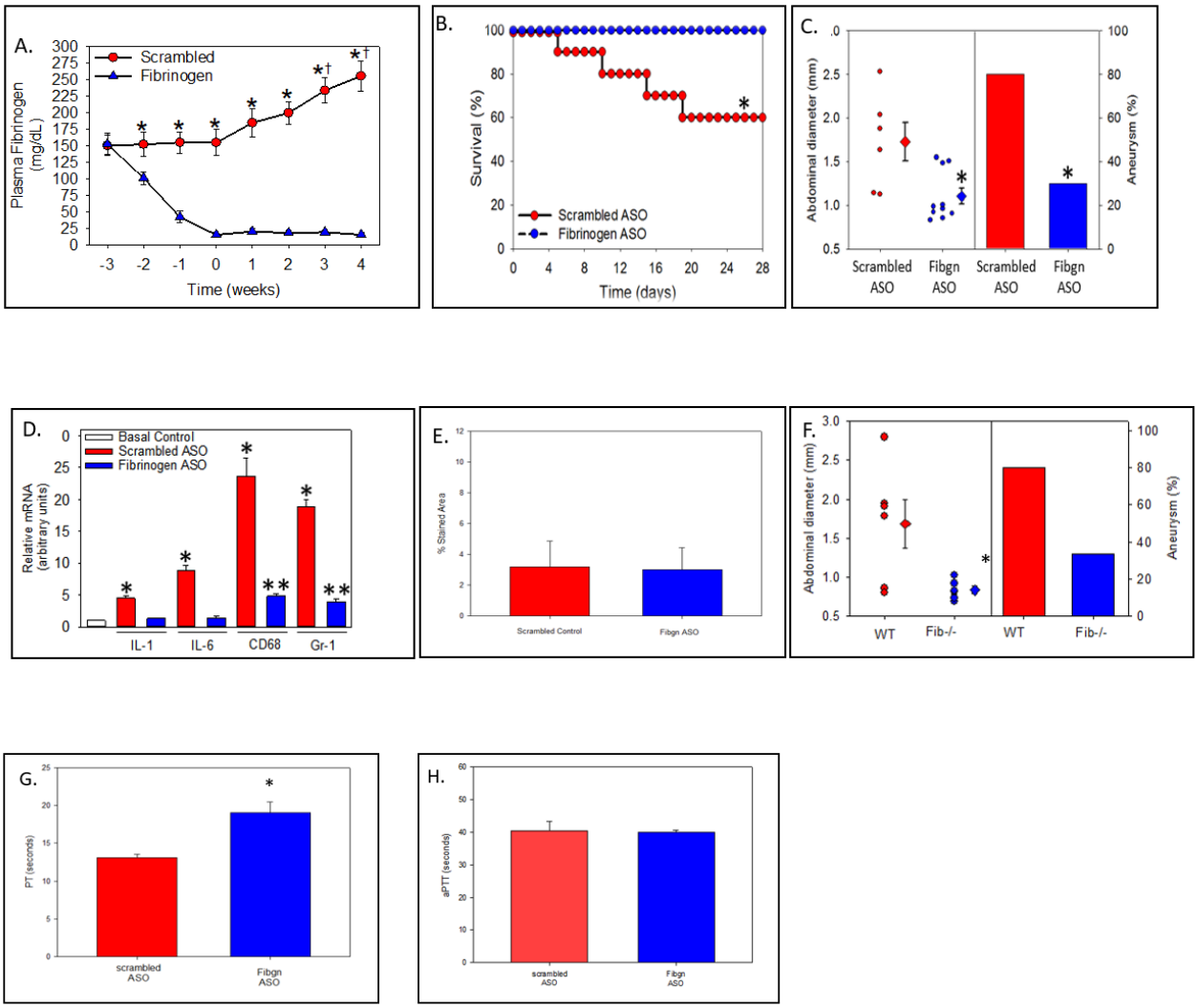

Fig 2: Fibrinogen suppression attenuates AAA. A) Plasma levels of fibrinogen in mice receiving antisense oligonucleotide to β-fibrinogen or scrambled control 3 weeks prior to and throughout the study period. Time 0 denotes administration of AngII. B) Survival analysis of mice throughout the study period. C) abdominal aortic diameter (left) and aneurysmal incidence (right) in mice receiving either Fibrinogen ASO or scrambled control. D) Relative mRNA levels of IL-1, IL-6, CD68 and Gr-1 in mice receiving either fibrinogen ASO or scrambled control. E) Fibrinogen deposition in aortas of mice after aneurysm induction, and either scrambled control or fibrinogen ASO (p=1.00) F) Aneurysmal burden mice of WT mice or mice possessing genetic deficiency of fibrinogen G) PT measurements of mice treated with scrambled or fibrinogen ASO H) PTT measurements of mice treated with scrambled or fibrinogen ASO I) Histological sections of mouse aorta receiving either fibrinogen ASO or control

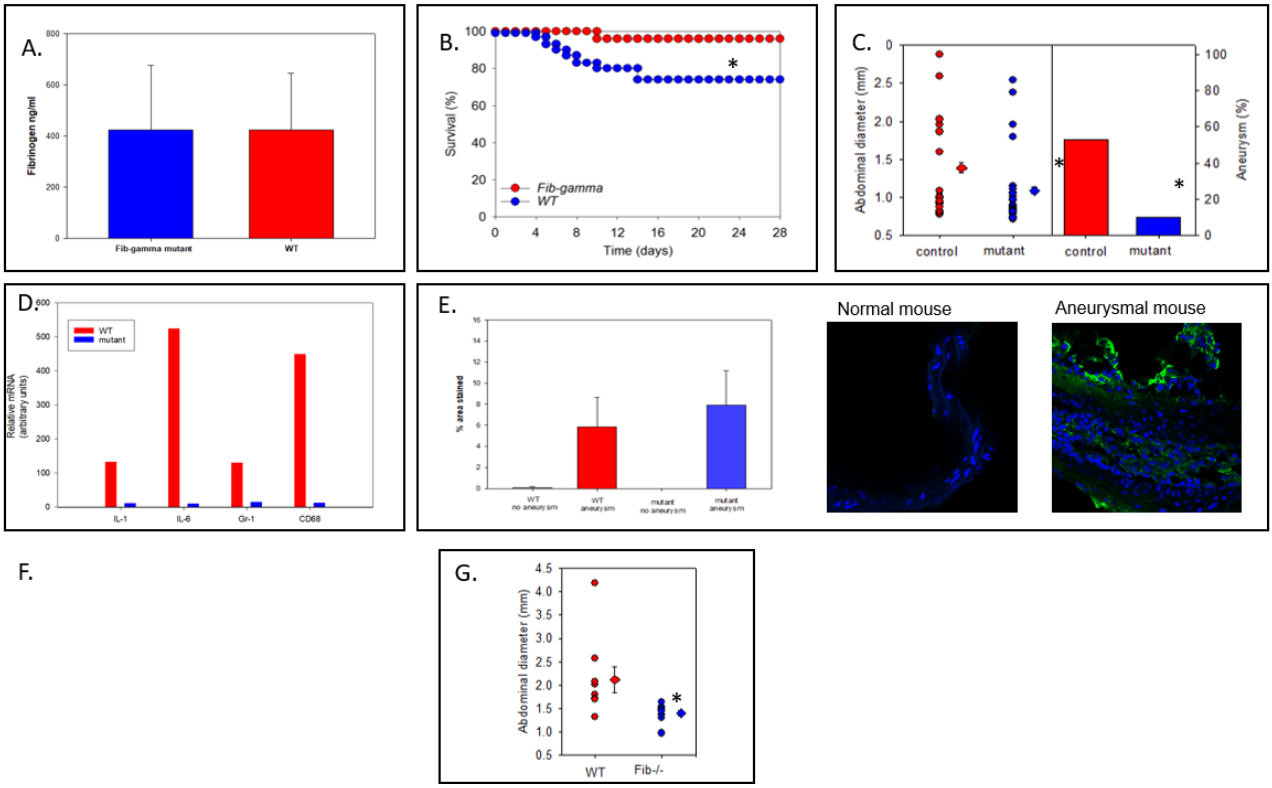

Fig 3: Mutation of the Mac-1 binding site on the Fibγ chain attenuates AAA. A) mean plasma fibrinogen levels of *Ldlr*$^{-/-}$*Fibγ*$^{390\text{-}396A}$ or *Ldlr*$^{-/-}$*Fibγ*^{WT} mice after aneurysm induction. B) survival analysis of mice throughout the study period. C) abdominal aortic diameter (left) and aneurysmal incidence (right) in either *Ldlr*$^{-/-}$*Fibγ*$^{390\text{-}396A}$ or *Ldlr*$^{-/-}$*Fibγ*^{WT} mice after AngII infusion. D) Relative mRNA levels of IL-1, IL-6, CD68 and Gr-1 in either *Ldlr*$^{-/-}$*Fibγ*$^{390\text{-}396A}$ or *Ldlr*$^{-/-}$*Fibγ*^{WT} mice at the end of the study period. E) Histological sections of mouse aorta from either mutant or wild type mice after aneurysm induction F) abdominal aortic diameter in either *Ldlr*$^{-/-}$*Fibγ*$^{390\text{-}396A}$ or *Ldlr*$^{-/-}$*Fibγ*^{WT} after topical elastase induction of aneurysm.

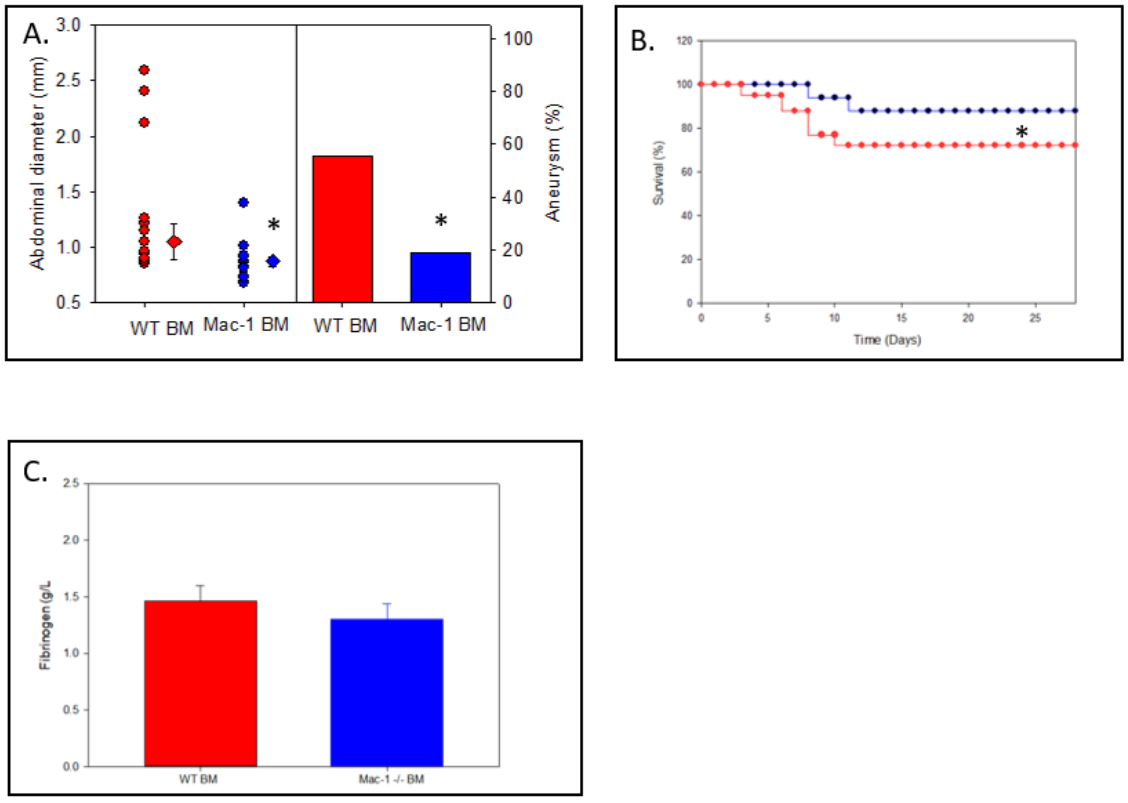

Fig 4: Removal of the Mac-1 integrin in hematopoietic cells attenuates AAA.. A) abdominal aortic diameter (left) and aneurysmal incidence (right) in *Ldlr*$^{-/-}$ mice receiving either *Mac-1*$^{-/-}$ or *Mac-1*$^{+/+}$ bone marrow. B) Survival analysis of mice throughout the study period. C) mean plasma fibrinogen levels at completion of study

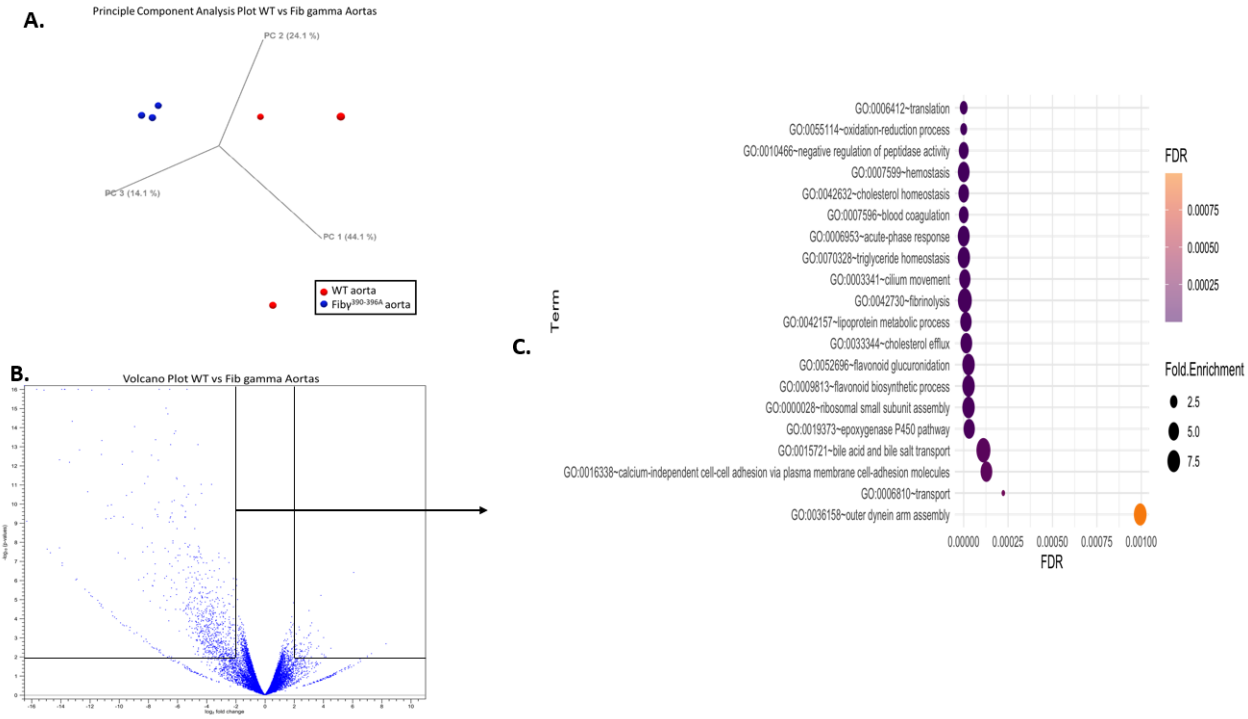

Fig 5: RNAsequencing demonstrates decreases in inflammatory, lipid homeostasis and hemostatic processes A) Principle component analysis of mice with or without Fibγ$^{390\text{-}396A}$ mutation, 3 days after AngII aneurysm induction. B) Volcano plot demonstrating upregulated and downregulated genes. C) Go pathway analysis of downregulated genes in Fibγ$^{390\text{-}396A}$ mice

Gene	correlation to thrombin	p-value
F2	1	4.25E-78
Ahsg	0.999803165	2.33E-18
Apoa4	0.999231383	2.11E-15
Plg	0.999180057	2.91E-15
Apon	0.999123155	4.08E-15
Itih1	0.999082508	5.11E-15
Serpina1e	**0.998551297**	**5.01E-14**
Serpina3k	**0.998080512**	**2.05E-13**
Cpn1	0.997938776	2.92E-13
Apom	0.997107537	1.59E-12
Cyp2a12	0.996564251	3.75E-12
Masp2	0.996535487	3.91E-12
Hpx	0.996143478	6.67E-12
Ces3a	0.996098295	7.07E-12
Hrg	0.995787388	1.04E-11
Cyp2c50	0.994757491	3.09E-11
Azgp1	0.994503448	3.91E-11
Ugt1a1	0.994050538	5.81E-11
Lcat	0.993640173	8.11E-11
Rdh7	0.993605348	8.33E-11
Etnk2	0.993309798	1.04E-10

Table 3. WGCNA analysis to thrombin of genes in RNAseq analysis

CHAPTER 4

Platelet Activation is a Critical Component of Abdominal Aortic Aneurysm Formation

ABSTRACT

Abdominal aortic aneurysm (AAA) is pathophysiologic degradation of the tunica media resulting in aortic dilatation, systemic inflammation, and dysregulated hemostasis. A persistent feature of AAAs is the formation and constant presence of a laminated nonocclusive intraluminal thrombus (ILT) along the aneurysmal aorta. While the ILT is known to contain platelets, it is unknown whether these platelets contribute to the initial and ongoing pathogenesis of AAA. In this work, we find that platelet transcripts are highly enriched in the ILT and the platelet collagen receptor glycoprotein VI is upregulated and predictive of aneurysm growth rate in human patients. We show that platelets arrive within a day of aneurysm initiation and that platelet factor 4 is highly correlated to aortic diameter and growth in a mouse model of AAA. Genetic deficiency of platelet receptors or mice given anti-platelet therapies resulted in no difference in abdominal aortic diameter but augmented rupture-induced death versus littermate and placebo controls, respectively, in the angiotensin II model of AAA formation. Systemic platelet depletion results in pervasive rupture-induced death in several mouse strains and with three different mouse models of AAA. In sum, inhibition of platelet function may be detrimental in an expanding aortic lumen resulting in catastrophic hemodynamic failure.

Introduction

Abdominal aortic aneurysm (AAA) is a degenerative vascular disease characterized by a progressive inflammatory infiltrate and medial wall degradation resulting in a permanent localized dilatation of the aortic wall. Affecting more than 1 million American adults, the prevalence is ~5 – 8% in males and 1 – 2% of females over the age of 65, resulting in ~9,928 deaths per year in the US. [155] Currently, no therapies or pharmaceutical treatments can prevent the development and growth of AAA, with only endovascular grafting or invasive open surgical intervention as a means to prevent aortic rupture. Understanding the multifactorial complexities of the disease and identifying high risk patients with predictive biomarkers is necessary to improve translational treatment strategies for aneurysm patients.

