Hemostasis and thrombosis in zebrafish models of human congenital fibrinogen disorders

Freire Sanz, Cristina

UNIVERSITÉ DE GENÈVE FACULTÉ DES SCIENCES

Département de médecine génétique et développement FACULTÉ DE MÉDECINE

Professeur Marguerite Neerman-Arbez

HEMOSTASIS AND THROMBOSIS IN ZEBRAFISH MODELS OF HUMAN CONGENITAL FIBRINOGEN DISORDERS

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Cristina FREIRE SANZ

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ABSTRACT

The ultimate goal of hemostasis is to stop bleeding by the conversion of soluble fibrinogen into insoluble fibrin to form a blood clot. Fibrinogen is a 340 kDa hexameric glycoprotein composed of two copies of three polypeptide chains: Aα, Bβ and γ, which are encoded by three different genes: *FGA*, *FGB* and *FGG*, respectively, clustered on human chromosome 4 (4q32). Each gene is transcribed and translated separately; chains are assembled as a hexamer and secreted into the bloodstream by hepatocytes. In a subclass of fibrinogen molecules, the two common Aα chains are replaced by two AαE isoforms, rendering a fibrinogen molecule of 420 kDa. The AαE isoform accounts for 1–3 % of circulating fibrinogen and arises from splicing out the last 15 codons of exon 5 and intron 5 and keeps exon 6. As this form is present throughout the vertebrate kingdom from lampreys to humans, it may have an important (and still unknown) physiological function. Normal levels of fibrinogen vary from 1.5 to 4 g/L. Congenital fibrinogen disorders (CFDs) are rare bleeding disorders caused by mutations in the three fibrinogen encoding genes. These mutations affect the quantity (afibrinogenemia and hypofibrinogenemia), quality (dysfibrinogenemia), or both aspects (hypodysfibrinogenemia) of the fibrinogen molecule. These disorders are associated with bleeding and thrombotic events; however, their precise risks are difficult to predict.

In the first part of this thesis, we studied the pathophysiology of afibrinogenemia, dysfibrinogenemia and hypodysfibrinogenemia disorders using zebrafish as a model organism. We generated zebrafish models for each disorder by using genome editing technologies. To evaluate hemostasis, we used a laser-induced vascular endothelial injury method in zebrafish larvae (3 and 5 days post-fertilization (dpf)). This method allowed us to study venous occlusion by measuring time to vascular occlusion (TTO), and thrombocyte recruitment by measuring thrombocyte adhesion/aggregation at the

injury site. Afibrinogenemic fish showed prolonged TTO at 3dpf and formation of unstable thromboembolic aggregates at 5dpf. Dysfibrinogenemic and hypodysfibrinogenemic larvae showed prolonged venous TTO at 3dpf and reduced thrombocyte adhesion/aggregation at 5dpf. The phenotypes seen in the three zebrafish models are consistent with the bleeding and thrombotic phenotypes observed in patients with these CFDs. Therefore, we concluded that the zebrafish model is useful to study the molecular basis of the pathophysiology of afibrinogenemia, dysfibrinogenemia and hypodysfibrinogenemia disorders.

In the second part of this thesis, we investigated the roles of the common Aα chain and the AαE isoform in embryonic hemostasis using different zebrafish strains (AB, TU and TL strain). It is known that the proportion of the fibrinogen-420 (AαE-containing fibrinogen) varies through life, being more abundant in a new-born human's blood than in adults. Similarly, zebrafish produce the AαE-containing fibrinogen predominantly in larval stages. We observed that larvae from the AB strain had a prolonged TTO when comparing to larvae from the TU or TL strains. Interestingly, these larvae did not produce AαE isoform due to a mutation occurring in the AαE-specific coding region of *fga*. We questioned if the lack of AαE in AB larvae was the reason for their prolonged TTO. We showed that the AαE isoform rescued venous occlusion in fibrinogen deficient mutants, while surprisingly the common Aα chain was less efficient. Furthermore, we studied the contribution of Aα chain and AαE isoforms to thrombosis after performing a vessel injury. We showed differences in the contribution of the two chains to promote thrombosis linked to the availability of different cell types for clotting. Taken together, we identified a genetic modifier of venous thrombosis, demonstrated a role for the AαE isoform in embryonic

hemostasis and differences in the contribution of the two isoforms to promote thrombosis dictated by the cell type availability.

RESUME EN FRANÇAIS

Le but ultime de l'hémostase est d'arrêter un saignement par la conversion de fibrinogène soluble en fibrine insoluble pour former un caillot sanguin. Le fibrinogène est une glycoprotéine hexamérique de 340 kDa composée de deux copies de trois chaines polypeptidiques, Aα, Bβ et γ. Ces trois chaines sont codées par trois gènes différents : *FGA*, *FGB* et *FGG* respectivement, regroupés chez l'humain sur le chromosome 4 (4q32). Chaque gène est transcrit et traduit séparément ; les chaînes sont assemblées en hexamère et sécrétées dans la circulation sanguine par les hépatocytes. Dans une sous-classe de molécules de fibrinogène, les deux chaines Aα principales sont remplacées par deux isoformes AαE, donnant lieu à une molécule de fibrinogène de 420 kDa. Cet isoforme AαE représente 1-3% du fibrinogène circulant, et provient de l'épissage des 15 derniers codons de l'exon 5 et de l'intron 5, en gardant l'exon 6 intact. Cette forme étant présente chez les vertébrés de la lamproie aux humains, elle pourrait avoir une importante (et toujours inconnue) fonction physiologique. Les niveaux normaux de fibrinogène varient de 1.5 à 4 g/L.

Les anomalies congénitales du fibrinogène (Congenital fibrinogen disorders - CFDs) sont des maladies rares de la coagulation causées par des mutations dans les trois gènes codant pour le fibrinogène. Ces mutations affectent la quantité (afibrinogénémie and hypofibrinogénémie), la qualité (dysfibrinogénémie), ou les deux aspects (hypodysfibrinogénémie) de la molécule de fibrinogène. Ces anomalies sont associées à des saignements et des incidents thrombotiques ; cependant, leurs risques précis sont difficiles à prédire.

Dans la première partie de cette thèse, nous avons étudié la pathophysiologie de l'afibrinogénémie, la dysfibrinogénémie et l'hypodysfibrinogénémie en utilisant le poisson-zèbre comme organisme modèle.

Nous avons généré des modèles de poisson-zèbre pour chaque phénotype en utilisant des technologies d'édition génomique. Pour évaluer l'hémostase, nous avons induit des lésions endothéliales vasculaires par laser à des larves de poisson-zèbre (3 et 5 jours post-fertilisation (jpf)). Cette méthode nous a permis d'étudier l'occlusion veineuse en mesurant le temps d'occlusion vasculaire (time to vascular occlusion - TTO), et le recrutement de thrombocytes en mesurant l'adhésion et l'agrégation de thrombocytes à l'endroit de la lésion.

Les poissons afibrinogénémiques avaient un TTO prolongé à 3jpf et formaient des agrégats thromboemboliques instables à 5jpf. Les larves dysfibrinogénémiques et hypodysfibrinogénémiques avaient un TTO prolongé à 3jpf et une réduction de l'adhésion/ agrégation de thrombocytes à 5jpf. Les phénotypes observés dans ces trois modèles de poisson-zèbres sont compatibles avec les saignements et les phénotypes thrombotiques observés chez les patients avec ces CFDs.

Par conséquent, nous avons conclu que le poisson-zèbre est un modèle utile pour étudier les bases moléculaires de la pathophysiologie des troubles d'afibrinogénémie, dysfibrinogénémie et hypodysfibrinogénémie.

Dans la seconde partie de cette thèse, nous avons investigué les rôles des chaines Aα et l'isoforme AαE dans l'hémostase embryonnaire en utilisant différentes souches de poisson-zèbres (AB, Tu et TL). Il a été démontré que la proportion de fibrinogène-420 (fibrinogène contenant l'isoforme AαE) varie au cours de la vie, étant plus abondante dans le sang d'un nouveau-né humain que chez un adulte. De la même façon, les poisson-zèbres produisent majoritairement du fibrinogène-420 aux stades larvaires.

Nous avons observé que les larves AB avaient un TTO prolongé comparé aux larves Tu et TL. De manière intéressante, ces larves ne produisaient pas l'isoforme AαE, à cause d'une mutation dans la région codant pour la chaine AαE du gène FGA. Nous nous sommes demandés si le manque de AαE dans les larves AB était la raison pour laquelle leur TDO était prolongé. Nous avons montré que l'isoforme AαE rétablit l'occlusion veineuse chez les poissons mutants déficients en fibrinogène, tandis que la chaine Aα commune était moins efficace. De plus, nous avons étudié la contribution des isoforms Aα et AαE à la thrombose induite par une lésion vasculaire. Nous avons trouvé des effets différents de ces deux isoformes sur la thrombose, liés à la disponibilité de différents types cellulaires pour la coagulation.

Dans l'ensemble, nous avons identifié un modificateur génétique de la thrombose veineuse, démontré un rôle de l'isoforme AαE dans l'hémostase embryonnaire et des différences de contribution de ces deux chaines pour promouvoir la thrombose, dictées par la disponibilité des types cellulaires.

LIST OF ABBREVIATIONS

AαC Aα C-terminal Domain

AαE Aα extended isoform

AαEC Aα Extended C-terminal domain

APC Activated protein C

aPTT Activated partial thromboplastin time

APP Acute phase protein

APR Acute phase response

BBB Blood-brain barrier

BK Bradykinin

cDNA Complementary DNA

C/EBP CCAAT/enhancer binding protein

CFD Congenital Fibrinogen disorder

CVD Cardiovascular diseases

DIC Disseminated intravascular coagulation

dsRed Red fluorescent protein

dpf Days post fertilization

EC Endothelial cells

ECM Extracellular matrix

EGFP Enhanced green fluorescent protein

EN-RBD European network of rare bleeding diseases

ER Endoplasmic reticulum

FBG Fibrinogen-like globe

FFP Frozen plasma fibrinogen

FGA Fibrinogen α-chain gene

FGB Fibrinogen β-chain gene

FGG Fibrinogen γ-chain gene

Fib340 Fibrinogen 340

Fib420 Fibrinogen 420

FpA Fibrinopeptide A

FpB Fibrinopeptide B

γ’ Gamma prime isoform

GC Glucocorticoid

HMWK High molecular weight kininogen

HNF-1, HNF-3 Hepatocyte nuclear factor -1, -3

HSF Hepatocyte stimulating factor

ICAM-1 Intercellular adhesion molecule 1

IL-1, IL-6 Interleukin -1, -6

IL-6RE Interleukin 6 responsive element

IRES Internal ribosome entry site

Kb Kilobase

kDa Kilodalton

KI Knock-in

KO Knock-out

Mac-1 Macrophage-1 antigen

MI Miocardial infarction

MO Morpholino

MS Multiple Sclerosis

nt Nucleotide

PKK Plasma prekallikrein

PK Plasma kallikrein

PPAR Peroxisome proliferator activated receptor

PS phosphatidylserine

PT Prothrombin time

RA Rheumatoid arthritis

RBC Red blood cell

Serpin Serine protease inhibitor

STAT Signal transducer and activator of transcription

SNP Single nucleotide polymorphism

TAFI Thrombin activatable fibrinolysis inhibitor

TF Tissue factor

TFPI Tissue factor pathway inhibitor

TG Transglutaminase

TM Thrombomodulin

TNF-α Tumor necrosis factor alpha

tPA Tissue type plasminogen activator

TSS Transcription start site

TT Thrombin time

uPA Urinary type plasminogen activator (urokinase)

VTE Venous thromboembolism

vWF Von Willebrand factor

WFH World federation of hemophilia

ZFNs Zinc-finger nucleases

INTRODUCTION

HEMOSTASIS

Hemostasis (from the Greek-words *aima*, meaning blood and *stasis*, meaning halting) is a rapid and efficient mechanism that stops bleeding in response to vascular damage by the formation of a blood clot (Figure 1). Hemostatic balance is controlled in space and time and is maintained by the vascular endothelium, platelets, blood coagulation factors, coagulation inhibitors, and fibrinolysis. Imbalances in this tightly regulated process can result in thrombotic or hemorrhagic episodes [1].

Hemostasis can be divided into three sequential processes: primary hemostasis, secondary hemostasis and fibrinolysis [2]. Primary hemostasis refers to platelet aggregation and platelet plug formation, where platelets become activated in a multifaceted process and resultingly adhere to the site of injury (adhesion) and to each other (aggregation), plugging the injury. Secondary hemostasis refers to the propagation of the coagulation cascade; and it concludes with the formation of a fibrin mesh that holds the platelet plug, strengthening and stabilizing the blood clot [3] [4]. Fibrinolysis refers to the dissolution and removal of the blood clot, establishing the return to the normal architecture of the vascular endothelium [2].