Platelets are crucial mediators of vascular hemostasis and initiators of thrombosis. [156] Collagen and tissue factor (TF) in the subendothelial matrix facilitate both the maintenance of a contained circulatory system via the initiation of the coagulation system upon the breach of the vessel wall. Endothelial damage and exposed collagen interact with platelet glycoprotein VI (GPVI) while collagen-bound von Willebrand factor (vWF) reacts with GP1b, GPV, and GPIX. [156, 157] However, it is GPVI that is the major agonist for the initial activation of platelets, subsequent alpha granule release, and primary platelet adhesion. [158, 159] This initial platelet activation via collagen is independent of thrombin-mediated agonism, which occurs with TF propagation of the coagulation cascade and culminates with thrombin-mediated cleavage of protease-activated receptors (PARs). [157] Thrombin-induced platelet activation via PAR1 (humans)

and PAR4 (mice) results in the release of adenosine diphosphate (ADP) and thromboxane A_2 (T_XA_2), which subsequently activate the $P2Y_{12}$ and thromboxane receptors, respectively, leading to thrombus propagation. [160]

The formation of a tri-layered intraluminal thrombus (ILT) in 70% of AAAs at the site of expansion is indicative of platelet accumulation. [54, 106] Platelet activation products primarily appear in the outermost layer of the thrombus (luminal layer) where it is considered biologically active. [57, 107, 108] $P2Y_{12}$ inhibitors administered to mice block the initiation and progression of AAA. [109] Furthermore, the GPIIb-IIIa inhibitor abciximab attenuates thrombus size and luminal dilation in a rat xenograft model of AAA. [161] While platelet inhibitors are associated with decreased mortality in AAA patients, several metaanalyses have demonstrated little to no benefit from these drugs. [17, 19, 46] Conversely, some studies have shown that platelet inhibition can lead to worsening outcomes and rupture in patients with aortic dissection. [162, 163] While platelets likely play a causal role in human AAAs, the contribution of platelets to the initiation and pathophysiology of AAAs is still poorly understood.

Previous work from our group has identified a role for platelet activation in the progression of vascular degeneration and rupture in a mouse model of AAA with a confirmatory increased survival in aneurysm patients treated with antiplatelet therapeutics.[13] Here, we directly assess the role of platelets in the initiation and early progression of AAA. Using next generation RNA-sequencing, we identify the platelet transcriptome is significantly increased in the ILT compared to the adjacent aneurysm wall and to control aortas. The platelet collagen receptor, glycoprotein VI (GPVI), is significantly upregulated and increased on the platelets of AAA patients with soluble

GPVI (sGPVI) predictive of AAA growth rate and diameter changes. Genetic (*Par4*$^{-/-}$ and *P2y*$_{12}$$^{-/-}$) and pharmacologic (aspirin, dabigatran etexilate, clopidogrel) inhibition of platelets increases the incidence of rupture in a mouse model of AAA. Importantly, platelet depletion results in rupture-induced death in >80% in three different mouse models of AAA. Our data suggests platelet activation is critical for both the formation and hemostatic stabilization of AAAs.

Results

The platelet transcriptome is amplified in the ILT of human AAAs

Next generation RNA sequencing was performed on age-matched control aortic tissue (n = 5), AAA aortic tissue (n = 4), and AAA ILT (n = 3). All three groups demonstrate spatial separation via principle component analysis (PCA Figure 1A) and heatmap clustering (Figure 1B) with several unique AAA wall and thrombus-specific genes in each subset comparison (Supplemental Figure 1). Platelet-specific transcripts all primarily cluster within the AAA thrombus versus the control and AAA aortic tissue (Figure 1C). Differential expression analysis of the AAA thrombus versus the control aortic wall (Figure 1D) and AAA wall (Supplemental Figure 1B) demonstrates several platelet-specific transcripts are in the upper quartile of significantly upregulated genes (noted with red dots) with the volcano plot demonstrating several significantly upregulated platelet-specific transcripts (Figure 1D – red dots). Finally, platelet transcripts comprise 8 out of the top 30 significantly enriched genes in the comparison of AAA thrombus to control aortic wall (Figure 1E, platelet genes bolded).

The platelet-specific collagen receptor GPVI is upregulated in AAA patients

The biocollagen GPVI is expressed only on platelets and platelet precursor cells (megakaryocytes) and contains two extracellular Ig domains, a short transmembrane domain and a cytoplasmic tail. Our RNA profiling suggests that GPVI transcripts are upregulated in the AAA thrombi versus both control and AAA aortic walls (Figure 1 and Supplemental Figure 1). As such, we examined the protein levels of platelet GPVI isolated from human AAA patients versus age-matched controls. Resting platelet GPVI protein is significantly increased as measured by both Western blotting and surface expression via flow cytometry (Figure 2A – 2B).

We previously demonstrated that platelet factor 4 (PF4) levels are highly variable in human plasma, which is likely due to sample processing. [119] Importantly, soluble glycoprotein VI (sGPVI) is shed from the platelet surface and is a highly reproducible marker of platelet activation. As such, we examined the amount of sGPVI in a cohort of control subjects compared to slow-growing or fast-growing AAAs (defined previously). [119] Baseline characteristics of the study participants are shown in Table 1. The mean (standard deviation (SD)) age of participants in the control, slow-growing AAA, and fast-growing AAA groups was 67 (2.6), 73 (6.6), and 73 (6.8) years, respectively. Smoking, CVD, CHD, hypertension, and COPD were more common among patients with AAA than controls. Additionally, AAA cases were more likely to report aspirin and statin use than controls. The median (25^{th}, 75^{th} percentile) sGPVI for controls, slow-growing AAAs, and fast-growing AAAs was 14.9 ng/mL (8.7, 34.9), 42.2 ng/mL (29.0, 62.7), and 102.9 ng/mL (72.9, 132.9), respectively (Figure 2C).

In a comparative analysis, AUC values from the ROC analysis of plasma sGPVI samples predicts the occurrence of fast-growing AAAs with 91% sensitivity (97%

specificity), where ≥49 mg/mL achieves significance (Figure 2D). In patients with slow-growing AAA, the mean (SD) difference in diameter was 3.1 (3.8) mm and the mean (SD) growth rate was 0.8 (1.0) mm/year. In patients with fast-growing AAA, the mean (SD) difference in diameter was 12.1 (6.5) mm and the mean (SD) growth rate was 3.7 (1.6) mm/year. After adjustment, per doubling of sGPVI, the β-coefficient for difference in diameter was 5.6 (95% CI: 4.9, 6.4) and the β-coefficient for growth rate was 1.6 (95% CI: 1.4, 1.8) (Figures 2E – 2F).

After adjustment, per doubling of sGPVI, the odds of being in a higher outcome group compared to a lower outcome group was 4.1 (95% CI: 3.0, 5.8) (Table 2). In participants with sGPVI levels above 38 ng/mL, the odds of being in a higher outcome group compared to a lower outcome group was 11.6 (95% CI: 6.2, 21.9) after adjustment for all covariates (Figures 2G and Supplemental Figure 2).

AngII infusion results in a time-dependent increase in plasma parameters of coagulation and platelet activation

To determine whether our mouse model of aneurysm formation also had robust platelet activation, we examined the levels of PF4, thrombin-antithrombin (TAT), and D-dimer in saline infused mice compared to AngII-infused mice (Figure 3A). Infusion of AngII resulted in a time-dependent increase in plasma PF4, TAT, and D-dimer (Figures 3B, 3D, 3F). Interestingly, all coagulation variables were highly correlated with abdominal aortic diameter (Figures 3C, 3E, 3G).

Platelet accumulation in the abdominal aorta of AngII-infused mice precedes macrophages

To date, the medial accumulation of macrophages and subsequent degradation of elastin fibers is thought to be one of the earliest events in the abdominal aorta after AngII infusion. [11] However, platelet accumulation in mouse models of aneurysm formation has not yet been studied. Therefore, platelet and macrophage accumulation were measured at different times in Ang II-infused mice. We utilized *Ldlr*$^{-/-}$ mice fed a high fat/high cholesterol diet (HFD/Western) for 1 week prior to and throughout AngII infusion (1,000 ng/kg/min) for 0-3, 5, 7, and 28 days. Mice had similar cholesterol, lipid fractions, and systolic blood pressures at all time-points (data not shown). Platelets rapidly accumulated in the abdominal aorta of AngII-infused mice beginning at day 1 and time-dependently increasing up to day 28 (Figure 4A). Macrophages accumulated with delayed kinetics relative to platelets with first observation at day 2 and a significant increase at day 3 (Figure 4B). Mice receiving control agents (IgG and nanoparticles) labelled with 700 and 800nm reagents had no recordable fluorescent signal (Supplemental Figure 3). Aneurysm formation also resulted in the hyperactivation of blood platelets with after 28 days of AngII infusion (Supplemental Figure 4).

Genetic deficiency of platelet activation receptors augments AAA rupture.

Thrombin activation of platelets is a crucial step in hemostasis. To determine the effect of the thrombin platelet receptor PAR4 on the initiation of AAA, we utilized male *Ldlr*$^{-/-}$*/Par4*$^{+/+}$ or *Ldlr*$^{-/-}$*/Par4*$^{-/-}$ littermates fed a HFD for 1 week prior to and throughout AngII infusion for 28 days. We found that *Ldlr*$^{-/-}$*/Par4*$^{-/-}$ mice had no change in abdominal aortic diameter but significantly increased the incidence of AAA and rupture-induced death (Figure 5A and 5B). To determine if these effects were due to bone marrow cells (i.e. platelets), *Ldlr*$^{-/-}$*/Par4*$^{+/+}$ and *Ldlr*$^{-/-}$*/Par4*$^{-/-}$ mice were irradiated and

repopulated with *Par4*$^{+/+}$ or *Par4*$^{-/-}$ bone marrow to create 4 chimeric groups. After 4 weeks, repopulation of the bone marrow was verified by isolation and treatment of platelets with a Par4 agonist peptide (P4AP). All mice with hematopoietic Par4 deficiency were unresponsive to stimulation with P4AP (Supplemental Figure 5A). Mice were fed a HFD for 1 week prior to and throughout AngII infusion for 1 week. No difference was found among any groups regarding body weight, plasma cholesterol, lipoproteins, or systolic blood pressure (data not shown). Similar to whole body deficiency, Par4 deficiency in bone marrow-derived cells had no effect on abdominal aortic diameter but significantly increased the rate of AAA rupture (Figure 5C and 5D). While PAR1 is the major platelet receptor in humans, it is not found on the platelets of mice. As such, examination of *Ldlr*$^{-/-}$*/Par1*$^{+/+}$ or *Ldlr*$^{-/-}$*/Par1*$^{-/-}$ littermates yielded no difference in aneurysm formation (Supplemental Figure 6). Administration of the thrombin inhibitor dabigatran etexilate (10 g/kg diet, efficacy of dabigatran – Supplemental Figure 5B) given 1 week prior to and throughout AngII infusion for 28 days recapitulated the effects of *Par4*$^{-/-}$ in *Par4*$^{+/+}$ mice, but did not result in any additive differences versus *Par4*$^{-/-}$ alone (Figure 5E and 5F).

Next, we examined the effect of a deficiency of *P2Y*$_{12}$ in hematopoietic cells on AAA formation and rupture. We irradiated male *Ldlr*$^{-/-}$ mice and repopulated them with *P2Y*$_{12}$$^{+/+}$ or *P2Y*$_{12}$$^{-/-}$ bone marrow. Transplanted mice were allowed to recover for 4 weeks and then fed a HFD for 1 week prior to and throughout AngII infusion for 28 days. Successful incorporation of *P2Y*$_{12}$ deficient BM was verified by P4AP or convulxin stimulation of isolated platelets. Mice with hematopoietic deletion of *P2Y*$_{12}$ had a markedly reduced integrin activation, as measured by JON/A, with P4AP or convulxin

compared to $P2Y_{12}^{+/+}$ BM mouse platelets (Supplemental Figure 5C). There was no difference in body weight, plasma cholesterol, lipoproteins, or systolic blood pressure (data not shown). In mice with a deficiency of $P2Y_{12}$ in hematopoietic cells we found no effect on abdominal aortic diameter, a non-significant increase in incidence and a significant difference in AAA incidence compared with controls (Figure 6).

Effect of platelet inhibitors, thrombin inhibition, or platelet deficiency on AAA formation and rupture.

To determine the effect of platelet inhibitors on AngII-induced AAAs, male $Ldlr^{-/-}$ mice were given either placebo or ASA (30 mg/L) via drinking water for one week prior to AngII pump implantation and throughout the study. In addition, another group of mice were fed placebo diet or a diet containing clopidogrel bisulfate (50 mg/kg) for one week prior to AngII pump implantation and throughout the study. Platelet inhibition was verified in ASA treated mice by examining aracidonic acid (AA) and thromboxane B2 (T_XB_2) platelets whereas inhibition in clopidogrel bisulfate treated mice was measured in ADP-treated platelets. Clopidogrel bisulfate resulted in almost complete abrogation of ADP-mediated JON/A activation, while ASA significantly reduced AA-stimulated platelets and completely suppressed T_xB_2 secretion (Supplemental Figure 5D – 5F). These results occurred with the absence of platelet inhibitors having any effects on body weight, plasma cholesterol, lipoproteins, or systolic blood pressure (data not shown). We found that platelet inhibition had no effect on abdominal aortic diameter or AAA incidence (Figure 6A and 6C). There was a non-significant increase in AAA incidence with ASA or clopidogrel bisulfate. However, administration of ASA or

clopidogrel bisulfate markedly increased rupture-induced death versus placebo controls (Figure 6B and 6D).

In contrast to our results, a recent publication found that administration of clopidogrel bisulfate to AngII infused *apoE*$^{-/-}$ mice resulted in decreased abdominal aortic diameter and AAA incidence with no change in rupture. [164] We observed no change in body weight, plasma cholesterol, lipoproteins, or systolic blood pressure (data not shown). However, this study delivered clopidogrel bisulfate via intraperitoneal injections (IP, 30 mg/kg). To determine if the different results could be explained by the route of administration, we repeated our study with IP administration of clopidogrel bisulfate (30 mg/kg). Similar to our study with oral administration of clopidogrel bisulfate, we found that IP clopidogrel bisulfate had no effect on abdominal aortic diameter or AAA incidence (Supplemental Figure 7A) but increased AAA rupture-induced death (Supplemental Figure 7B). Interestingly, we found that oral administration of clopidogrel bisulfate was much more effective at inhibiting platelet function in a tail bleeding assay and integrin activation assay (Supplementary Figure 7C and 7D).

In mice thrombin activates platelets by cleavage of *Par4*. Therefore, we examined the effect of thrombin inhibition on AAA formation and rupture. *Ldlr*$^{-/-}$ mice were fed a HFD containing either placebo or dabigatran etexilate (10 g/kg diet) 1 week prior to and throughout AngII infusion for 28 days. We found that administration of dabigatran etexilate significantly increased the activated partial thromboplastin time (aPTT; placebo: 0 ng/mL plasma dabigatran; dabigatran etexilate: 289 ± 32 ng/mL plasma dabigatran). Mice had similar cholesterol, body weights, lipid fractions, and

systolic blood pressures (data not shown). Dabigatran etexilate had no effect on abdominal aortic diameter or AAA incidence but significantly increased the rate of AAA rupture (Figure 7 E and 7F).

Platelet depletion results in catastrophic rupture of the abdominal aorta

As we determined that inhibition of platelets resulted in an increase in rupture-induced death, we examined the effect of platelet depletion in the AngII model of AAA. Platelets were globally depleted in *Ldlr*$^{-/-}$ mice and mice were given HFD and infused with AngII, as described above. Platelet depletion with anti-CD42b antibody (5 μg/g) significantly increased rupture-induced death versus irrelevant IgG control with no mice surviving past day 12 (Figure 8A). This result was recapitulated in another model of hyperlipidemia (apolipoprotein E – *apoE*$^{-/-}$) as well as normolipidemic *C57BL/6J* mice infused with AngII (Figure 8B and 8C). This result was not confined to the AngII model of AAA, as the deoxycorticosterone acetate (DOCA) salt model of aneurysm also recapitulated rupture (Figure 8D). [165] Next, we examined the topical elastase mouse model of AAA with concomitant TGFβ depletion with or without platelet depletion. [8] Importantly, this model forms a spontaneous ILT, as opposed to the AngII and DOCA models. The topical elastase TGFβ model resulted in similar rupture-induced death compared to all other strains and models (Figure 8E), suggesting platelets play a critical role in the initiation and stabilization phase of aneurysm formation.

Removal of the LNK receptor on platelets results in catastrophic rupture comparable to platelet depletion

Because deficiency of the LNK receptor has been previously correlated to aortic dissection in mice [166], and has been associated with cardiovascular disease in GWAS studies, [167] we investigated whether platelet deficiency of LNK resulted in a similar phenotype. Platelets were depleted in *Ldlr*$^{-/-}$ mice by busulfan and mice were given HFD and infused with AngII as described above. After 14 days, mice underwent adoptive transfer of platelets either deficient or proficient in LNK receptor, or no platelet control. Platelet injections were repeated on days 5, 10, 15, 20, and 25 and mice were euthanized on day 28 (Fig 9a). Mice receiving *LNK*$^{-/-}$ platelets fared similarly to control mice who received no platelets, and to platelet depleted mice in previous experiments, showing catastrophic aneurysm failure and high rates of death. However, mice receiving *LNK*$^{+/+}$ platelets were rescued from catastrophic death and showed rupture rates that would be expected in *Ldlr*$^{-/-}$ mice in the AngII model (Fig 9d).

Discussion

The AngII mouse model of AAA has the greatest conformity to human aneurysm pathology with regard to luminal dilatation, medial elastin breaks, accumulation of atherosclerosis, infiltration of inflammatory cells, proteolytic destruction, intact medial layer, and the penultimate rupture [11, 168]. However, it is also an acute model which results in gross medial dissections of the aortas leading to creation of both true and false lumens that are associated with vascular and adventitial hematoma formation [11, 169]. Importantly, the AngII model presents with a consistent rupture phenomenon similar to human aneurysms. Our data clearly demonstrates that inhibition of platelet activation increases rupture indicating that formation of a platelet-rich hemostatic plug is crucial to

protect against AngII-induced rupture during the early medial dissection. Similar results were observed with thrombin inhibition. In addition, we found that a deficiency of tissue factor is associated with increased rupture in this model [170]. These results indicate that mice with impaired hemostasis have increased rupture in the early phase of the AngII mouse model.

Platelets are considered one of the earliest cells infiltrating the vessel wall in a variety of vascular diseases. For example, it was recently demonstrated that platelet adhesion is critical for the initiation of atherosclerosis in a mouse model [171]. We found that PF4, a prototypical platelet activation marker, is increased in a time-dependent manner and was significantly correlated with abdominal diameter in AngII-induced AAAs. Furthermore, we observed that platelets accumulate first and more expediently than macrophages in the abdominal aorta of AngII infused mice. It was recently demonstrated that monocyte/macrophage infiltration is necessary for progression to rupture in AngII infused mice [172]. We speculate that early platelet accumulation is necessary to prevent monocyte/macrophage induction of rupture by providing a hemostatic plug.

While platelets are protective at an early time-point, the continued accumulation of platelets and macrophages can result in proteolytic destruction of the aorta via MMPs. Platelets [173, 174] and macrophages [130] are a robust source of MMPs. Further, platelet-derived chemokines can regulate the expression of MMPs from VSMCs [175] and macrophages [176]. Here, we demonstrate clopidogrel bisulfate and ASA intervention reduce the amount of platelet and macrophage infiltration, and ultimately the amount of active MMP-2 and MMP-9 in the abdominal aortas. While it is uncertain whether these

MMPs are derived from platelets or macrophages, it is possible they are derived from both sources.