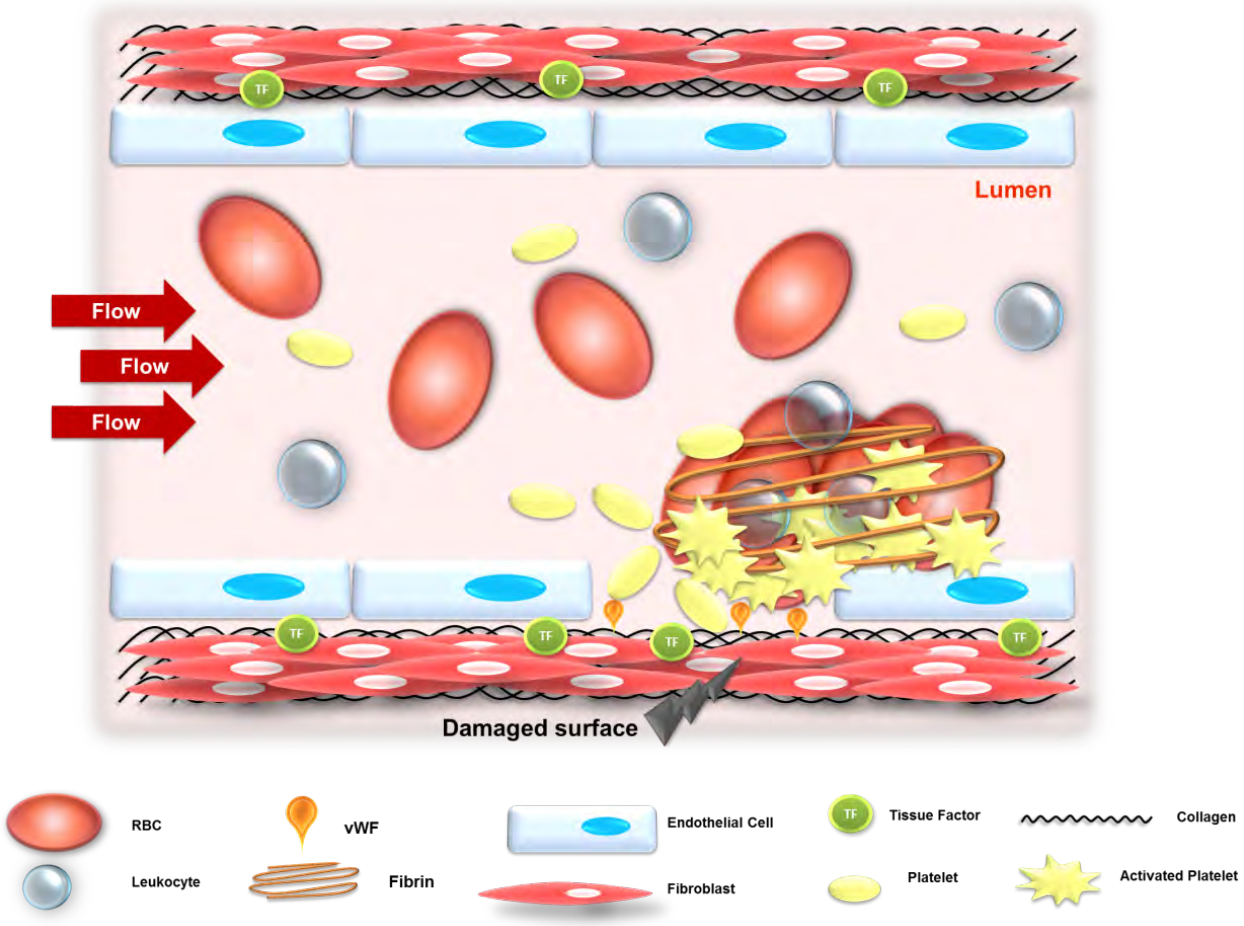

Figure 1. *Hemostasis. When an injury occurs in the vasculature, it triggers the release and or exposure of clotting factors. First the formation of a platelet plug occurs and then fibrin fibers adhere to the clot to encapsulate the platelets, red blood cells and leukocytes creating an insoluble blood clot.*

I. Primary Hemostasis

Vascular endothelium

All blood vessels have three elements [5]. First they have an inner tunica intima that is composed of the endothelium and sub-endothelium. The vascular endothelium is a cellular monolayer lining the circulatory system that acts as a thromboresistant physical barrier separating the blood's components from reactive sub-endothelial structures. It produces various negative mediators that prevent the initiation of hemostasis, such as heparin sulphate, thrombomodulin, prostaglandin I2, NO, and tPA. It regulates hemostasis and modulates vascular tone and permeability. Beneath the endothelium is the sub-endothelium. The sub-endothelium is thrombogenic in nature and consists of collagen fibers, elastin, and other proteins such as von Willebrand factor (vWF) and tissue factor (TF). Second, they have a tunica media which consists of smooth muscle cells, collagen fibrils, and an elastic layer (arteries). The third element is an outer tunica adventitia that is constituted of collagen and fibroblasts, which plays a protective and structural role. After tissue damage, the integrity of the endothelial membrane is disrupted and the underlying sub-endothelium is exposed, causing a shift to a more pro-thrombotic and pro-inflammatory state. This pro-thrombotic state is promoted by endothelial expression and the synthesis of several molecules and proteins involved in coagulation, such as integrin receptors, TF, chemokines and fibrinolysis inhibitor plasminogen activator inhibitor PAI-1 [6] [7].

Platelets

Platelets are enucleated cells produced by megakaryocytes in the bone marrow and lungs that travel along the vessel for five to ten days before they are eliminated by the spleen [8] [9]. Platelets are involved in the maintenance of normal hemostasis by contributing to both the first and the second waves of hemostasis. Indeed, they are central to obstructing bleeding in forming a plug at the site of injury (platelet activation, adhesion and aggregation) (Figure 2) but also in activating cell-based thrombin generation processes, amplifying the blood coagulation cascade (seen below in Secondary Hemostasis).

The first step in primary hemostasis involves rapidly moving platelets interacting with the exposed sub-endothelial extracellular matrix (ECM) so they can adhere to limit hemorrhaging and promote tissue healing. Stable platelet adhesion is a coordinated process that involves tethering, rolling, activation and firm adhesion [10] [11] [12] [13]. The local rheological conditions will dictate the initial interactions between platelets and ECM. At low shear rates (< 1000 s−1, i.e., in veins and large arteries) platelet adhesion involves binding to collagen, fibronectin (through α5β1 integrin binding) and laminin (through α6β1 integrin binding). Platelet-collagen interactions are mediated by two receptors: glycoprotein VI (GPVI) and the integrin α2β1. Both of these induce a strong intracellular outside-in signaling [14] that leads to activation of the $\alpha_{IIb}\beta_3$ integrin receptor, which is the main receptor in binding fibrinogen and fibrin to platelets [2]. Apart from its role in platelet aggregation, fibrinogen also mediates the adhesion of platelets to the damaged endothelium. Interestingly, soluble fibrinogen does not bind resting $\alpha_{IIb}\beta_3$ integrin receptors while immobilized fibrinogen does [15] [16]. Moreover, after fibrinogen to fibrin conversion, fibrin retains its ability to adhere platelets [17]. At high shear rates (> 1000 s−1, i.e., in the microvasculature and small

arteries), the interaction between the platelet surface receptor glycoprotein Ibα (GPIbα) and VWF, either in the extracellular matrix or immobilized on exposed collagen, is essential in slowing down the platelets in circulation. The final step of platelet adhesion is firm arrest on the ECM. Although the $\alpha_{IIb}\beta_3$ integrin receptor is considered to be the main platelet aggregating receptor, it also mediates firm adhesion of platelets [18] [19].

Platelet activation is caused by the binding of various agonists (e.g., thrombin, thromboxane A2, ADP, collagen, arachidonic acid) to specific receptors, which triggers the activation of signaling pathways and ultimately leads to integrin activation, which involves degranulation and the release of soluble agonists [20] [21] [22]. Normally, $\alpha_{IIb}\beta_3$ is in a resting state, but upon platelet activation it gets activated in a process called inside-out. Activated $\alpha_{IIb}\beta_3$ displays high affinity for fibrinogen and VWF [23]. In addition, platelets can be activated by the direct interaction of the $\alpha_{IIb}\beta_3$ receptor with fibrinogen and VWF, through the RGD or AGD ligand motifs, directly activating $\alpha_{IIb}\beta_3$ in a process called outside-in signaling [14] [10] [18].

Platelet adhesion and activation is followed by platelet aggregation. This is a complex and dynamic process involving many ligands such as fibrinogen, fibronectin, and VWF, receptors such as GPIbα and $\alpha_{IIb}\beta_3$, and platelets in different activation states. The major interactive mechanism for platelet aggregation occurs between $\alpha_{IIb}\beta_3$ and fibrinogen. As fibrinogen is a bivalent ligand it can bind to $\alpha_{IIb}\beta_3$ on two platelets, leading to platelet aggregation. As for platelet adhesion rheological properties will dictate the type of platelet aggregation: At low shear rate (< 1000 s−1), platelet aggregation is mainly mediated by $\alpha_{IIb}\beta_3$–fibrinogen interactions, and at shear rates between 1000 and 10,000 s−1, aggregation depends on the adhesive function of GPIbα and $\alpha_{IIb}\beta_3$. At high shear rates (> 10,000 s−1), platelet aggregation is

mediated by VWF–GPIbα interactions and can occur in the absence of $\alpha_{IIb}\beta_3$ activation or even platelet activation [24] [25].

Following platelet binding and activation a controlled release reaction takes place. Platelet granules contain a multitude of bioactive molecules that are important for adhesion, hemostasis, inflammation, angiogenesis, and wound healing. Their paracrine and autocrine nature causes the activation of nearby platelets, resulting in direct amplification of the platelet response, i.e., platelet aggregation and thrombus formation. Three types of granules can be distinguished: α-granules, which contain over 300 different proteins such as VWF, fibrinogen, $\alpha_{IIb}\beta_3$, plasmin, and plasminogen; dense granules (δ granules), which contain ATP, ADP, serotonin, histamine and calcium among others; and lysosomes (λ-granules) [9] [10] [26].