Alternatively, as LNK deficiency also showed catastrophic failure, this would suggest that other components of platelet signaling are not on their own responsible for protection against rupture. The LNK receptor is involved in outside-in signaling of fibrinogen to platelets and LNK deficiency is associated with poor clot structure and retraction [105]. This may suggest that the role of platelets in AAA rupture protection in animal models is due to biomechanical stabilization of the aneurysmal area. The thrombus in AAA has been associated with stabilization of the aneurysmal wall and platelets may be crucial to stable development of this thrombus, as suggested by our findings in LNK deficiency.

In complete contrast to our results with orally-administered clopidogrel, one study surprisingly reported that IP-administered clopidogrel reduced aneurysm diameter without affecting rupture in an AngII model with *apoE*$^{-/-}$ mice [164]. However, we found that IP-administered clopidogrel increase AAA rupture in *Ldlr*$^{-/-}$ mice in a similar manner to orally administered clopidogrel and that ASA gave comparable results. Furthermore, depletion of platelets in AngII treated apoE$^{-/-}$ mice increased rupture. Together, these results indicated that platelets prevent rupture at an early stage in the AngII model of AAA.

Methods (Expanded in supplementary material)
Mice and diet:

Male *Ldlr*$^{-/-}$ or *apoE*$^{-/-}$ mice were obtained from The Jackson Laboratory. *Par4*$^{-/-}$ mice (Sambrano et al. 2001) were crossed into the *Ldlr*$^{-/-}$ background to create *Ldlr*$^{-/-}$*/Par4*$^{+/+}$ and *Ldlr*$^{-/-}$*/Par4*$^{-/-}$ mice. *P2Y*$_{12}$$^{+/+}$ and $^{-/-}$ mice, originally from Portola Pharmaceuticals [134], were used as donors in bone marrow transplant experiments. *ApoE*$^{-/-}$ mice were fed a normal laboratory diet throughout experimentation. To induce hypercholesterolemia, *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 this infusion.

Platelet and thrombin inhibition:

Platelets were depleted in *apoE*$^{-/-}$ mice by injecting a rat anti-mouse GP1bα (5μg/g bodyweight, R300, Emfret Analytics, n = 5), via tail vein, twice weekly for 1 week prior to and throughout AngII infusion. Rat IgG (5μg/g bodyweight, C301, Emfret Analytics, n = 5) was similarly administered for negative control. Platelet depletion was confirmed by Hemavet analysis (Hemavet 950 LV, Drew Scientific).

For inhibition of thrombin, *Ldlr*$^{-/-}$ mice were fed a custom-made HFD containing peanut butter (10 g/kg diet) with or without dabigatran etexilate (10 g/kg diet, purchased from UNC pharmacy: 150 mg capsules from Boehringer Ingelheim) 1 week prior to and throughout AngII infusion (Dyets Inc., based on Harlan Teklad TD.88137). Dabigatran-induced thrombin inhibition in the plasma was measured with a HEMOCLOT Thrombin Inhibitors kit (Aniara).

For P2Y$_{12}$ platelet inhibition, *Ldlr*$^{-/-}$ mice were fed either 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) or intraperitoneally injected with 30 mg/kg clopidogrel bisulfate 1 week prior to and throughout AngII infusion (Dyets Inc., based on Harlan Teklad TD.88137). Clopidogrel bisulfate inhibition of *P2Y*$_{12}$ was determined by platelet agonism by ADP or tail bleeding time. ASA (30 mg/L) was administered to *Ldlr*$^{-/-}$ mice via water 1 week prior to and throughout AngII infusion, as described previously [137]. Effective ASA absorption was determined by plasma levels of thromboxane B2.

Platelet Isolation and Immunoblotting:

Healthy patients were volunteers without any known medical history, aneurysmal disease, or on antiplatelet agents were recruited in studies approved by the Institutional Review Board at the Cleveland Clinic. All AAA patient samples were collected by venipuncture from pre-operative patients under serial imaging surveillance. For each subject, blood was drawn by a medical professional into citrate plasma tubes, then centrifuged in a tabletop centrifuge at 1100 rpm for 15 mins. Platelet rich plasma (PRP) well above the buffy coat was decanted and the final platelet centrifugation step at 2600 rpm for 5 mins was conducted with a final concentration of 10 μM PGI_2 (in Tris Buffer, pH 9.0). The final washed platelet pellet from one human plasma citrate tube was resuspended in 1000 μL of fresh Tyrode's solution which was diluted 1:10 in fresh Tyrode's solution. This was aliquoted into 100 μL platelet samples before reducing SDS-PAGE and Immoblotting as described by us previously [138, 139]. Blocking buffer was 3% bovine serum albumin/Tris-buffered saline–Tween 20 for 60 minutes at room temperature with agitation. TBST-T Primary anti-GPVI antibody (ABCAM, # ab129019) was used in a 1:4000 dilution in 3% bovine serum albumin/Tris-buffered saline–Tween

20 for 12 hours at 4 degrees C with agitation. GAPDH antibody (Cell Signaling Technology #5174S) was used in a 1:000 dilution in 3% bovine serum albumin/Tris-buffered saline–Tween 20 for 12 hours at 4 degrees C with agitation. Secondary antibody (GE Healthcare, Buckinghamshire, UK) was used in a 1:2000 titer in 5% milk/Tris-buffered saline–Tween 20 for 1 hour at room temperature with agitation. Final autoradiographic films (Bioblot BXR, Laboratory Product Sales, Rochester NY) were quantified by densitometry using ImageJ software (National Institutes of Health).

Platelet surface GPVI Quantification by Flow Cytometry:

Isolated 100 µL platelet samples were incubated with 1 µL of labeled GPVI antibody (BD Biosciences, Clone HY101**,** #565241 in the dark for 30 minutes.) This reaction was then stopped by adding 100 µL of 2% formalin to each reaction, and then quantification of platelet surface GPVI was made possible using an Accuri C6 Plus Flow Cytometer (BD Biosciences) at 10K events per sample. Data was then processed through FloJo (Ashland, Oregon). Platelet surface GPVI was quantified using the geometric mean.

Time-course study:

Ldlr$^{-/-}$ mice were fed a Western diet for 1 week prior to and throughout AngII infusion for 2, 4, 7, and 28 days (n = 20 each time point). In vivo platelet and macrophage labelling and ex vivo examination are detailed in the online supplement.

Intervention study:

Ldlr$^{-/-}$ mice (n = 60) were fed a Western diet for 1 week prior to and throughout a 28 day AngII infusion. Mice were stratified by aneurysm size, via ultrasonography, into 4 groups with equivalent aortic diameter: placebo Clopidogrel bisulfate (50 mg/kg

cellulose filling in diet), Clopidogrel bisulfate (50 mg/kg Clopidogrel in diet), placebo ASA (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, certain mice were retro-orbitally injected (daily) with an anti-GPIX mouse antibody (700 nm). One day prior to sacrifice, all mice were retro-orbitally injected with a solution of dextran-coated nanoparticles (800 nm). For MMP analysis, mice were retro-orbitally injected with a MMP-sense solution (680 nm, 150 nmol/kg body weight, Perkin Elmer), one day prior to sacrifice, as described previously [140]. Aortas were imaged and quantified as described in the online supplement.

ELISAs and cytokine array:

The following commercially available kits were used: mouse PF4 (R&D systems), thromboxane B2 (Cayman Chemical), thrombin-antithrombin Enzygnost micro kit (Dade Behring/Siemens), MMP-2 and MMP-9 Biotrak Activity Assay System (GE Healthcare).

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.

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 previously

described [12]. Pump implantation for the intervention study is described in an earlier section.

Bone marrow transplantation:

This procedure was performed as described previously [177]. Irradiated mice were re-populated with bone marrow (2 x 10^6 cells per animal) harvested from *Par4*$^{+/+}$ (n = 17), *Par4*$^{-/-}$ (n = 16), *P2Y*$_{12}$$^{+/+}$ (n = 16), or *P2Y*$_{12}$$^{-/-}$ donor mice via retro-orbital injection. Mice were allowed to recover for 4 weeks and were then fed diet and infused with AngII, as referenced in the previous 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 [141].

Aortic tissue and plasma collection:

Twenty-eight days after pump implantation, mice were terminated, and blood was drawn from the inferior vena cava and plasma processed as described previously [178, 179]. Aortas were perfused with saline, extracted, and placed into formalin (10% wt/vol) until dissection.

Measurements of abdominal aortic diameters:

Abdominal aortas were visualized with high-frequency ultrasound (Vevo 2100, VisualSonics, Toronto, ON, Canada) on day 0 and 27, as described previously [9, 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.

Plasma lipid analysis:

Mouse plasma lipids were analyzed with the following commercially kits: total plasma cholesterol (Total Cholesterol E), triglycerides (L-Type TG M), and HDL-C (L-Type HDL-C) from Wako Chemicals (Richmond, VA). LDL-C was calculated using the Friedewald equation. 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. Values of $P < 0.05$ were considered statistically significant.

Study approval:

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

Acknowledgements

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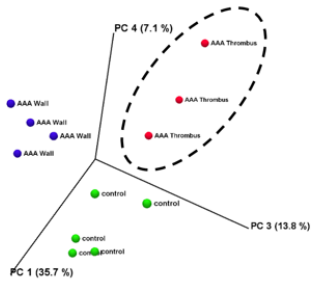
A.



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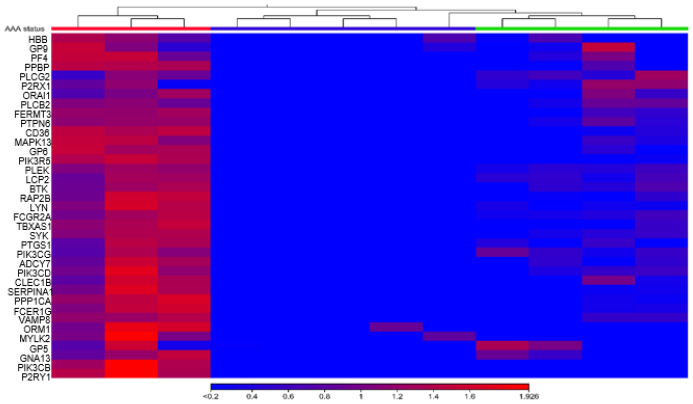

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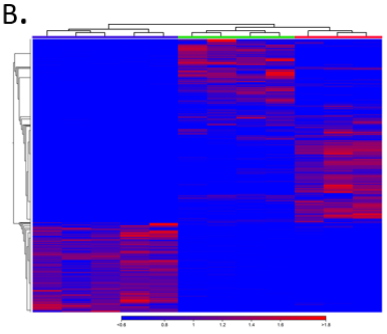
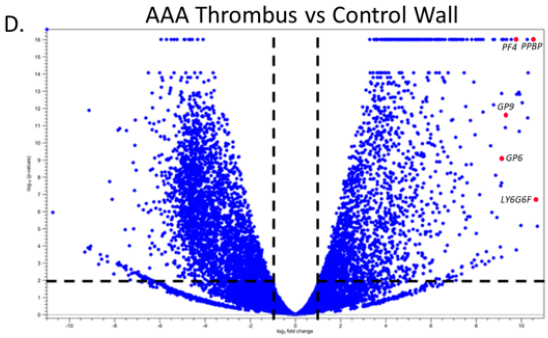


D.



Top 30 Upregulated Genes

E.

Gene symbol	Gene name	Log_2 fold change	FDR p-value
LY6G6F	**Lymphocyte antigen 6 family member G6F**	**10.72**	**7.55E-06**
PPBP	**Pro-platelet basic protein**	**10.54**	**8.53E-15**
ADGRE3	Adhesion G protein-coupled receptor E3	10.3	8.53E-15
HBM	Hemoglobin subunit Mu	10.28	3.67E-12
MMP12	Matrix metalloproteinase 12	10.26	8.53E-15
HBB	Hemoglobin subunit beta	10.03	4.78E-13
CLC	Charcot-Leyden crystal galectin	9.99	6.45E-06
HBA1	Hemoglobin subunit alpha 1	9.93	1.53E-13
TMEM40	Transmembrane protein 40	9.91	2.28E-11
HBA2	Hemoglobin subunit alpha 2	9.89	1.18E-13
CXCL5	CXC chemokine ligand 5	9.79	8.53E-15
PF4	**Platelet factor 4**	**9.76**	**8.53E-15**
CXCR1	C-X-C motif chemokine receptor 1	9.55	8.53E-15
GP9	**Platelet glycoprotein IX**	**9.3**	**1.36E-11**
SLAMF9	SLAM family member 9	9.22	8.53E-15
CXCR2	C-X-C motif chemokine receptor 2	9.15	8.53E-15
CLEC1B	**C-Type lectin domain family 1 member B**	**9.15**	**8.49E-10**
DEFA1	Defensin alpha 1	9.13	1.35E-13
GP6	**Platelet glycoprotein VI**	**9.09**	**3.21E-08**
CABP5	Calcium binding protein 5	8.92	2.20E-06
FCGR3B	Fc fragment Of IgG receptor IIIb	8.82	8.53E-15
HBD	Hemoglobin subunit delta	8.77	6.26E-13
MMP9	Matrix metalloproteinase 9	8.75	8.53E-15
HEMGN	Hemogen	8.69	1.16E-06
TCL1A	T Cell leukemia/lymphoma 1A	8.65	1.56E-10
HCAR3	Hydroxycarboxylic acid receptor 3	8.31	8.53E-15
CCR3	C-C chemokine receptor type 3	8.31	1.71E-12
SPP1	Secreted phosphoprotein 1	8.1	8.53E-15
PF4V1	**Platelet factor 4 variant 1**	**7.57**	**1.90E-09**
ITGA2B	**Integrin subunit alpha 2b**	**7.54**	**7.92E-13**

Figure 1: *Platelet-specific gene expression is upregulated in human AAA thrombi.* RNA recovered from age-matched control aortic wall (n = 5), AAA aortic wall (n = 4), and AAA thrombi (n = 3) underwent next generation RNA sequencing. (A) Represents the principal component analysis (PCA) of the RNA-seq results from control wall, AAA wall, and AAA thrombus samples. (B) Heat map of gene expression differences in control wall, AAA wall, and AAA thrombus samples. (C) Venn diagram showing overlap in the number of transcripts with significant differences in all three examined groups. (D) Volcano plot showing total fold change and p-value of significant AAA thrombus genes versus control wall, with highlighted platelet-specific genes (red dots). (E) Heat map of recognized platelet genes in all three examined groups. (F) A list of the top 30 significantly enriched genes in AAA thrombus versus control wall. Platelet-associated genes are bolded.

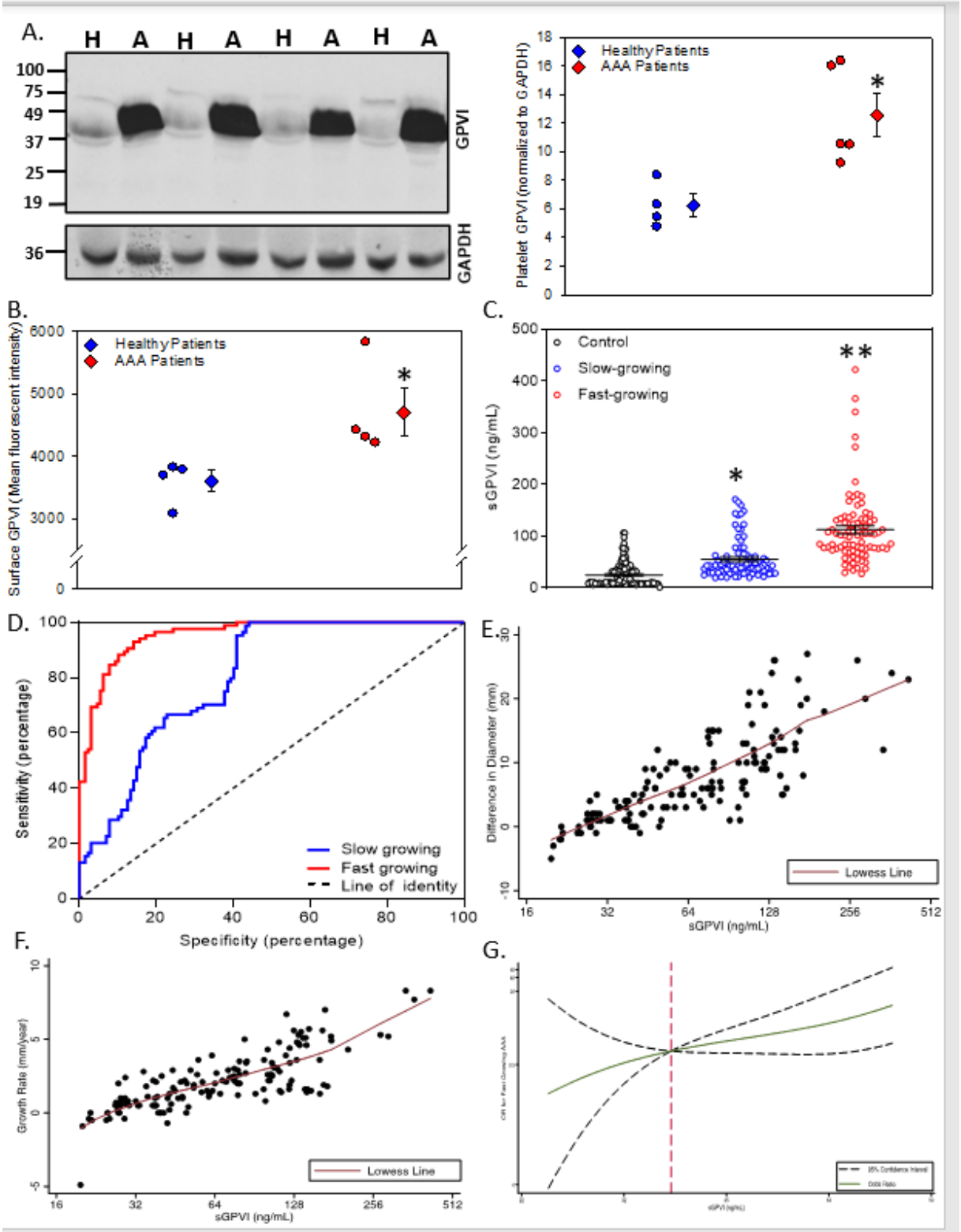

A.
H A H A H A H A
100
75
49
37
25
19
36
GPVI
GAPDH
Platelet GPVI (normalized to GAPDH)
Healthy Patients
AAA Patients
B.
Surface GPVI (Mean fluorescent intensity)
Healthy Patients
AAA Patients
C.
sGPVI (ng/mL)
Control
Slow-growing
Fast-growing
D.
Sensitivity (percentage)
Specificity (percentage)
Slow growing
Fast growing
Line of identity
E.
Difference in Diameter (mm)
sGPVI (ng/mL)
Lowess Line
F.
Growth Rate (mm/year)
sGPVI (ng/mL)
Lowess Line
G.