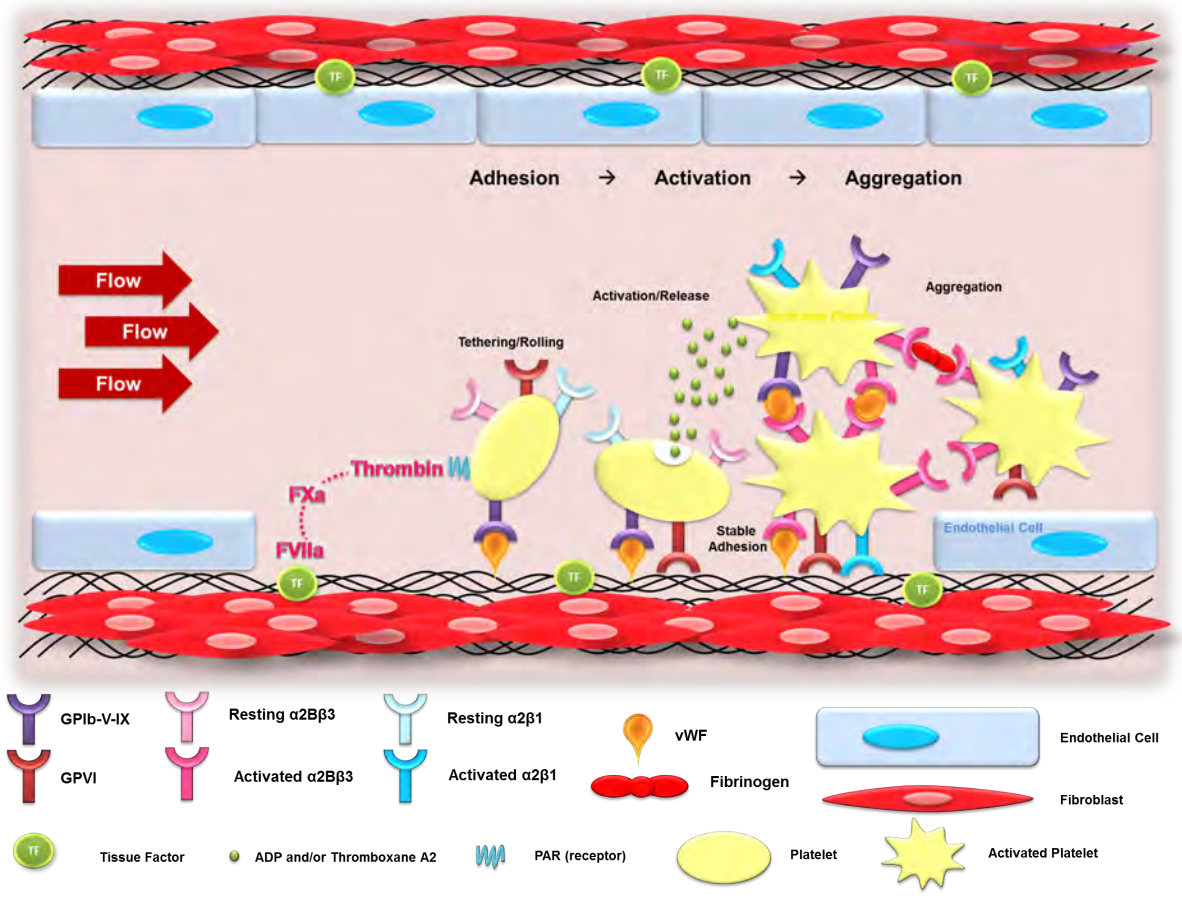

Figure 2. *Platelet adhesion and aggregation. The first interaction enables platelet tethering and it is mediated by the binding of vWF with the receptor complex GPIb-V-IX. This interaction enables GPVI binding with collagen. This leads to a shift in the integrin receptors to a high affinity state (activation), and to a release of ADP and thromboxane A2 (TXA2). At the same time, tissue factor (TF) triggers thrombin activation which leads to platelet activation due to its binding to PAR receptors on the platelet surface.*

II. Secondary hemostasis

The coagulation cascade, or waterfall model, was originally proposed by McFarlane in 1964 [27]. It is defined as a complex series of biochemical enzymatic reactions, wherein each reaction results in the activation of the next one in a sequential manner, like a cascade or a waterfall. In 2001, a cell-based model of hemostasis was presented by Hoffman [28], which better emphasizes the important role of specific cellular receptors for the binding of clotting proteins, where cells with similar phospholipid content can play different roles in hemostasis depending on their surface receptors. This model proposes that coagulation does not occur as a cascade, but rather in three overlapping stages: initiation, amplification, and propagation. Both models begin in response to vessel injury and culminate in the conversion of fibrinogen into fibrin, to form a blood clot.

Traditional model of coagulation

This model is divided into two different pathways that are independently capable of initiating coagulation: the intrinsic, or contact activation pathway, and the extrinsic, or tissue factor pathway. Both pathways then converge into a common pathway (Figure 3). The traditional model demonstrates some limitations because it does not take into consideration the cellular contribution and regulation provided by the endothelial cells and platelets. However, this model remains useful for *in vitro* diagnostic tests, such as plasma-based laboratory tests [2].

All coagulation proteins have been identified as zymogens, pro-cofactors and substrates with no enzymatic activity. The coagulation cascade involves the activation of inert zymogens into catalytically active serine proteases (i.e., factors IIa

(thrombin), VIIa, IXa, Xa, XIa and XIIa), with the exception of factor XIII which is a transglutaminase. The pro-cofactors (i.e., Ca^{2+}, and factors V and VIII) are converted into active cofactors so that they can bind to a serine protease to act as catalysts in the reactions [29].

The extrinsic pathway requires tissue factor (TF) for its activation. The majority of TF is in an inactive state, but after endothelial damage TF becomes exposed to blood flow leading to its activation. The extrinsic pathway is initiated by the interaction of exposed TF to FVII or FVIIa forming the TF/FVII or FVIIa complex, or extrinsic tenase complex [30] [31] [32] [33], which in turn activates FIX and FX. Activated factors IX and X must assemble with their own protein cofactors, FVIIIa and FVa, respectively, leading to a large burst of thrombin, which promotes the clotting cascade [34] [29]. The intrinsic pathway starts with the activation of protease factor XII through its contact with exposed endothelial collagen upon endothelial damage. Factor XIIa activates plasma prekallikrein (PK) to kallikrein (K), which liberates the peptide bradykinin (BK) from the protease high molecular weight kininogen (HMWK). Factor XIIa in combination with HMWK activates factor XI, which then activates factor IX. Factor IXa in combination with factor VIIIa forms the intrinsic tenase complex, activating FX to FXa. Factor VIII is activated by both factor Xa and thrombin. Thrombin also increases the generation of factor IXa via the activation of factor XI [29]. The final common pathway to blood clotting starts with the activation of factor X to factor Xa by both tenase complexes. Factor Xa, when linked to factor Va forms the prothrombinase complex, which converts prothrombin (factor II) to activated thrombin (factor IIa) ultimately converting fibrinogen (factor I) to fibrin (factor Ia) and activating factor XIII. FXIIIa forms covalent bonds that crosslink the fibrin polymers to form a more stable fibrin mesh clot.

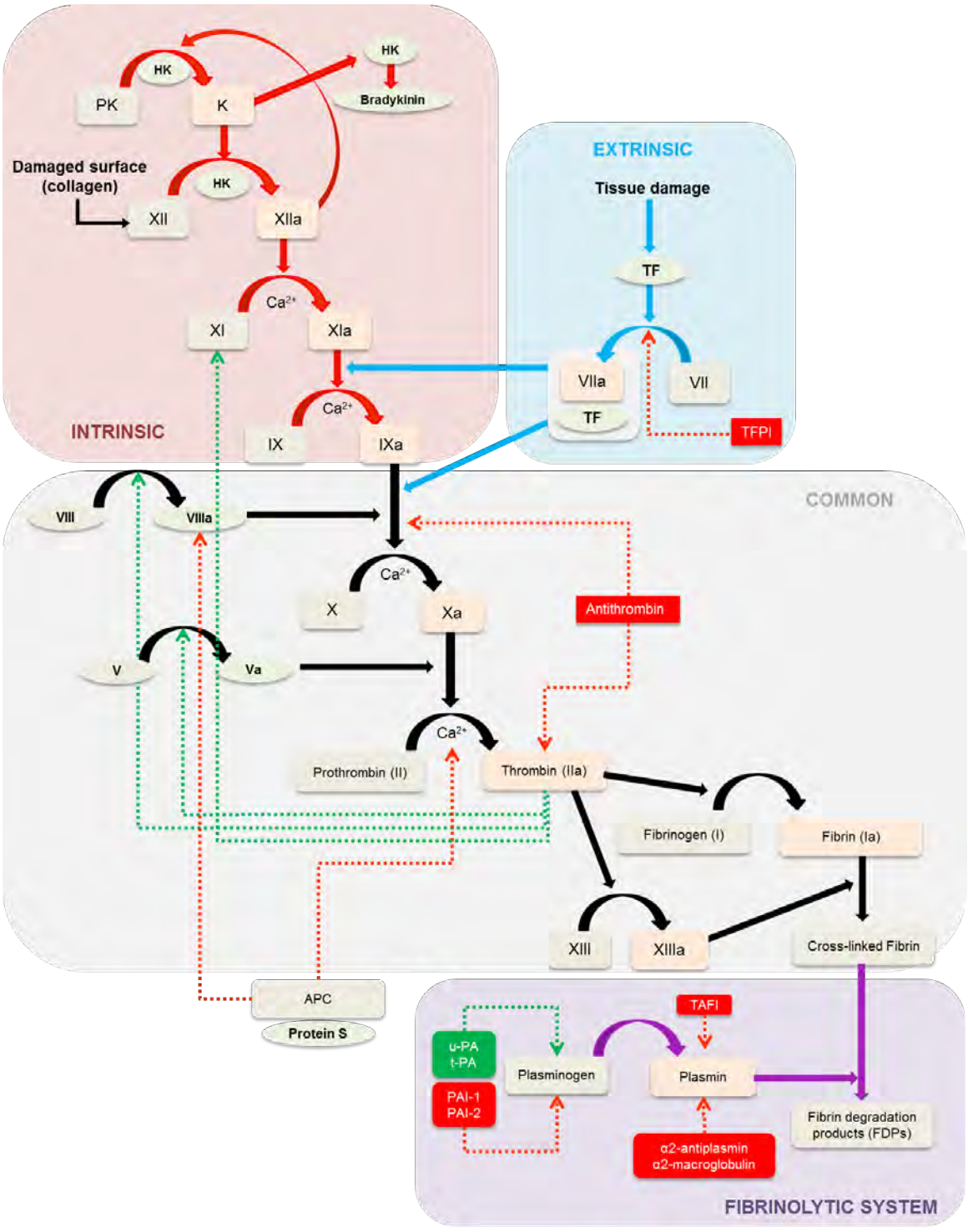

***Figure 3**. The Coagulation Cascade. In the diagram appears the traditional model of coagulation cascade (intrinsic, extrinsic and common pathways) and the fibrinolytic system. Arrows in green represent the positive feedback. Arrows in red represent the negative feedback.*

Cellular based model of coagulation

The cell-based model of coagulation explains that hemostatic coagulation can proceed when TF-bearing cells are brought into close proximity with activated platelets and a source of coagulation factors. Platelets adhere to sites of injury, become activated and undergo a number of changes that allow them to serve as a surface for the assembly and activity of the coagulation complexes (Figure 4) [28].

This model is divided into three stages. First, the initiation phase occurs in TF-bearing cells when they are exposed to blood components after a blood vessel is damaged. Circulating FVII or FVIIa binds to exposed TF, resulting in the assembly of the TF-FVII complex. The TF/FVIIa complex cleaves small amounts of both FIX and FX. FIXa, FXa, and thrombin can activate FV, which binds FXa at the platelet surface to form the prothrombinase complex generating the small amount of thrombin necessary for the activation of platelets and FVIII during the amplification phase [35].

In the next stage, amplification, small amounts of thrombin generated on TF-bearing cells amplify the initial procoagulant signal by enhancing platelet activation and adhesion to the injury site. Platelet activation results in an increase in phosphatidylserine (PS) exposure on the outer membrane, the secretion of partially activated FV from alpha granules and the activation of surface receptors. Some of the thrombin bound to GPIb/IX on the surface of platelets, remains active and can activate other coagulation factors and cofactors on the platelet surface. Additionally, the GPIb/IX complex binds to vWF. This binding promotes the adhesion of inactivated platelets at sites of vascular injury, as well partial platelet activation. When vWF bound to FVIII (vWF)/FVIII binds to GPIb/IX complex in the platelet surface is cleaved by thrombin to activate FVIII and release it from vWF. Activated cofactors V and VIII bound to activated platelet surfaces promote the assembly of procoagulant

complexes and a burst of thrombin generation [28] [35]. In the third stage, propagation, the active proteases combine with their cofactors on the platelet surface where large amounts of thrombin are generated and ultimately lead to fibrin clot formation [35].