Figure 2: *The thrombotic platelet receptor GPVI is increased in human AAA patients.* Platelets from healthy (n = 4) and AAA patients (n = 5) were analyzed for the platelet receptor GPVI. Additionally, plasma was assessed for soluble GPVI (sGPVI) from healthy elderly control patients (n = 115), slow-growing AAAs (< 4mm/yr; n = 83), or fast-growing AAAs (> 4mm/yr; n = 84). Platelets isolated form healthy patients or patients with AAA have (A) increased total GPVI protein expression by Western blot (P = 0.0016 by Student's *t*-test), and (B) by platelet surface flow cytometry (P = 0.028 by Mann-Whitney *U*). (C) Plasma from elderly healthy individuals, slow growing AAAs, or fast-growing AAA was assessed for the soluble platelet GPVI receptor which (D) predicts fast growing AAA with 91% sensitivity, 97% specificity, area under the curve (AUC) of 95% with a cut point of > 49 ng/mL, arrow (P <0.0001 vs healthy). sGPVI is log base-2 transformed for each analysis. (E) Scatterplot and Lowess smoothing line for the relationship between sGPVI and difference in diameter. (F) Scatterplot and Lowess smoothing line for the relationship between sGPVI and growth rate. (G) OR for fast-growing AAA from the 25^{th} to 75^{th} percentiles of log-transformed sGPVI modeled as restricted cubic splines. Referent value is set at the median value of sGPVI for fast-growing AAA. Models are adjusted for age, smoking years, diabetes, CHD, aspirin use, hypertension, and COPD. MFI=mean fluorescence intensity. GAPDH=glyceraldehydephosphate dehydrogenase).

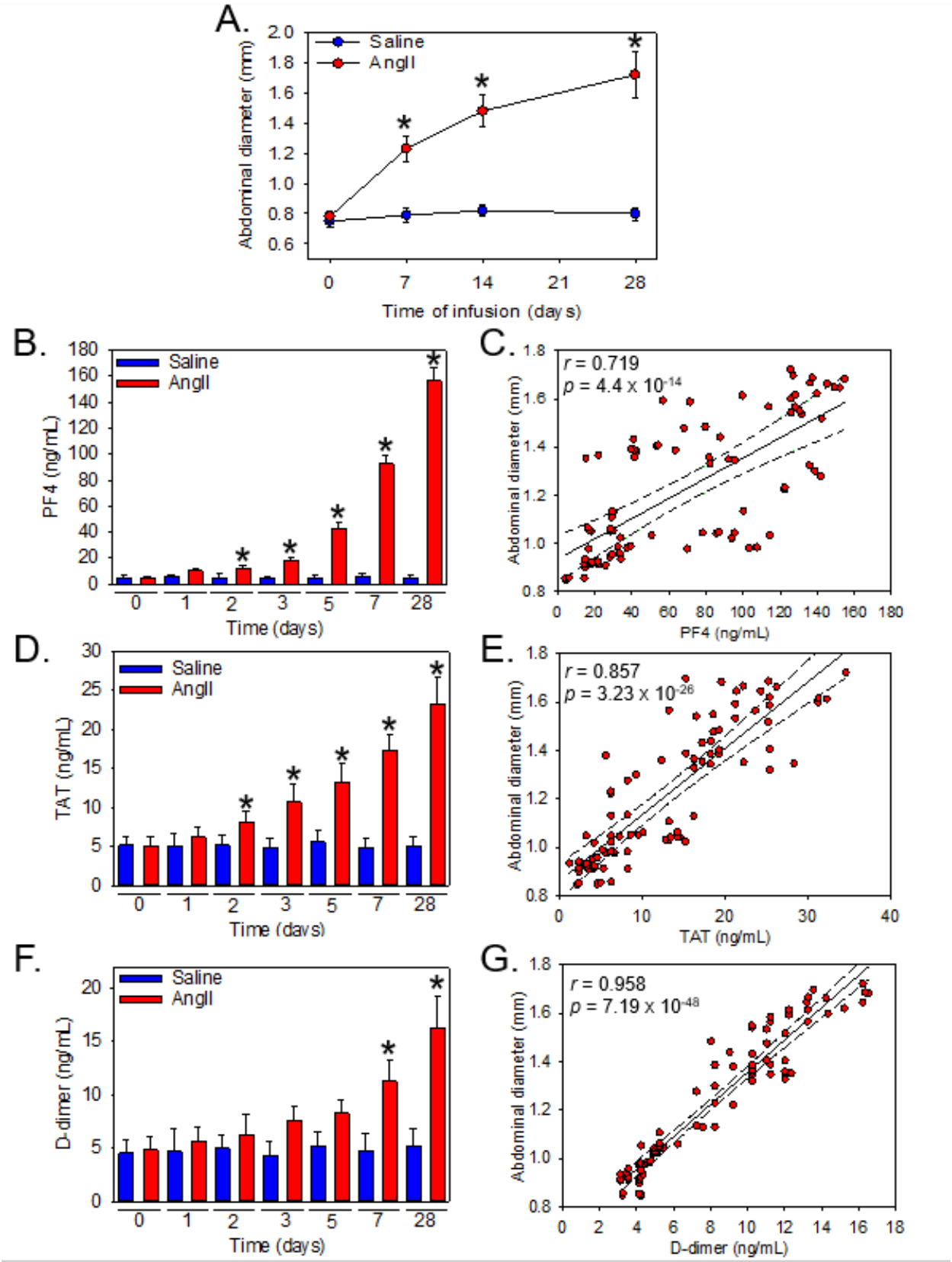

A.
Saline
AngII
Abdominal diameter (mm)
Time of infusion (days)
B.
PF4 (ng/mL)
Time (days)
C.
r = 0.719
p = 4.4 x 10-14
Abdominal diameter (mm)
PF4 (ng/mL)
D.
TAT (ng/mL)
Time (days)
E.
r = 0.857
p = 3.23 x 10-26
Abdominal diameter (mm)
TAT (ng/mL)
F.
D-dimer (ng/mL)
Time (days)
G.
r = 0.958
p = 7.19 x 10-48
Abdominal diameter (mm)
D-dimer (ng/mL)

Figure 3: *AngII induction of AAA augments plasma concentrations of hemostatic proteins and increases abdominal aortic platelet accumulation*. Male *Ldlr*$^{-/-}$ mice (8 – 10 weeks old) were fed a high fat/cholesterol 'Western' diet for 1 week prior to and throughout AngII infusion (1,000 ng/kg/min) for 0, 1, 2, 3, 5, 7, and 28 days (n = 10 – 15 each time-point). (A) Ultrasonically measured maximal luminal diameters of in vivo suprarenal aortas were measured on days 0, 7, 14, and 28. Citrated plasma was collected from the inferior vena cava and (B) platelet factor 4 (PF4, ng/mL), (D) thrombin-antithrombin (TAT, ng/mL), and (F) D-dimer (ng/mL) were measured by commercially available ELISA. In a second cohort of mice fed similar 'Western' diet and similar AngII implantation, platelets (5 days before sacrifice) and macrophages (24 hours before sacrifice) were labelled with anti-GPIX conjugated 700nm fluorophore (red) or and dextran-coated nanoparticles conjugated to DyLight 800 fluorophore (green), respectively. (H) Representative platelet, macrophage, merged, and grayscale images, (I) subsequent quantification. The abdominal aorta within the dotted yellow lines were analyzed for total fluorescent signal in panel I. Histobars represent means ± SEM. (C, E, G) Abdominal diameter was significantly correlated with plasma measures of hemostatic proteins utilizing the Pearson product-moment correlation coefficient ($4.4 \times 10^{-14} < P < 7.19 \times 10^{-48}$). *P < 0.05 versus 0 days, repeated measures ANOVA on ranks with Dunn's post hoc analysis.

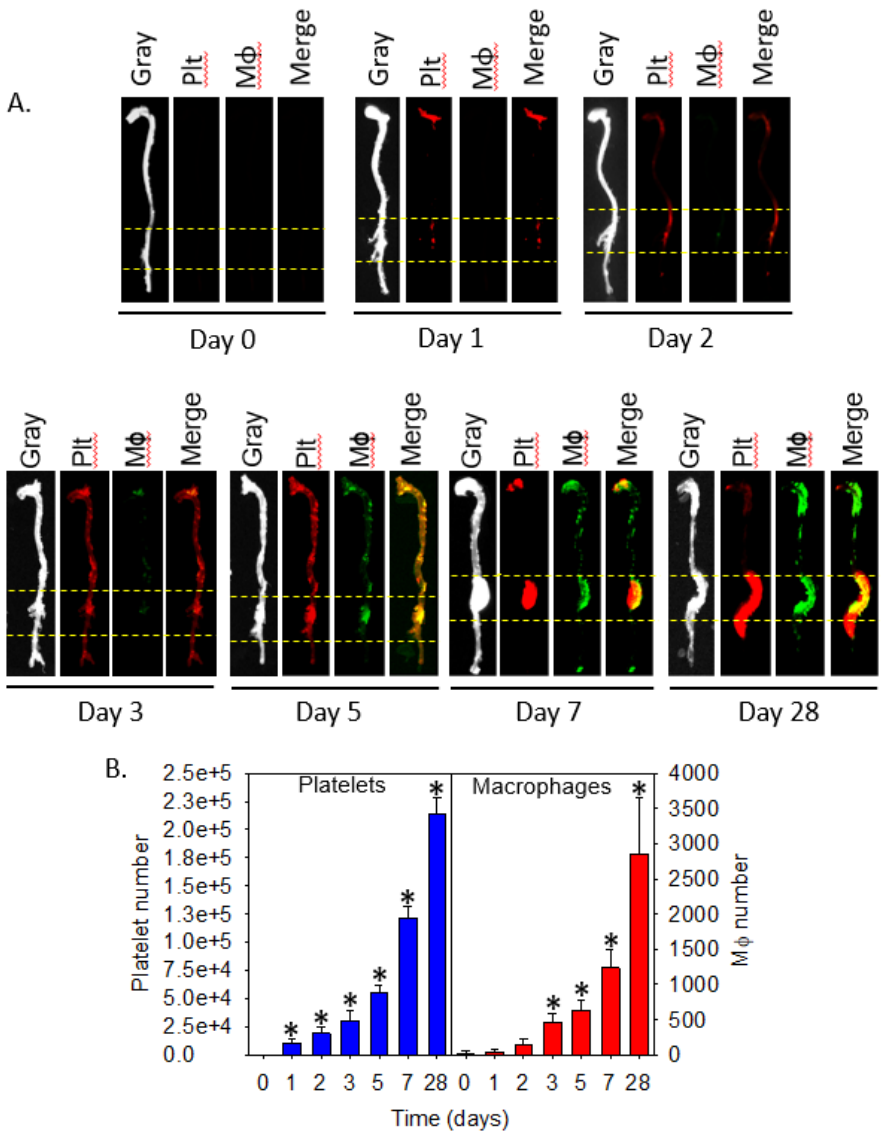


Figure 4: *AngII infusion increases abdominal aortic platelet accumulation.* *Ldlr*$^{-/-}$ mice (male; 8 – 10 weeks old) were fed a high fat/cholesterol 'Western' diet for 1 week prior to and throughout AngII infusion (1,000 ng/kg/min) for 0, 1, 2, 3, 5, 7, and 28 days (n = 10 – 15 each time-point). Platelets (5 days before sacrifice) and macrophages (24 hours before sacrifice) were labelled with anti-GPIX conjugated 700nm fluorophore (red) or and dextran-coated nanoparticles conjugated to DyLight 800 fluorophore (green), respectively. (A) Representative platelet, macrophage, merged, and grayscale images, (B) subsequent quantification. Histobars represent means ± SEM of 10 mice. The abdominal aorta within the dotted yellow lines were analyzed for total fluorescent signal in panel B. *P < 0.01 placebo versus treatment groups. Data were analyzed with a One Way ANOVA on Ranks with Dunn's post hoc.

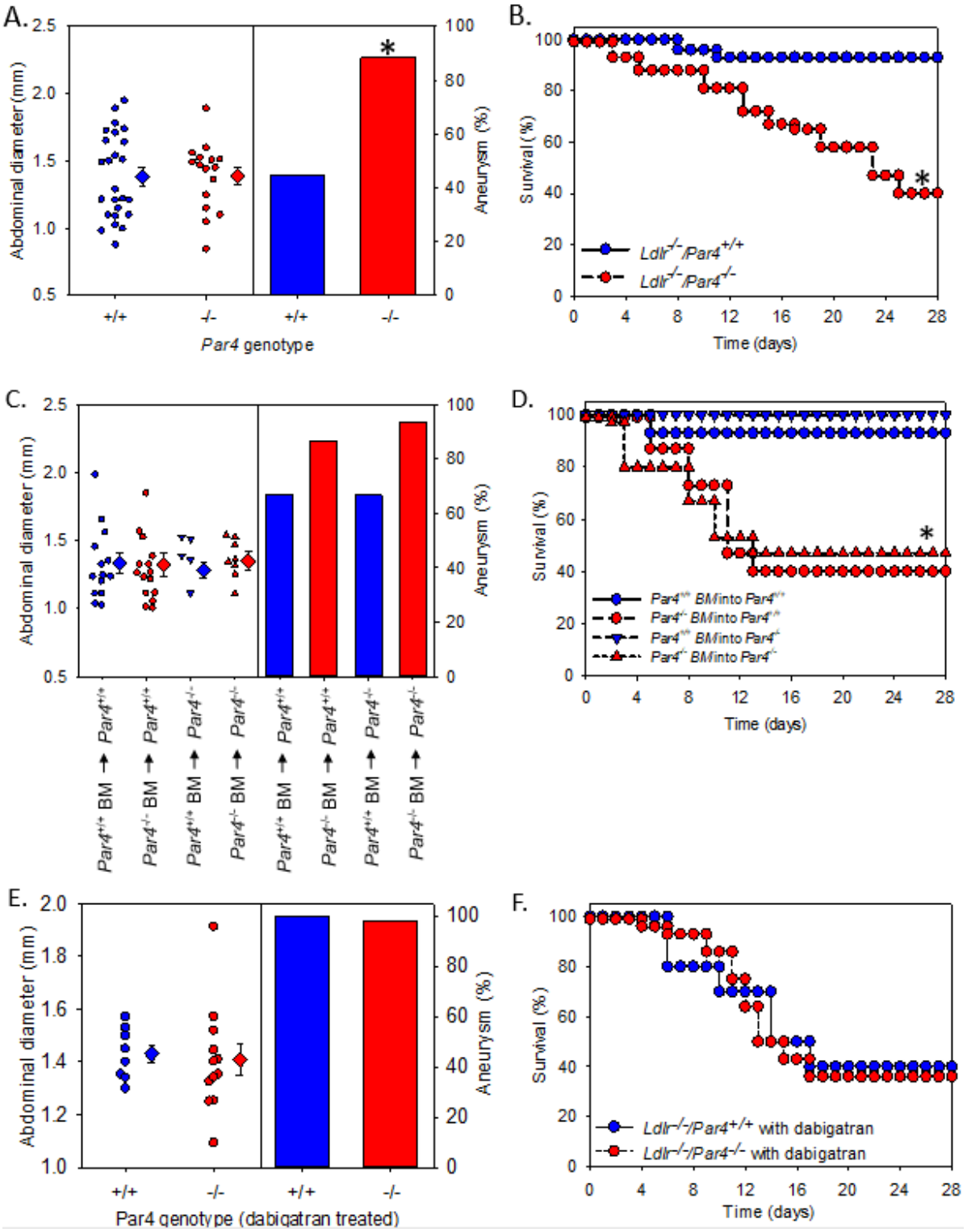

A.
Abdominal diameter (mm)
Aneurysm (%)
Par4 genotype
+/+
-/-
+/+
-/-
*
B.
Survival (%)
Time (days)
Ldlr-/-/Par4+/+
Ldlr-/-/Par4-/-
*
C.
Abdominal diameter (mm)
Aneurysm (%)
Par4+/+ → Par4+/+
Par4-/- → Par4+/+
Par4+/+ → Par4-/-
Par4-/- → Par4-/-
Par4+/+ BM
Par4-/- BM
D.
Survival (%)
Time (days)
Par4+/+ BM into Par4+/+
Par4-/- BM into Par4+/+
Par4+/+ BM into Par4-/-
Par4-/- BM into Par4-/-
*
E.
Abdominal diameter (mm)
Aneurysm (%)
Par4 genotype (dabigatran treated)
F.
Survival (%)
Time (days)
Ldlr-/-/Par4+/+ with dabigatran
Ldlr-/-/Par4-/- with dabigatran

Figure 5: *Hematopoietic cell Par4 deficiency augments AAA rupture via thrombin activation*. *Ldlr*$^{-/-}$*/Par4*$^{+/+}$ or $^{-/-}$ male mice (8 – 10 weeks old) were either irradiated and repopulated with *Ldlr*$^{-/-}$*/Par4*$^{+/+}$ or $^{-/-}$ bone marrow (BM; n = 15 each group) and fed a 'Western' diet, or *Ldlr*$^{-/-}$*/Par4*$^{+/+}$ or $^{-/-}$ male mice (+/+: n = 20; -/-: n = 31) were fed a modified 'Western' diet with dabigatran etexilate (10 g/kg diet) for 1 week prior to and throughout AngII infusion (1,000 ng/kg/min) for 28 days. Ultrasonically measured maximal luminal diameters of in vivo suprarenal aortas were measured and AAA incidence determined in irradiated (A) and dabigatran etexilate fed mice (C). Survival curves were also determined in these groups (B, D). Circles represent individual mice, diamonds represent means, and bars represent SEM. Vertical bars represent percent incidence of AAA. *P < 0.001, with both *Par4*$^{-/-}$ chimeras versus *Par4*$^{+/+}$ chimeras. Data was analyzed with a Fisher's exact test or a Kaplan Meier estimator.