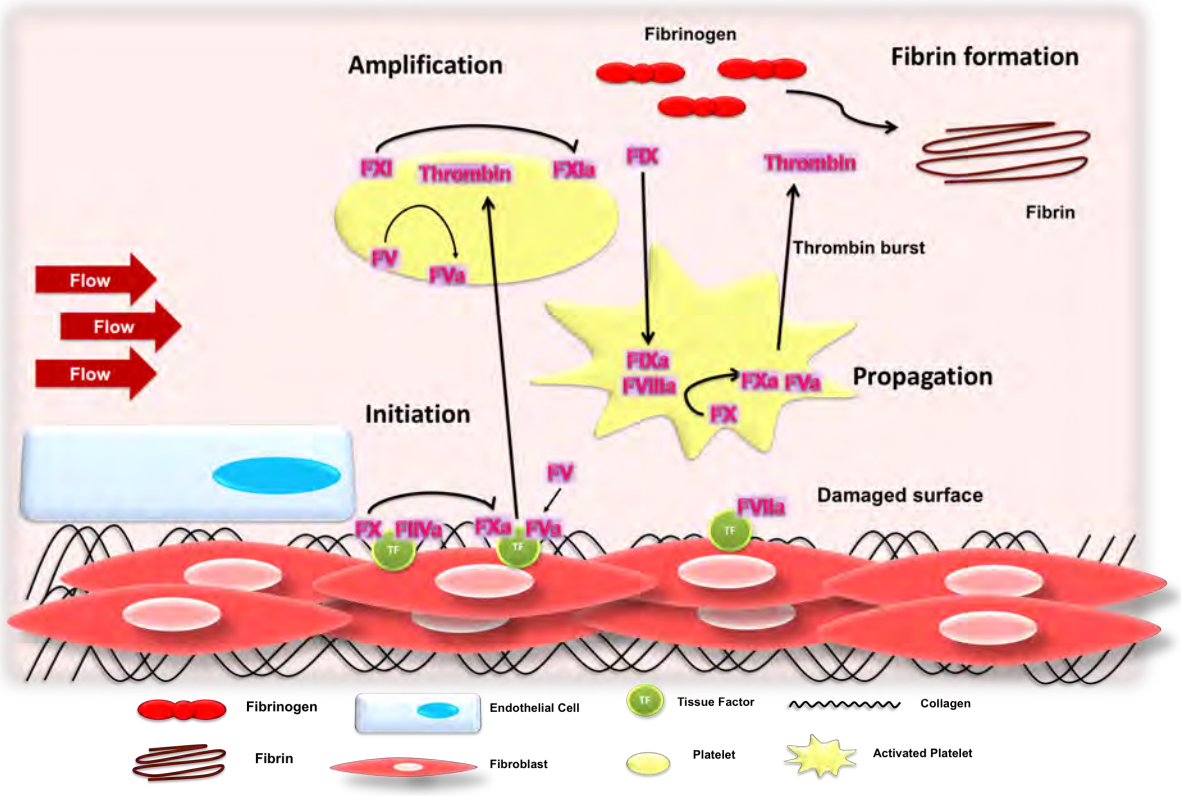

Figure 4. *The cell-based model of coagulation is divided in three overlapping phases. (I) The initiation phase starts when an injury exposes TF (from TF-bearing cell) to blood flow, leading to generation of small amounts of thrombin and FIXa. (II) In the amplification phase, the small amounts of thrombin are sufficient to activate platelets and to generate FVa, FVIIIa, and FXIa. (III) In the propagation phase, all the activated coagulation factors generated in the other two phases now bind on the platelet surface to form the intrinsic tenase complex which generates FXa on the*

platelet surface. Subsequently, the prothrombinase complex is formed on the platelet surface, where large amounts of thrombin are generated.

Regulation of coagulation and termination of clotting cascade

The hemostatic response is rapid and localized to the injury site. It is also potentially explosive, and, if uncontrolled, could lead to thrombosis, vascular inflammation, and tissue damage. To avoid these issues, coagulation is modulated by a number of mechanisms such as the dilution of procoagulants in the blood, removal of activated factors in the liver, and natural antithrombotic pathways anchored on vascular endothelial cells [29]. These pathways include the tissue factor pathway inhibitor (TFPI) which inhibits both complexes TFPI-FXa and TF/VIIa, antithrombin which neutralizes thrombin, Xa, TF:VIIa complex, IXa, XIIa and factor Xia, and activated protein C (APC) which in association with protein S inactivates factors Va and VIIIa, inactivating the prothrombinase and the intrinsic X-ase, respectively. APC itself is subject to regulatory inhibition by the protein C inhibitor, plasminogen activator inhibitor type 1.

III. Fibrinolysis

After a clot has been formed, it is dissolved so that blood flow is restored. This process is called fibrinolysis (Figure 3). The serine protease which lyses the fibrin clot is plasmin. Plasmin is generated from the zymogen plasminogen, which is activated by binding either tissue plasminogen activator (tPA), which binds fibrin to two different sites (Aα-(148-160) and γ(312-324) chains), or urokinase (uPA) [36]. After proteolysis, fibrin is degraded to fibrin degradation products (FDPs), which are important mediators in the inflammatory response [37]. Circulating plasmin and plasminogen activators are neutralized by serine or non-serine protease inhibitors, non-serpins such as α2-macroglobulin, or serpins such as PAI-1, PAI-2, and α2-antiplasmin, which are present in excess concentrations and are important in preventing excess unregulated plasminogen activity.

FIBRINOGEN

I. Fibrinogen

Fibrinogen structure

Fibrinogen—or coagulation factor I—is a 340 kDa hexameric glycoprotein composed of two copies of three polypeptide chains: Aα, Bβ and γ which are held together by 29 disulfide bonds [38] [39] [40]. These chains are encoded by three different genes: *FGA*, *FGB*, and *FGG* respectively [41] [42], clustered together on human chromosome 4 [43] [44]. Each gene is transcribed and translated separately and then assembled in the endoplasmic reticulum. The three polypeptide chains are expressed in hepatocytes, assembled as a hexamer and secreted by hepatocytes to the circulation. Fibrinogen is the coagulation factor with the highest concentration in blood plasma, varying between 1.5 – 4 g/L in normal conditions [45], and increasing 2 to 10-fold following an inflammatory stimulus. Therefore, the expression of the three fibrinogen genes is regulated at basal levels and during the acute phase response [46].

The *FGA* gene consists of six exons, and two different mRNA products are produced: the major mRNA species, accounting for the 90% of the transcripts, encodes the common Aα-chain and contains exons 1 to 5, and the extended αE isoform which accounts for 1–2% of the transcript. The latter splices out the last 15 codons of exon 5 and intron 5 and keeps exon 6 [47] [48]. The N-terminal domains of each of the Aα, Bβ and γ chains are homologous, but the random coil C-terminal domain of the common Aα-chain is different from the highly conserved globular C-terminal domains present in Bβ and γ chains. The *FGB* gene has 8 exons and encodes a single mRNA

transcript. The *FGG* gene consists of 10 exons, and two different mRNA products are produced. The major mRNA species, accounting for 85-92 % of the transcripts, encodes the common γ-chain (γA-chain) and contains 10 exons. Only 4 residues are coded by exon 10: AGDV. The other 8% to 15% of plasma fibrinogen contains the alternative spliced variant or γ′-chain. This variant arises from the loss of exon 10 and the inclusion of a portion of intron 9 (also known as exon 9a) in the mRNA, leading to a γ′ C-terminus in which the AGDV residues are replaced by 20 residues, VRPEHPAETEYDSLYPEDDL (Figure 5) [49].