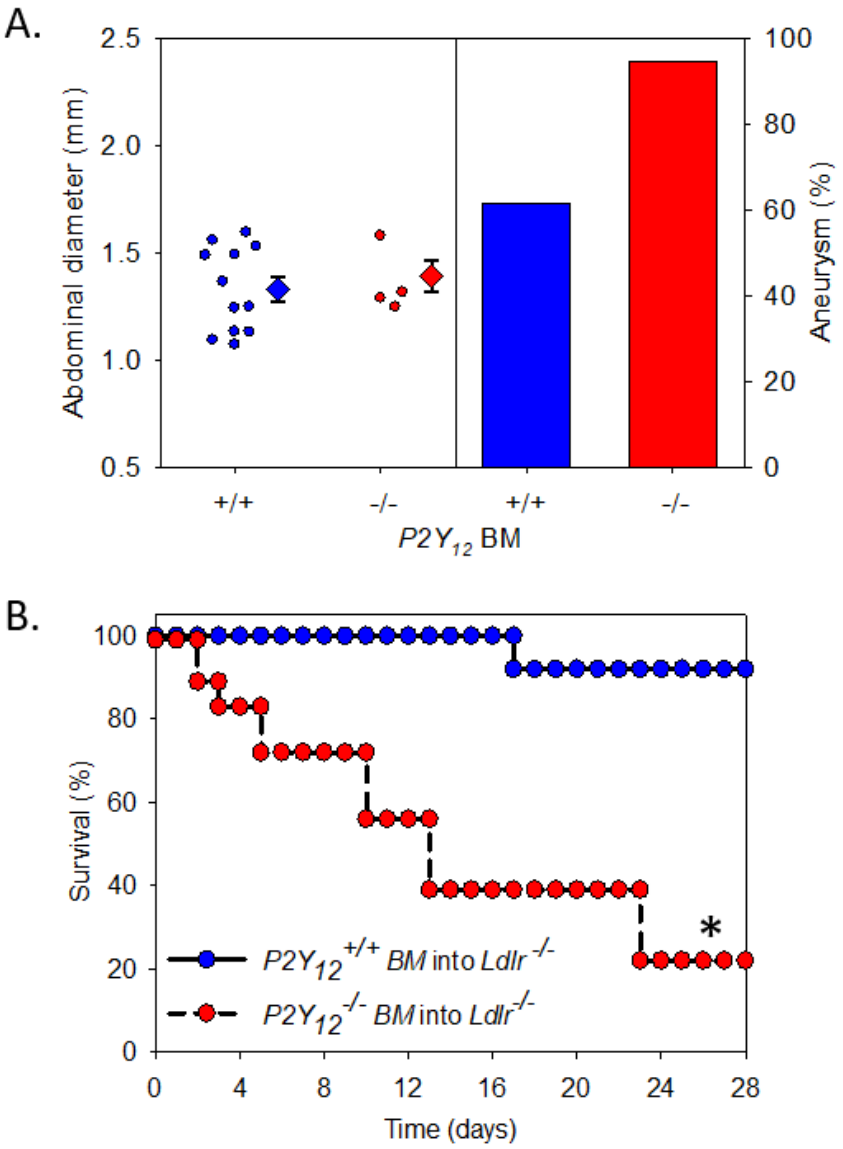


Figure 6: *Genetic platelet dysfunction increases AAA rupture.* Irradiated *Ldlr*$^{-/-}$ mice were repopulated with *P2Y*$_{12}$ (+/+: n = 13, -/-: n = 18) bone marrow (BM), were fed a high fat/cholesterol 'Western' diet for 1 week prior to and throughout AngII infusion (1,000 ng/kg/min) for 28 days. Ultrasonically measured maximal luminal diameters of in vivo suprarenal aortas were measured and AAA incidence determined in *P2Y*$_{12}$ BM (A) mice. Survival curves were also determined in these groups (B). Circles represent individual mice, diamonds represent means, and bars represent SEM. Vertical bars represent percent incidence of AAA. *P < 0.01, versus other genotype. Data was analyzed with a Fisher's exact test or a Kaplan Meier estimator.

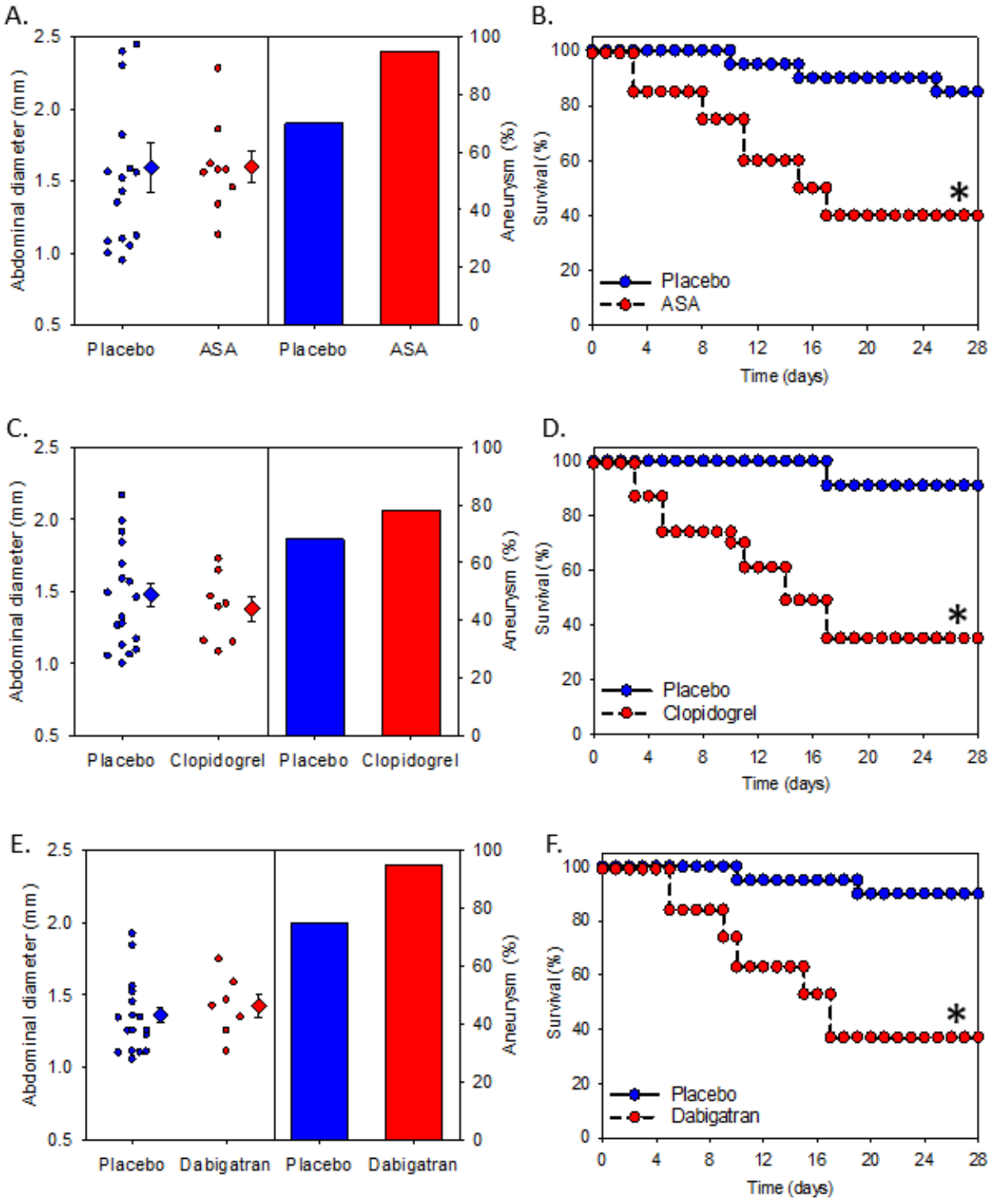


Figure 7: *Inhibition of platelet function increases AAA rupture.* *Ldlr*$^{-/-}$ mice (male; 8 – 10 weeks old) were fed a normal or modified high fat/cholesterol 'Western' diet for 1 week

prior to and throughout AngII infusion (1,000 ng/kg/min) and given placebo or acetylsalicylic acid (ASA) via water (n = 20 each group; started with diet), placebo (n = 22) or Plavix (50mg/kg diet; n = 23) via modified 'Western' diet for 35 days, or placebo (n = 20) or dabigatran etexilate (10 g/kg diet; n = 19) via modified 'Western' diet for 35 days. Ultrasonically measured maximal luminal diameters of in vivo suprarenal aortas were measured and AAA incidence determined in ASA (A), Plavix (C), and dabigatran etexilate (E) treated mice. Survival curves were also determined in these groups (B, D, F). Circles represent individual mice, diamonds represent means, and bars represent SEM. Vertical bars represent percent incidence of AAA. *P < 0.01 treatment groups versus controls. Data was analyzed with a Fisher's exact test or a Kaplan Meier estimator.

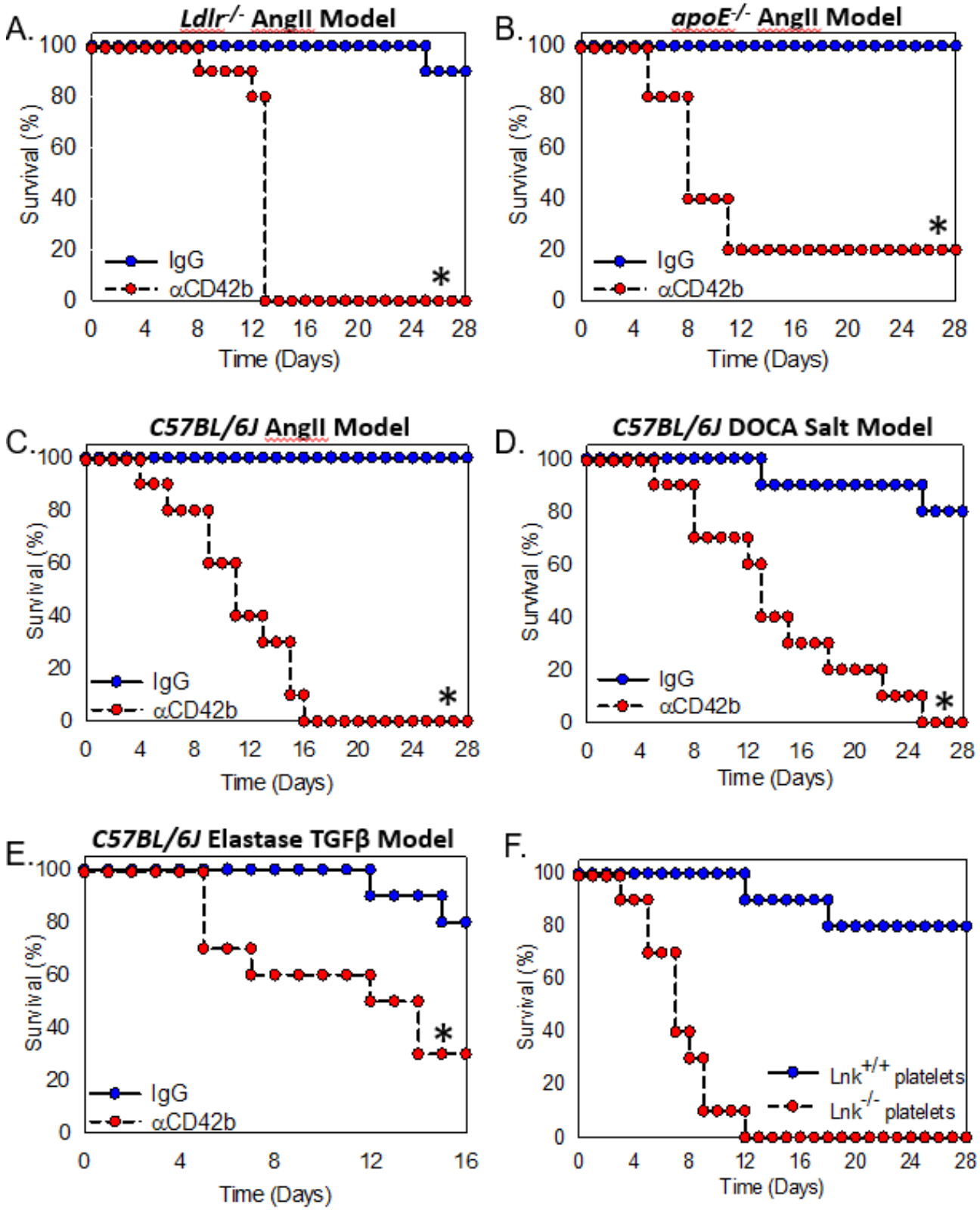

A.
Ldlr-/- AngII Model
Survival (%)
Time (Days)
IgG
αCD42b
*
B.
apoE-/- AngII Model
Survival (%)
Time (Days)
IgG
αCD42b
*
C.
C57BL/6J AngII Model
Survival (%)
Time (Days)
IgG
αCD42b
*
D.
C57BL/6J DOCA Salt Model
Survival (%)
Time (Days)
IgG
αCD42b
*
E.
C57BL/6J Elastase TGFβ Model
Survival (%)
Time (Days)
IgG
αCD42b
*
F.
Survival (%)
Time (Days)
Lnk+/+ platelets
Lnk-/- platelets

Figure 8: *Platelet depletion augments rupture-induced death in several models of aneurysm formation*. Male (A) *Ldlr*$^{-/-}$, (B) *apoE*$^{-/-}$, and (C – E) *C57BL/6J* mice (8 – 12 weeks old; D – 10 months old) were intraperitoneally (I.P.) injected with either non-immune rat IgG or αCD42b platelet depletion antibody (5μg/g body weight, every three days) and (A) fed chow or (B, C) a high fat/cholesterol 'Western' diet for 1 week prior to and throughout AngII infusion (1,000 ng/kg/min) for 28 days, (D) implanted with DOCA pellets (50mg 21-day release) and salt (0.9% NaCL plus 0.2% KCl) in drinking water, or (E) had their infrarenal aortas treated topically with elastase (10μl 100%) with anti-TGFβ injections (10mg/kg; 3 times/week, I.P.). (F) *Ldlr*$^{-/-}$ mice were platelet depleted and then adoptively transferred with lymphocyte adapter protein (*Lnk*)$^{+/+}$ or $^{-/-}$ platelets, fed a 'Western' diet and infused with AngII. Survival curves are represented. *P < 0.05; data was analyzed with a Kaplan Meier estimator.

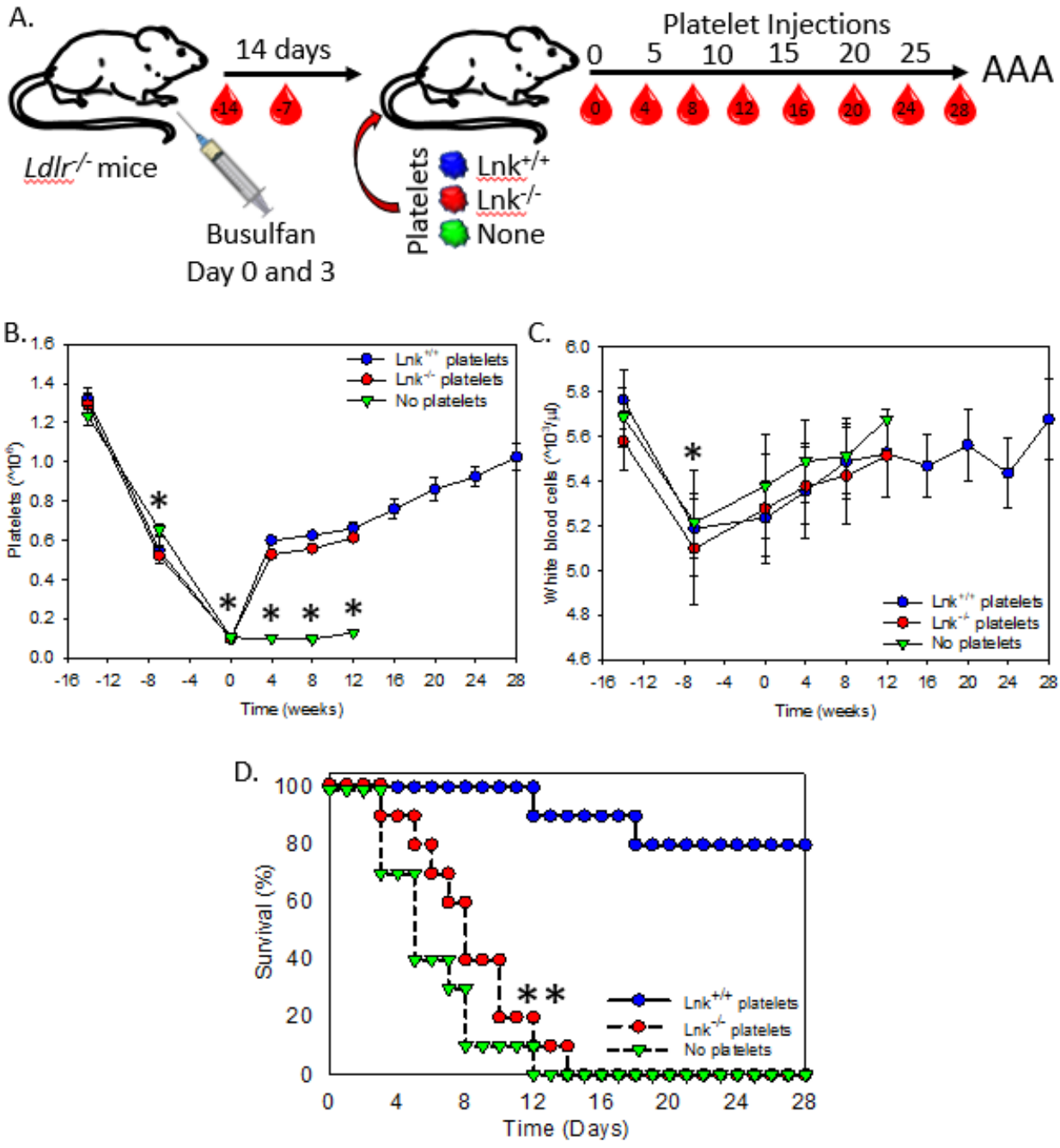


Figure 9. *Lnk deficient platelets augments rupture-induced death.* (A) Schematic representation of chronic platelet depletion with busulfan and reconstitution with Lnk+/+, Lnk-/-, or no platelets. Platelet depleted male Ldlr-/- mice were infused with Lnk+/-, -/-, or no platelets every 5 days, starting at day 0 with implantation of AngII pumps (1,000 ng/kg/min). Blood was drawn at various timepoints to examine (B) Platelets and (C) white blood cells. (D) Representative survival curve of 28 day AngII infusion experiment. *P < 0.05 – data analyzed by Repeated Measures ANOVA; **P < 0.05 – data analyzed by Kaplan Meier estimator.

Table 1. Participant characteristics by case definition (n=264)			
	Control (n=115)	Slow Growing (n=75)	Fast Growing (n=74)
Characteristic			
Age (years)	67.4 (2.6)	72.7 (6.6)	72.6 (6.8)
Height (cm)	177.1 (10.3)	181.8 (6.2)	180.7 (6.2)
Weight (kg)	84.1 (13.6)	90.2 (15.7)	89.3 (16.2)
CHD	10 (8.7)	27 (36.0)	25 (33.8)
Hypertension	56 (48.7)	51 (68.0)	44 (59.5)
CVD	5 (4.4)	18 (24.0)	7 (9.5)
Claudication	1 (0.9)	8 (10.7)	1 (1.4)
COPD	5 (4.4)	12 (16.0)	11 (14.9)
Renal Insufficiency	2 (1.7)	4 (5.3)	4 (5.4)
Diabetes	17 (14.8)	17 (22.7)	4 (5.4)
Aspirin Use	17 (14.8)	37 (49.3)	33 (44.6)
Statin Use	30 (26.1)	35 (46.7)	27 (36.5)
Current Smoker	6 (5.2)	12 (16.0)	25 (33.8)
Smoking Years	23.4 (13.7)	34.2 (13.0)	39.4 (14.3)

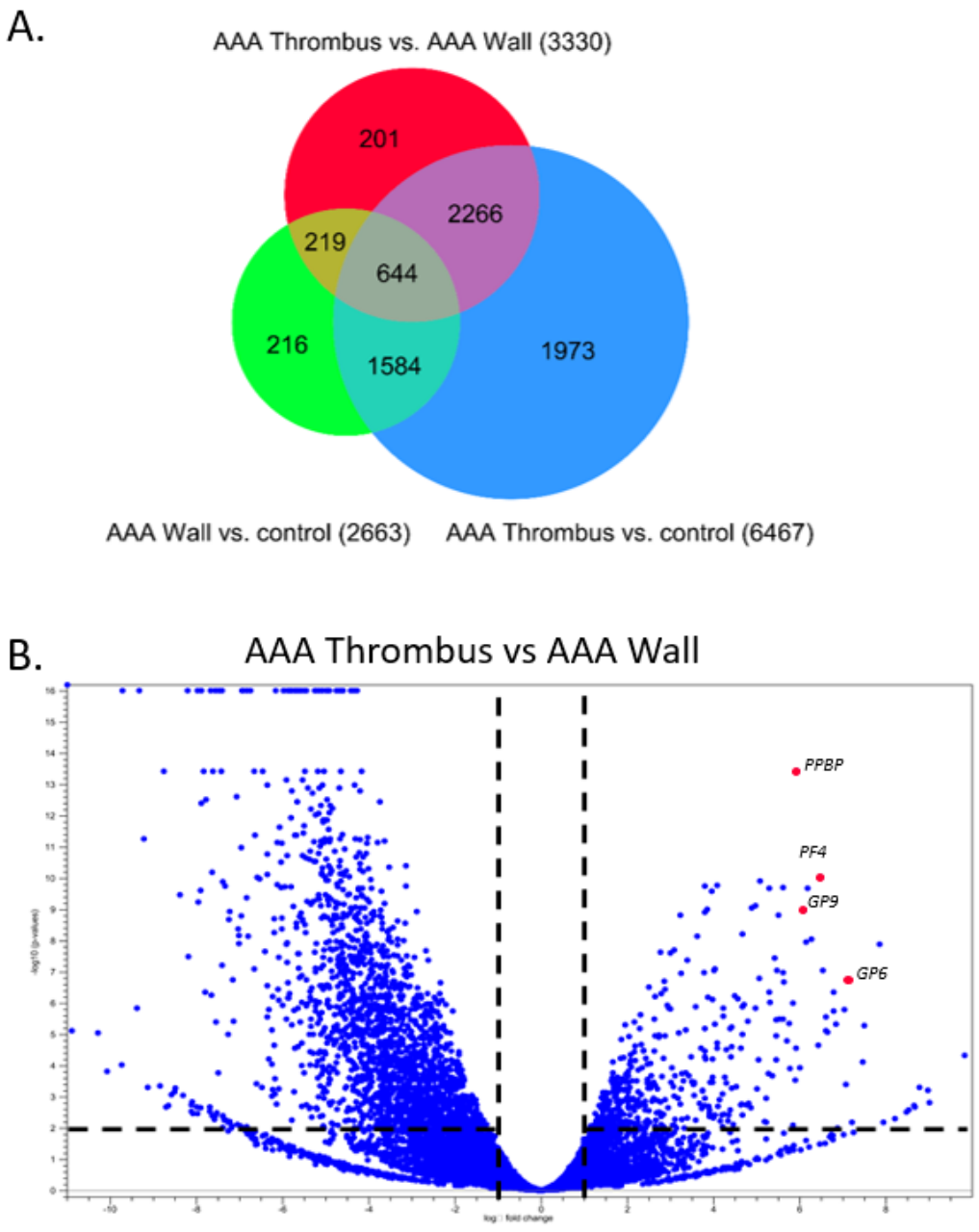
Note: Values are mean (SD) or N (%)

Abbreviations: S.D. standard deviation; sGPVI, soluble glycoprotein VI; cm, centimeters; kg, kilograms; ng, nanograms; mL, milliliters; CHD, coronary heart disease; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease,

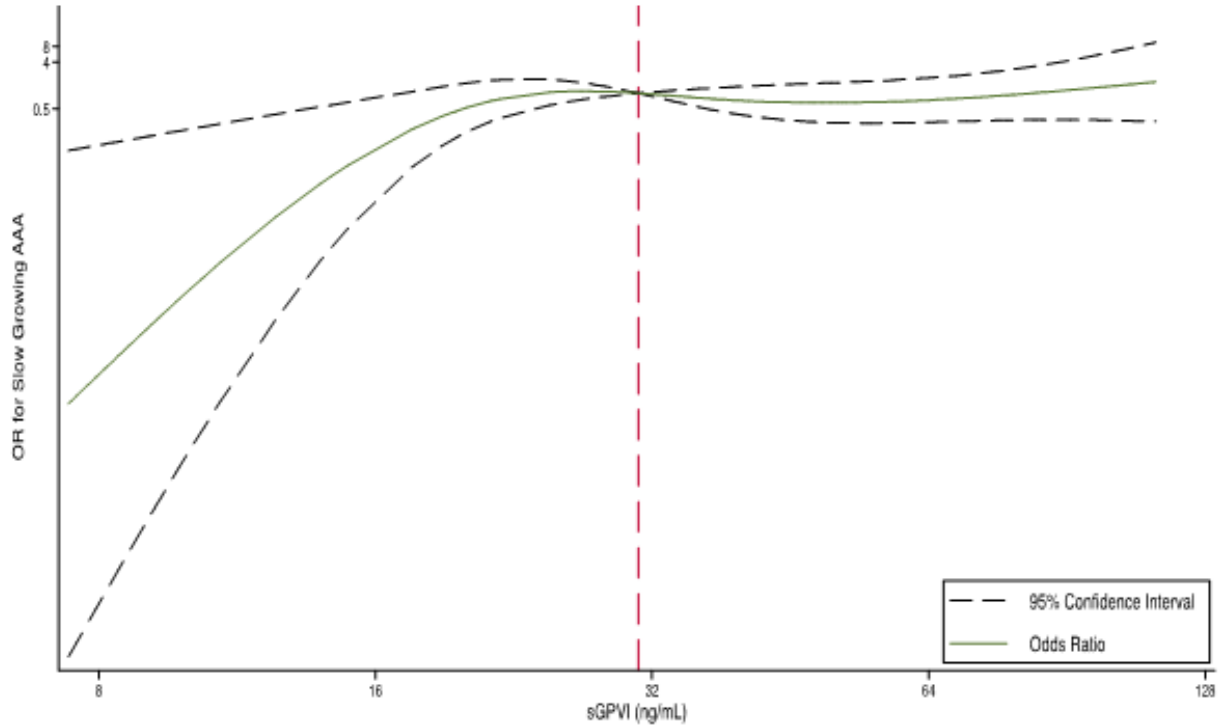
Table 2. OR by case definition	Control (N)	Slow AAA (N)	Fast AAA (N)	OR (95% CI)	Adjusted OR (95% CI)
Continuous					
sGPVI, per doubling	-	-	-	4.8 (3.5, 6.5)	4.1 (3.0, 5.8)
Dichotomous					
<38 ng/mL	92	31	4	1.0 (ref)	1.0 (ref)
≥38 ng/mL	23	44	70	16.0 (9.1, 28.3)	11.6 (6.2, 21.9)

Note: Odds ratios obtained from ordinal logistic regression; continuous sGPVI is log transformed; adjusted for age, smoking years, CHD, diabetes, aspirin use, hypertension, and COPD

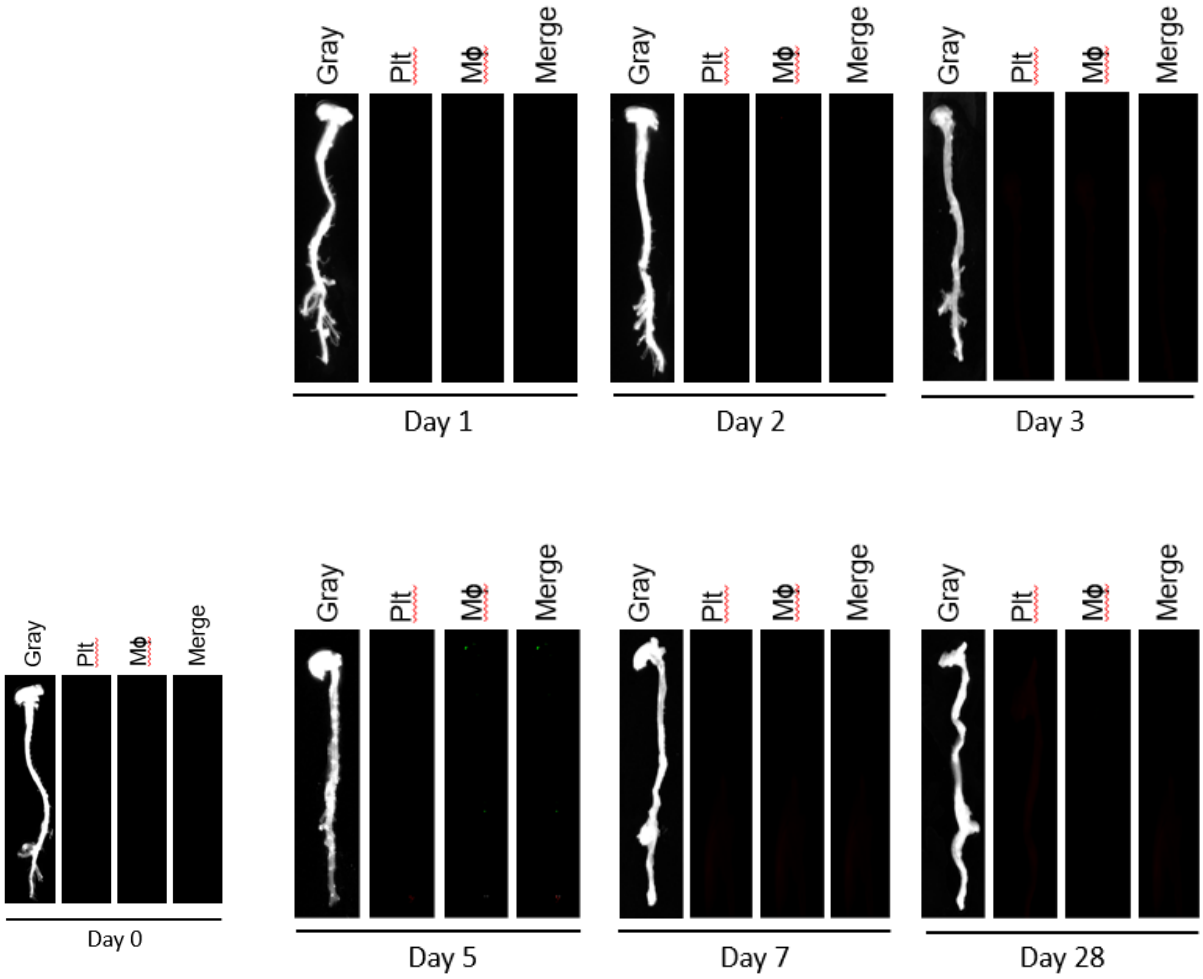
Abbreviations: C.I. confidence interval; O.R. odds ratio; sGPVI, soluble glycoprotein VI; C.H.D. coronary heart disease



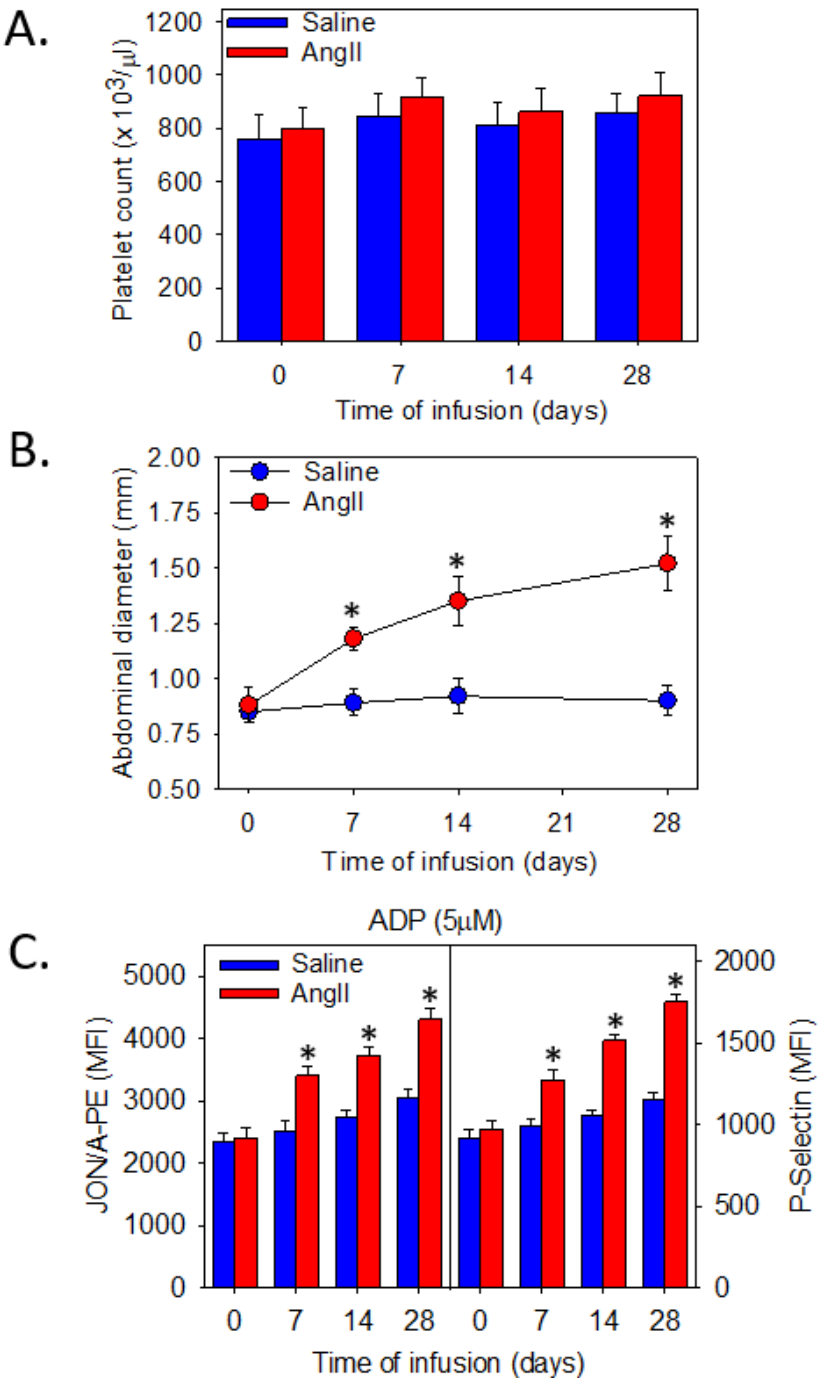
Supplementary Figure 1. Subset Comparisons of AAA thrombus and wall vs. controls



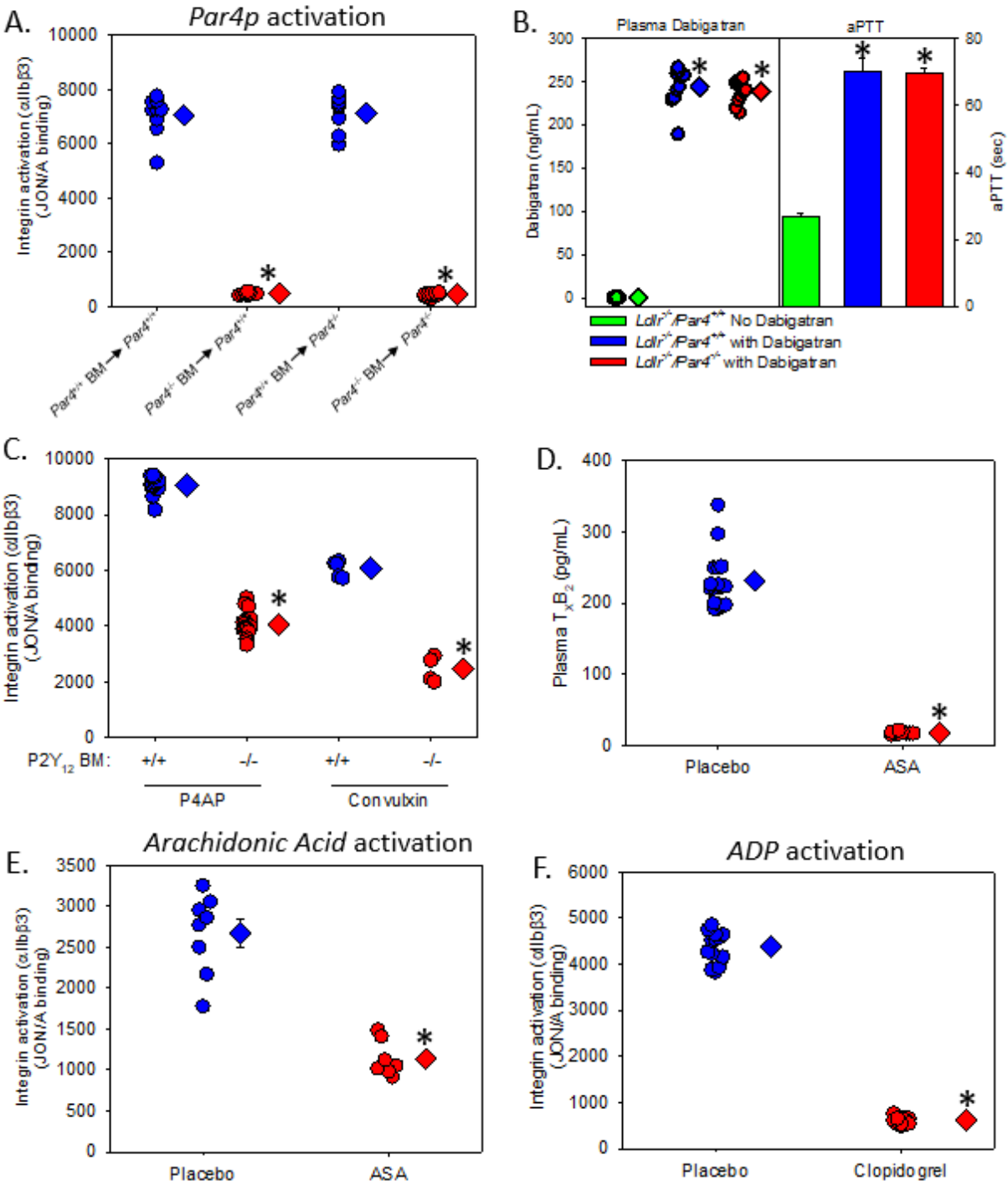
Supplementary Figure 2. Lowess Curve analysis of sGPVI Odds ratio in human AAA patients according to case status.



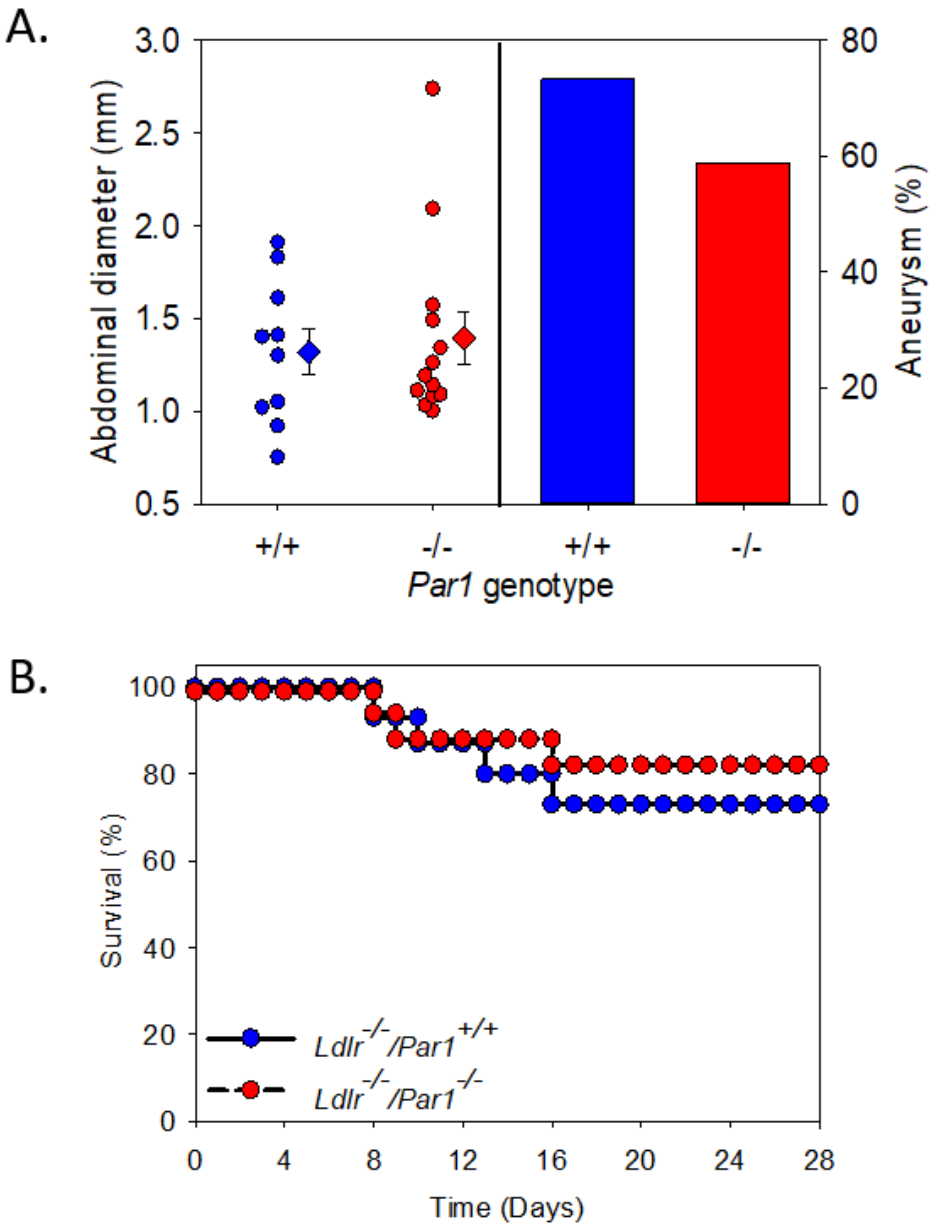
Supplementary Figure 3. Control injections with IgG and nanoparticle beads followed by AngII infusion.



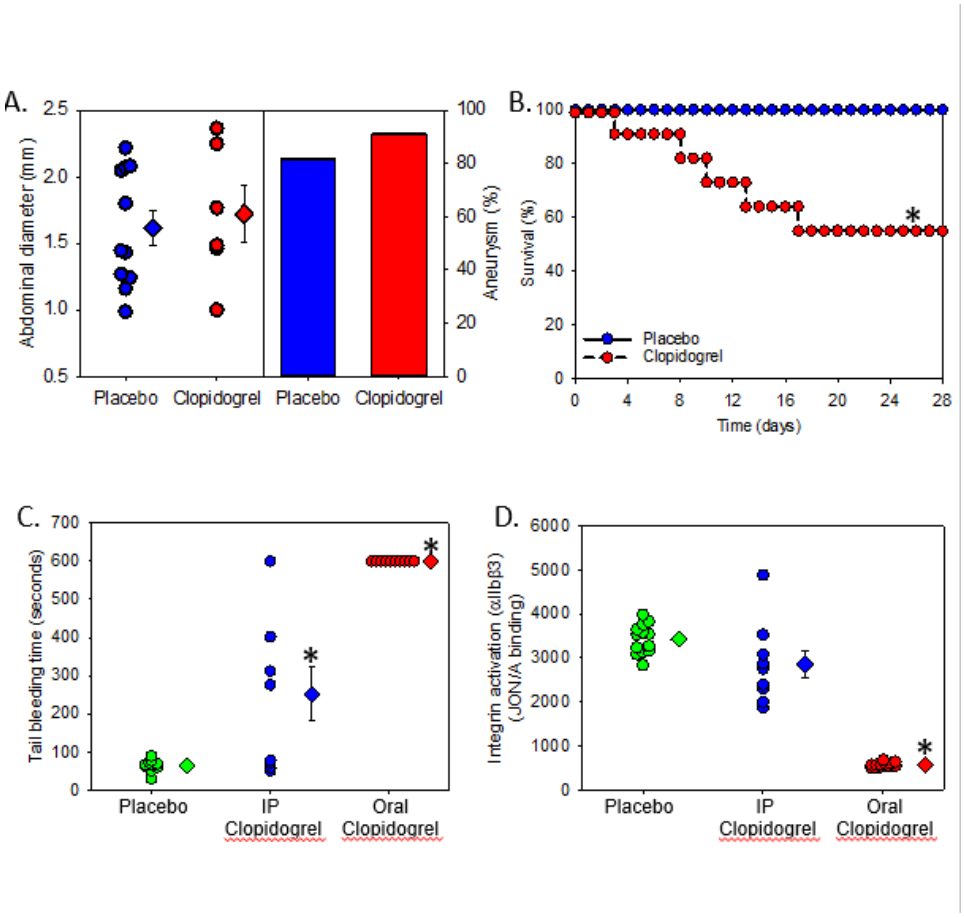
Supplementary Figure 4. Platelet responsiveness after AngII infusion



Supplementary Figure 5. Platelet aggregation of genetically deficient or pharmaceutically inhibited mice



Supplementary Figure 6. AngII-induced aneurysm in mice with genetic deficiency of Par1 compared to WT



Supplementary Figure 7. Analysis of clopidogrel response from oral and IP delivery route.

CHAPTER 5

5.1 Discussion

5.1.1 Proposed Mechanisms

It is known that macrophages are early responders to the site of elastin injury in aneurysm formation. [52] What this dissertation seeks to add to this understanding, is that platelets are earlier even in their response to the aneurysm formation. Furthermore, our data suggest that platelets are absolutely critical to the stability of an aneurysm in early development, with platelet impairment and depletion both being associated with significant amounts of rupture-related death in animal models of AAA. These results were replicated in many animal models of AAA, including models with mural thrombi and intraluminal thrombi, as well as low-penetrant models of AAA, and models that did not require hypercholesterolemia. These data suggest that the action of platelets are crucial to aneurysmal stability in early aneurysm.

One likely explanation for this can be found in the biomechanical stabilization that is presented by the ILT in aneurysm. The aneurysmal aorta presents an environment of structural stability for several reasons. As mentioned in this dissertation previously, the aneurysmal wall shows disorganized an extracellular matrix, as well as apoptosis and an associated degradation of the wall stability. [180] Furthermore, as aneurysmal dilation is localized, the widening of the aorta creates an unstable flow environment. [181] As blood flow proceeds from the relatively narrow healthy aorta into the dilated diseased aorta, and then back to the relatively narrow healthy aorta, the biomechanical flow is turbulent. Furthermore, LaPlace's law dictates that not only is the wall thickness important for

reducing wall stress, but also that the stress in a cylinder is notably less than that of a sphere, proportional to its radius [181]. Thus, initially, the ballooning of the aorta helps to alleviate the stress on the wall, but as the aneurysm progresses, returning to a cylindrical shape with a smaller radius results in less net wall stress. Also, in a spherical vessel, the wall thickness further decreases the tension on the wall and overall stress. As platelets facilitate the construction of an intraluminal thrombus, the overall stress is greatly alleviated. This is also supported by the finding that the majority of ILTs are found to be localized at the point of maximal dilation, [59, 60] though some data argues that presence or absence of an ILT is not relevant to rupture risk. [182]

Unfortunately, as the aneurysm progresses and the ILT grows, the ILT contributes to added extracellular matrix degradation and marked increase in wall hypoxia. It has also been noted that the wall is thinnest under the ILT, as compared to other points in the aneurysm. [180] Therefore, there is interest in determining the net effect of the ILT in aneurysm pathogenesis, and in evaluating the notion of targeting the ILT in aneurysm treatments.

As discussed previously, elevated fibrinogen is an exciting, but as of yet uninvestigated finding of population studies in AAA. [23, 24] First, we demonstrated that general suppression of fibrinogen expression in an AngII model of AAA showed improvement of the AAA phenotype. These results were recapitulated in mice with a genetic knockout of fibrinogen. Then, to identify possible mechanisms for this finding, aneurysm experiments were carried out in mice first with mutations in the fibrinogen γ-chain that are associated with significant defects in the immunomodulatory actions of fibrinogen, *Fibγ$^{390-396A}$.* Finding that there was similar improvement of disease burden in

these mice, we next moved to confirm our hypothesis that this effect was due to the leukocyte binding. As the 390-396 region of the γ-chain has been found to have multiple binding partners, [71] we removed the leukocyte binding partner of this fibrinogen binding region, integrin $\alpha_M\beta_2$, also known as ITGAM, complement receptor 3 (CR-3), or as we refer to it in our papers, Mac-1. Seeing that the results were similar in this model as they were the results of the mice with the mutated γ-chain, supported by mRNA data suggesting decreased inflammation in mice with a mutated γ-chain and in suppression of the whole fibrinogen protein, coupled with convincing RNAseq data suggesting a decrease in immune activation, our proposed mechanism is as follows: impairment of fibrinogen-mediated leukocyte recruitment results in an overall decrease in the inappropriate inflammatory response associated with AAA. Thus, fibrinogen is responsible for driving AAA pathogenesis, in part, by increasing the inflammation at the abdominal region of the aorta.

Our data suggest an active role for fibrinogen in aneurysm progression and initiation, and an important role for platelets in early stabilization of *in vivo* aneurysm models. Prior to our work, there was evidence of a correlation between fibrinogen and abdominal aortic aneurysm, however, no published work had looked into this relationship. In summary of our data, we showed that removal of fibrinogen from the biological system and then induction of abdominal aortic aneurysm results in a significant attenuation of disease burden in mice. We also show that impairment of fibrinogen-leukocyte interactions in the Mac-1 integrin axis result in similar improvement of aneurysm disease. Furthermore, our data show that fibrinogen can be correlated to aneurysm size in humans, and that fibrinogen levels are correlated to growth rate. Mice

with the same Fibrinogen γ-chain mutation have been shown to demonstrate impaired macrophage mobilization and migration, [151] and it has been proposed that macrophages migrate out of the bloodstream and localize to a point of inflammation with the assistance of this binding site on the γ-chain.

As discussed earlier, it is currently theorized that aneurysm begins with some unknown injury to the elastin fibers of the aorta and then inappropriate inflammatory response follows. [183] It is also important to note that, as demonstrated by our work in

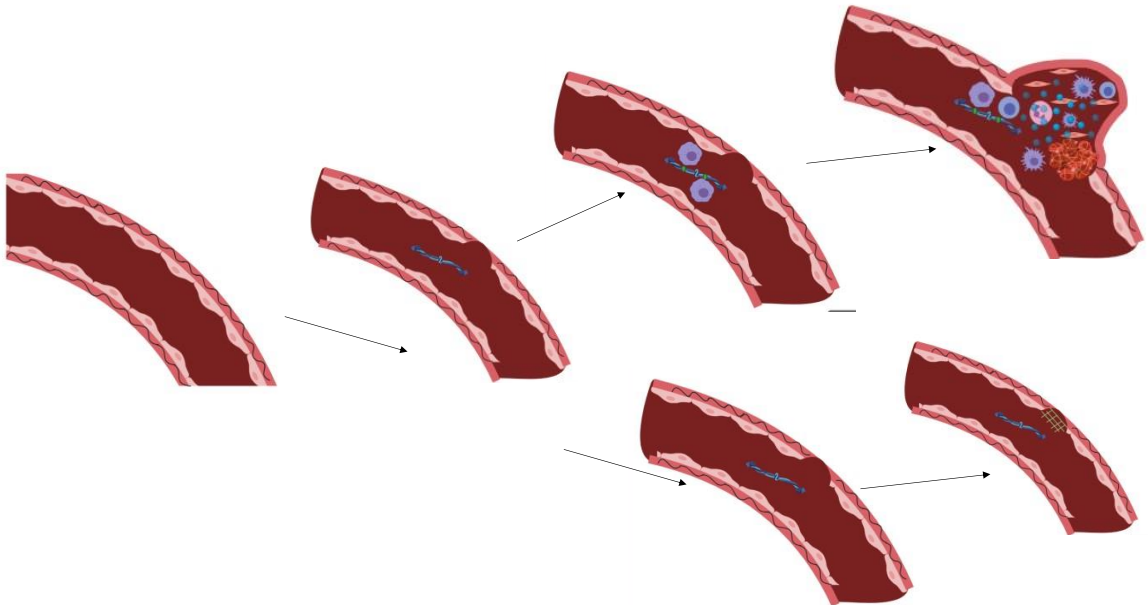


Fig 1 Proposed mechanism of fibrinogen γ contributions to abdominal aortic aneurysm and the accompanying protection of the *Fibγ*$^{390\text{-}296A}$ mutation. In normal AAA conditions, a break in elastin recruits fibrinogen to the site of injury, also recruiting and stabilizing the response of macrophages. These macrophages contribute to the ultimate, inappropriate inflammatory response that leads to aneurysm formation. When mutated fibrinogen is substituted, the site of injury is repaired without the accompanying, inappropriate inflammatory response.

platelets in this dissertation, platelet localization to the site of aneurysm injury occurs prior to that of macrophages.

Taking these findings into consideration, our proposed mechanism for the action of fibrinogen in abdominal aortic aneurysm is as follows: following platelet activation and aggregation at the theorized site of elastin injury, fibrinogen responds. The fibrinogen response after platelet activation helps facilitate leukocyte localization, migration and response at the aneurysm site. Together, platelets, leukocytes and fibrinogen amplify the inflammatory response, releasing cytokines, MMPs and other factors that perpetuate the destruction of the extracellular matrix and structural integrity of the vessel wall, leading to the progression of AAA.

1.1.2 Alternative Explanations and Limitations

Though fibrinogen's contributions to AAA have been largely uninvestigated, one other group has put forth a hypothesis in regards to fibrinogen in AAA. In this paper [184], Pham and colleagues demonstrated that IgG directed against fibrinogen can induce AAA formation in mice, and that depletion of lectin pathway mediators can reduce this AAA formation. In short, these researchers concluded that their data suggested an autoimmune pathway against fibrin(ogen) resulting in AAA. Pham and colleagues ultimately propose a pathway in which a fibrin clot initiates a lectin response, leading to neutrophil activation and AAA pathogenesis. Though similar in being an investigation of fibrinogen in AAA, their approach was fundamentally different than in this thesis, and their findings are not fully consistent with ours. Our preliminary data suggest a much more active role for fibrinogen alone, and demonstrate that AAA formation can be attenuated by depletion of the fibrinogen protein, not that it can be created by creating an improper immune response. We observed significantly higher AAA formation in

control mice compared to fibrinogen-depleted mice, without introducing any kind of anti-fibrinogen antibody. The Pham group does demonstrate the in vivo formation of anti-fibrinogen antibodies in the elastase model. It is possible that mice in our Angiotensin II-(AngII) induced AAA also develop anti-fibrinogen IgG spontaneously, but we find this to be a broad assumption. The elastase model results in an overwhelming immune response that, as demonstrated by Pham, results in antibodies directed against many self-proteins. The AngII-model, however, is an injury model, mediated by macrophages; we would not assume the same aberrant self-antibody formation. Though Pham's group potentially proposes an interesting model for AAA murine studies, we do not believe that their findings invalidate our line of inquiry nor make it redundant.

The work of Pham's group does, however, introduce an interesting question in regards to complement activation in AAA and fibrinogen. They suggest that fibrinogen may act in AAA pathogenesis via the lectin pathway (LP). However, fibrinogen is noted to have roles in hemostasis, multiple pathways of inflammatory response, and wound healing. This thesis did not hone in on the lectin pathway (LP) because we feared it premature to disregard the myriad potential roles for a versatile protein such as fibrinogen. The lectin pathway has been implicated previously in AAA pathogenesis [185], but the literature as a whole does support complement being solely responsible. AAA is believed to be caused by an activation of many inflammatory pathways, several of which fibrinogen is a participant. Our data presented in this dissertation would support the notion that if fibrinogen acts on AAA pathogenesis through the LP, it is not the only mechanism of action, and is unlikely to even be the primary mechanism of action. As such, though Pham's group demonstrated that LP mediators are crucial to AAA, and

that fibrinogen's interaction with the LP is an avenue for consideration, their conclusion that this can be equated to implicating anti-fibrinogen mediated inflammation is not given.

It is important to note that some literature has suggested that platelet inhibition is a viable avenue for aneurysm treatment, where our work here suggests that platelet inhibition results in high fatality. Furthermore, data in regards to the use of anti-platelet therapy in humans is markedly mixed, with some reports suggesting benefit, and others data reporting no effect, and some reporting catastrophic results, especially after EVAR repair. [186] Partially addressing these mixed data, previous work from our group have indicated that platelet inhibition in established mouse aneurysms are, in fact, protective against rupture and may blunt aneurysm growth. [112] This work also shows a modest improvement in aneurysm outcomes when patients are on anti-platelet drugs such as ASA and $P2Y_{12}$ inhibitors.

Taking the data from our previous work with our new data showing platelet protection in early aneurysm, we see a possible explanation for the mixed results of platelet inhibition in humans. Our data demonstrate an overall picture that platelets are early responders to the site of hypothesized pre-aneurysmal injury. In this early phase, the platelets show a stabilizing effect as they carry out hemostatic functions. However, as this thrombus continues to develop, fibrin deposition continues, white blood cells accumulate in the thrombus, and platelets begin to mediate inflammatory processes, contribute to MMP and cytokine release, and the thrombus begins to contribute to worsening wall stability, the overall effect of platelets on aneurysm stability begins to be negative. Since human data is rarely stratified into the stage of the aneurysm, with

some data sets being skewed to advanced aneurysms and others being skewed away from it, and human data in early aneurysm is markedly lacking, the dichotomous effect of platelets throughout the natural history of aneurysm results in a generally mixed view of the effectiveness of platelet inhibition in this disease.

Our work assumes that the effect of platelets is due to biomechanics. However, it is important to consider that platelets are not only biomechanical participants in the overall response of vasculature. Platelets, as mentioned previously, are potent mediators of inflammatory response in their own right and must be considered to be biologically active. One activity of platelets in particular that is important to consider is their contribution to transforming growth factor beta (TGF-β). TGF-β is a cytokine of the transforming growth factor family, which, after forming supercomplexes with other proteins, binds to TGF-β receptors to induce potent immune effects by differentially inhibiting or inducing cell proliferation and differentiation. [187] The first known function of TGF-β is inducing fibroblast adhesion during wound healing, [188] but over the years, TGF-β has been implicated in growth inhibition of epithelial cells, and in the general regulation of tissue remodeling. [189] Importantly, platelets have been identified as the main source of TGF-β in circulation. [190] Furthermore, TGF-β has been recognized to be crucially important in protecting against aneurysm formation in mice. Mallat and colleagues demonstrated that neutralization of TGF-β in normocholesterolemic C57Bl/6 mice negates resistance to AngII-induced aneurysm formation. In fact, after neutralization of TGF-β, these mice are comparably susceptible to aneurysm as $LDLR^{-/-}$, HFD-fed mice in the AngII-induced model. Further studies demonstrated that MMP-12

depletion could partially rescue the phenotype, suggesting that TGF-β is a crucial regulator of the anti-inflammatory responses that help stabilize aneurysm. [191]

Considering these findings, it's possible that the protection of platelets that is seen in our studies is not biomechanical in nature. Rather, the protection seen in platelets may be due to the protective effects of TGF-β being dampened by the depletion of platelets, the main source of TGF-β in circulation. Further experiments into TGF-β, as well as comprehensive analysis of TGF-β levels in these mice would be instructive in determining the true mechanism of platelet protection in early aneurysm.

As previously mentioned, there are other binding partners for the 390-396 region of the γ-chain. One of these that is particularly of note in our studies is FXIII. FXIII natively exists as a heterotrimeric protein of FXIII-A_2B_2. This form exists in blood, and circulates almost exclusively bound to fibrinogen, with some excess FXIII-B_2 circulating in the unbound form. It has recently been documented that FXIII-A_2B_2 uses the 390-396 binding motif. [149] Due to this, mice with the *Fibγ$^{390-396A}$* mutation do show moderate delays in FXIII activation, but are not rendered completely devoid of FXIII activity. [71] Furthermore, some preliminary research suggests that FXIII may be involved in AAA. Similar to fibrinogen, polymorphisms of FXIII have been demonstrated to be in negative disequilibrium in AAA patients as compared to the general population. [150] Unpublished, preliminary data from our lab also suggests that FXIII levels are increased in patients with AAA.

Taken together, this information makes clear the necessity of evaluating the effect when FXIII is not able to effectively circulate in our aneurysm models, and whether inhibition of FXIII may be a confounding aspect of our findings in the fibrinogen

γ 390-396 binding motif and Mac-1. However, preliminary studies in our laboratory have not found that FXIII is important in AAA formation. When mice were treated with a FXIII siRNA or a control siRNA, and then underwent aneurysm induction by elastase, we did not see significant increases in aneurysmal disease burden (p=0.3) between these groups. However, further investigation into this possible contribution is forthcoming.

Though there has been substantial investigation into the biomechanical stability of the ILT, data in regards to the true effect of ILT is mixed. [6] It has been observed that even considering the decreased wall stress, the size of the ILT can be correlated to increased rupture rate at a smaller diameter. This would suggest that the biomechanical advantages of the ILT do not outweigh the other disadvantages posed by the ILT such as increased hypoxia and wall damage. However, these observations are not in conflict with our proposed mechanisms on early platelet activity in AAA. Though this does further complicate the picture of platelets in aneurysm overall, it connects our work to earlier work from our lab in which platelet inhibition in an established animal model of aneurysm was protective of rupture. [46]

One important point for discussion is the viability of targeting any hemostatic proteins in aneurysm. As demonstrated, the arguments for and against platelet inhibition are both valid, and the answer is not clear. Furthermore, though some direct oral anticoagulants (DOACs), including fondaparinux, enoxaparin and rivaroxaban, [192, 193] have shown promise in treating aneurysm in mouse models, quality studies evaluating their use in humans are lacking. Interestingly, preliminary data from our groups have seen some increase in rupture-induced death with the use of rivaroxaban. Also, there is hesitancy to use anticoagulants in the treatment of AAA in humans, given their

association with bleeding, and the difficulties in reversal in the event of aneurysm rupture. However, anti-platelet therapy is being used at this time in aneurysm patients [110, 111, 194], and though there is need for comprehensive analysis of the bleeding risk associated with this, at this time, there is not a documented contraindication for the use of antiplatelet therapy in AAA. It is important to note that our studies in regards to fibrinogen did not require that the hemostatic effects of fibrinogen be impaired to yield a positive effect in AAA. Furthermore, even in the event that fibrinogen and platelets are not druggable targets in AAA, this work will contribute to the overall understanding of AAA disease pathology.

Though we have proposed a mechanism for fibrinogen that focuses on the localization of white blood cells to the site of inflammation, where the 390-396 binding motif facilitates mobilization of white blood cells and helps to anchor the white blood cells to the site of the ILT, there are other explanations. Studies investigating the effects of the same binding motif in sickle cell anemia have found that when $Fib\gamma^{390\text{-}396A}$ mice when crossed with or bone marrow transplanted into Berkeley sickle mice, the mutant mice show significant decreases in white blood cell derived reactive oxygen species (ROS). [195] Importantly, ROS has been recognized as a potent driver of AAA pathology. [196] Though this has not been tested in the studies presented here, there is a logical hypothesis that ROS could also be contributing to the findings presented in this dissertation.

Another alternative explanation is altered immune response triggered by the Mac-1/390-396 fibrinogen axis. As mentioned previously, the 390-396 binding motif of fibrinogen is under investigation for its importance in a myriad of diseases. For example,

recent work in allergy and asthma suggests that Mac-1 interactions with the 390-396 binding motif on fibrinogen cleavage products may be important in facilitating communication between Mac-1 and toll-like receptor 4 (TLR4). [197] Importantly, TLR4 has also been implicated in AAA processes. [198, 199] TLR4 is a pattern recognition receptor which is found membrane-bound on myeloid lineage leukocytes. Its activation is a crucial element of immune response. In animal models of allergy and asthma, removal of Mac-1 in mice protects from asthma and airway hyperreactivity to acetylcholine. Furthermore, removal of the 390-396 binding motif results in protection from airway hyperreactivity, but not from innate asthma. Splenocytes from Mac-1 and *Fibγ$^{390-396A}$* mice show impaired response to fungal challenge as well. It is hypothesized that the 390-396 region facilitates interaction of Mac-1 and TLR4 via fibrinogen cleavage products from fungal proteases. This could imply that our effects in AAA are seen due to incomplete interactions between Mac-1 and TLR4. However, the diseases studied in the mentioned study are all primarily driven by eosinophils, and eosinophils have not been shown to be an eosinophil-driven disease. Furthermore, though their work represents strong interactions between TLR4 and Mac-1 in asthma and allergy, the connection to the 390-396 binding motif of fibrinogen remains theoretical. Attempts to test this directly have not been successful in the literature as of yet, due to methodological limitations.

Another limitation to consider is the red-cell retention of *Fibγ$^{390-396A}$* mice. Because of the altered interactions with FXIII, mice with the *Fibγ$^{390-396A}$* mutation show altered red-cell retention in clots. [71] In this finding, it is important to consider the effects of red clots compared to white clots. In red clots, the major components are fibrin and red

blood cells, where red-cell retention is crucially important in maintaining overall clot stability. In white clots, however, the major components are mainly composed of platelets and other immune cell mediators, generally positioned atop an atherosclerotic lesion. Red clots are the major thrombus of venous vessels, where white clots are the major thrombus of arterial vessels. [200] Though this finding of reduced red cell retention is not insignificant, it is important to consider that the ILT in AAA is an arterial clot, and is therefore, a white clot. [54] Further investigation is required to fully evaluate the impacts of this observation, but the hypothetical impact of the reduced red blood cell retention would be less important in AAA.

5.2 Future Directions

As discussed previously, IL-6 is of great interest in AAA. Similarly, there is evidence in our RNAseq data of IL-6 being important in the changes between the aneurysmal disease process in WT verses *Fibγ*$^{390-396A}$ mice. This is scientifically consistent with both the known pathogenesis of AAA and the intrinsic connection of IL-6 and fibrinogen in inflammation. Our lab is currently working to evaluate plasma levels of IL-6 and associated cytokines in WT and *Fibγ*$^{390-396A}$ mice after AngII-induced AAA. Several IL-6R inhibitors have been used in AAA, reducing aneurysmal burden. [88, 89] However, these same inhibitors have never been used in *Fibγ*$^{390-396A}$ mice in the context of AAA. To investigate the contributions of IL-6 in our findings, it would be instructive to evaluate further how the IL-6 feedback loop and the 390-396 binding motif of the fibrinogen γ chain interact in AAA. Alternatively, using genetic knockouts of IL-6 or of the IL-6R in AAA could be instructive, as IL-6 blockade in AAA has to date, only been achieved using pharmacologic means.

RNAseq analysis also identified some pathways associated with neutrophils. This was somewhat unexpected, as the RNAseq analysis presented here was done on samples from the AngII-model, which is recognized as being a macrophage-driven model. This finding will be investigated further, with both neutrophil staining to analyze possible neutrophil deposition changes, and direct analysis of neutrophil elicitation through thioglycolate experiments.

Though some preliminary data suggests that FXIII is not important in aneurysm formation in our models, this work is early. There is still substantial elements of the FXIII story that are not yet answered, and this needs to be thoroughly analyzed and investigated. It cannot be ignored that FXIII levels and FXIII polymorphisms have been demonstrated to be important in AAA disease, and if FXIII does not participate in the formation or progression of AAA, another explanation for these findings needs to be proposed. These experiments are currently forthcoming in our laboratory.

Notably, our work in fibrinogen focuses mainly on inflammatory roles of fibrinogen in AAA. However, this still leaves a lot of investigation into the hemostatic properties of fibrinogen. Some literature suggests that fibrinogen polymorphisms that have been linked to altered fibrin clot structures may be in disequilibrium to the general population. [150] Furthermore, the high prevalence of the ILT in human aneurysms make the hemostatic implications of fibrinogen removal difficult to ignore. A future direction for this work would be to investigate the hemostatic components of fibrinogen in AAA progression. Our lab has access to mice with a mutation in fibrinogen, Fib^{AEK}, which

has a mutated cleavage site, rendering the mouse deficient of fibrin, but normal for fibrinogen. Of note, these mice have impaired secondary hemostasis, but do not show defects in platelet aggregation. Interestingly, these fibrin deficient-mice do show clinically significant changes in anti-microbial host defense; however, Mac-1 engagement in these mice is intact. [201] Whether these mice show a similar reduction in aneurysmal disease burden to the mice models that were presented in this dissertation is an important question for future studies. In the event that these mice also show reduced disease model in animal models of AAA, aneurysm pathogenesis is recognized to be multifaceted, and both fibrinogen inflammatory processes and hemostatic processes could be involved.

Furthermore, our WGCNA analysis showed that although many inflammatory processes are downregulated in our animal model of AAA in $Fib\gamma^{390\text{-}396A}$ mice, there are also significant decreases in many hemostatic pathway genes, as well as decreases in lipid homeostasis and cholesterol efflux. This suggests that there is further investigation to be done in these pathways. Importantly, mice with the $Fib\gamma^{390\text{-}396A}$ mutation have not been noted to have impaired coagulation function. However, our RNAseq and WGCNA analyses do not compare a WT mouse to a $Fib\gamma^{390\text{-}396A}$ mouse, but compares these two genotypes under the stress of aneurysm induction. This would imply that there may be underlying coagulation processes that drive aneurysm progression, and if aneurysm induction or progression is prevented, subsequent hemostatic processes are not initiated.

It has been noted that mice with the *Fibγ$^{390-396A}$* do show differences in adipocyte-mediated inflammation. [81] Our findings in regards to lipid homeostasis bring into question if changes in lipid accumulation or adipocyte mediation could be important in our aneurysm models. Of note, studies analyzing changes in diet-induced obesity in *Fibγ$^{390-396A}$* mice occurred over a longer period of time, and with a diet with a higher fat content than in our studies. Over the course of our 5-week HFD diet feedings in *Fibγ$^{390-396A}$* mice, no significant changes in weight-gain were observed.

As discussed above, there has been investigation of fibrinogen in AAA through the work of Pham and colleagues. Experiments with mice possessing the Fib^{AEK} mutation could also be valuable in addressing and evaluating their work and its relation to our own work. For example, in the pathway proposed by Pham, they hold that LP activation is directed against fibrin clot. Our FibAEK mutant mouse experiments would be invaluable in determining if the demonstrated role for fibrinogen is due to fibrinogen or fibrin. With so many possible mechanisms for fibrinogen in AAA formation, our research would progress the investigation of Pham, while not disregarding other possible pathways for fibrinogen, and explanations for our findings.

Another consideration that requires investigation is the connection between our fibrinogen work and our platelet work. Fibrinogen is also suggested to be important in these early steps, as mice with LNK$^{-/-}$ platelets have impaired response to fibrinogen signaling show a similar outcome to mice with depletions of platelets. It is known that fibrinogen does signal as a ligand to platelets via the LNK receptor in an outside in

mechanism, and LNK deficiency is associated with poor clot retraction and clot stability, [105] often attributed to the inability of fibrinogen to signal in these platelets.

Our previous work in platelets and AAA demonstrate that inhibition of platelets through ASA or clopidogrel, [112] showed improvements in aneurysmal disease progression. Taken with our work in this dissertation, it would appear that the effects of platelets is highly dependent on the stage of aneurysm development. Therefore, considering the temporality of the effects in platelets, it would be valuable to investigate this same concept in fibrinogen, and test the contributions of fibrinogen in early aneurysm, verses an established aneurysm. An intervention study with fibrinogen impairment through ASO or similar would be instructive in this question. This would also be instructive in determining the possibility of pharmaceutical interventions for AAA via fibrinogen. If the positive effects of fibrinogen suppression are not observed in an established aneurysm model, our work in fibrinogen, though instructive about the pathobiology of fibrinogen, would be less promising to the direct treatment of AAA.

Notably, we did not see the same high mortality in fibrinogen depletion as we did in the platelet inhibition and depletion studies, and in adoptive transfer of *LNK*$^{-/-}$ platelets. This raises some question in regards to the disparate results between LNK deficient platelet mice and Fibrinogen deficient or suppressed mice in animal models of AAA. It would be a reasonable hypothesis that, if LNK deficiency results in major death because of the weak clot properties, due to impaired fibrinogen signaling, a similar effect of major death would be expected. This finding needs to be addressed. Further investigation into the mechanisms of LNK deficiency in aneurysm are necessary,

particularly identifying the interactions of fibrinogen and LNK deficiency directly in aneurysm.

There is an interesting avenue of research that would further investigate the connections between the two halves of our work. As referenced above, fibrinogen facilitates the formation of a stable platelet plug. However, our lab has access to a mutant mouse, Fib$\gamma^{\Delta 5/\Delta 5}$ that possesses and altered form of fibrinogen which is not capable of correct interactions with platelets, due to truncation of the γ-chain which removes interactions of the platelet $\alpha_{IIb}\beta_3$ to fibrinogen [68]. Questions in regards to some of the hemostatic fibrinogen contributions in AAA, and questions about LNK importance, as well as clot stability in platelets could be addressed in experiments with these mice.

As addressed previously, ROS is a known important factor in AAA pathogenesis, and the fibrinogen alterations studied in this dissertation have been shown to demonstrate improvements in white blood cell ROS in sickle cell anemia. [195] As such, experiments investigating the ROS production of *Fibγ$^{390-396A}$* mice in the context of AAA would be a valuable next step in fully elucidating the contributions of fibrinogen in AAA.

It is also important to consider the impacts of TLR4. Because the 390-396 region of the fibrinogen-γ chain has been potentially implicated in the Mac-1 mediated activation of TLR4, and TLR4 has been identified as being involved in AAA pathogenesis, there is potential for investigating the relationship of Mac-1, Fibrinogen-γ and TLR4 in AAA. More in depth analysis into the TLR4 pathway and its downstream targets, such as STAT6 and NFκB, in AAA would be informative in this question.

Another avenue for further research would be to investigate neutrophils further in this phenotype. Different aneurysm animal models are known to be driven by different immune cells. As the AngII model is recognized as being primarily driven by macrophage action, not by neutrophil action, the prominence of neutrophil markers in our RNAseq analysis was surprising. We have begun experiments to investigate these findings further, analyzing the thioglycolate elicitation in these animals, and looking into the co-localization of leukocytes and fibrinogen. We are also hoping to analyze neutrophil deposition in the aortic sections of our experimental tissues to hopefully learn potential explanations for the RNAseq findings.

5.3 Conclusion

Prior to the beginning of our work, there was substantial data demonstrating that the there was an association between fibrinogen and abdominal aortic aneurysm. Therefore, we investigated the contributions of fibrinogen as a whole protein to abdominal aortic aneurysm, as well as a possible mechanism of fibrinogen in abdominal aortic aneurysm due to the leukocyte-binding properties of the 390-396 binding motif on the γ-chain of fibrinogen. As a result of our studies, we have demonstrated that fibrinogen is a causal agent in abdominal aortic aneurysm pathogenesis, and that leukocyte binding between the 390-396 binding motif of fibrinogen and the Mac-1 integrin of hematopoietic cells contributes to the formation and progression of AAA. Further investigation must be undergone to further investigate the molecular mechanisms and signaling that result in this effect.

Similarly, prior to our work, there was some literature suggesting that platelets have an important role in abdominal aortic aneurysm. However, previous literature suggested

that platelets were participants in the progression of AAA. But there was not a body of data to describe platelets in early aneurysm or aneurysm formation. We worked to characterize the effects of platelets in this stage of aneurysm. As a result, we were able to demonstrate that platelets are among the first responders to the site of aneurysm formation, and that platelet activities are crucial to the stable formation of the aneurysm structure. Removal of platelets from the biological system results in a failure of the aneurysm to form in response to injury, and ends in structural failure and aneurysm rupture-induced death. Our work demonstrates that changes in platelets that result in poor clot formation also recapitulate the phenotype seen in platelet removal. This suggests that platelet and thrombus formation is important in early aneurysm stability.

The aneurysm field has been hesitant and somewhat unable to fully investigate the importance of hemostatic and coagulation proteins in abdominal aortic aneurysm. There has also been little investigation into fibrinogen in AAA, even given the clear correlations that AAA shows with fibrinogen levels. Our work demonstrates that there are clear impacts of both fibrinogen and platelets in AAA aneurysm formation, and impacts of fibrinogen in AAA aneurysm progression. There are clear avenues for further research in fibrinogen and platelets in AAA pathogenesis. Some of these include investigation into the hemostatic impacts of fibrinogen, further investigation into fibrinogen's partner IL-6, further investigation into neutrophils in fibrinogen mutants, analysis of the delicate balance of platelets, and investigations into the values of anti-fibrinogen therapy as an intervention in AAA.

Overall, this work has been a novel investigation into fibrinogen and platelets in AAA formation and has contributed significantly to understandings of both the inflammatory and hemostatic processes of AAA.

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