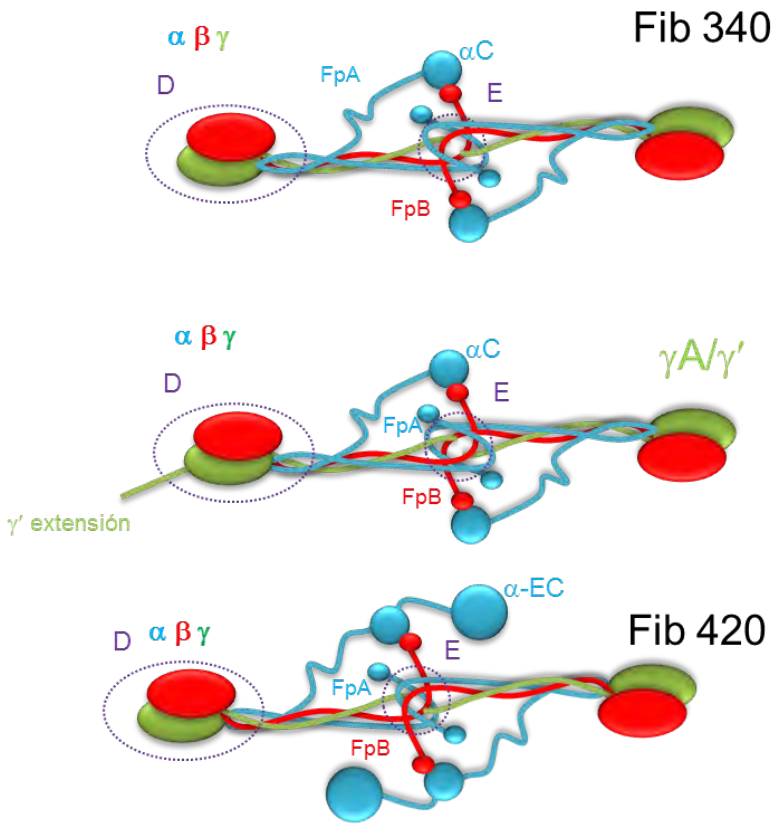

Figure 5. *Fibrinogen structure. Aα chains are shown in blue. Bβ chains are shown in red and γ chains are shown in green. FpA and FpB are fibrinopeptides A and B, respectively. E is the E central region and D is the D domain. The common fibrinogen*

form is named as Fib 340. There are two fibrinogen variants occurring due to alternative splicing which are: Fibrinogen γ' and the fibrinogen 420 (Fib420).

Evolution of vertebrate fibrinogen

The fibrinogen genes are located on human chromosome 4 (4q23-4q32) clustered in a region of 65kb arranged in the order *FGG*, *FGA* and *FGB.* The *FGA* and *FGG* genes are oriented in the same direction (on the same negative DNA strand) while the *FGB* gene is oriented in opposite direction (on the positive DNA strand) [50] [51]. All vertebrates possess fibrinogen molecules composed of two copies of three different polypeptide chains Aα, Bβ and γ [50]. Due to the similarities in amino acid [50] [51] and nucleotide [52] [53] [54] sequences among the three fibrinogen genes, it is clear that they have arisen due to duplication and evolutionary divergence from a common ancestor gene. The lamprey is the most primitive form of vertebrate and shares a common ancestor with humans between 400-500 years ago. Since fibrinogen chains from lampreys shared homology to human fibrinogen chains [55], the gene duplication that gave rise to the individual chains must have happened more than 400-500 million years ago [50] [56]. The divergence between γ and Bβ chains is more recent because the homology between these chains is higher when compared to the Aα-chain. This fact implies that Aα, pre-Bβ and pre-γ genes are originated from the duplication of an ancestral gene around 1 billion years ago. The opposite gene orientation of Bβ gene can be explained by the inversion of Aα and γ genes or inversion of Bβ gene during evolution [57].

II. Fibrinogen gene regulation

Basal fibrinogen gene expression

Compared to other coagulation factors, fibrinogen is expressed at relatively high levels under normal basal conditions at a concentration of 1.5 to 4 g/L in healthy individuals. Fibrinogen is mainly expressed by hepatocytes in the liver, but there are other non-hepatic tissues that express fibrinogen chains in small amounts (lung, brain and bone marrow) [46]. The fibrinogen genes are transcribed in a tightly coordinated manner since elevating the expression of any of the three fibrinogen genes leads to a simultaneous increase in the transcription of the three genes [58]. The mechanism for the fibrinogen transcriptional control seems to be due to the activity of proximal promoters and local enhancer elements. In the promoter region of the three fibrinogen genes, a TATA-like consensus sequence has been identified for the initiation of transcription. Moreover, there are binding sites in the promoter regions for hepatic-specific constitutive expression, while *FGA* and *FGB* expression is mostly dependent on the transcription factor HNF-1; the *FGG* expression is dependent on the transcription factor MLTF (Figure 6) [59] [60]. Furthermore, at basal levels, there seems to be an imbalanced expression of the three genes in both hepatic and non-hepatic cell lines [61]. It was suggested that synthesis of Bβ chain might be rate-limiting in the production of fibrinogen, thus leading to differential expression of the three genes [58] [59]. Other mechanisms influencing fibrinogen levels could be by the presence of (I) regulatory sequences in fibrinogen, (II) signaling pathways affecting fibrinogen gene expression regulation; and by (III) gene-environment interactions. In this sense, fibrinogen gene expression was studied after stimulation with pro-inflammatory agents; and a coordinated up-regulation of the three fibrinogen genes

was observed [61] [59]. The mechanism by which this coordinate fibrinogen expression is regulated is not yet completely understood.

Acute phase response fibrinogen gene expression

The acute phase response (APR) is a systemic defense system that is activated following tissue damage, infection, stress, neoplasia and inflammation. Pro-inflammatory (cytokines) and anti-inflammatory molecules (i.e., glucocorticoids) control and modulate the acute phase response. TNF-α and IL-1β are two of the first pro inflammatory cytokines that are produced and the ones that increase the expression of IL-6 [62]. These cytokines act on the liver to change the expression of proteins known as acute phase proteins or APPs. Within a few hours, the pattern of protein synthesis in the liver is altered: there is a decrease in the synthesis of normal blood proteins (negative APP) like transferrin and albumin and an increase in protein synthesis (positive APP) stimulated by cytokines. The APP genes are classified into Type I and Type II according to their cytokine responsiveness according to whether an IL-1β-controlled pathway is needed or not for their regulation. Therefore, IL-1, IL-6, TNF-α and glucocorticoids up-regulate Type I genes while IL-6 and GCs up-regulate Type II genes [63]. It is important to mention that this classification is simplified because cross-talks of TNF-α, IL-1β and IL-6 pathways occur as well in the hepatocyte. The liver-specific expression of APP genes is controlled by different transcription factors. Type I APP genes are regulated by the transcription factors CCAAT/enhancer-binding protein (C/EBP) and the nuclear factor (NF)-κβ which are activated by IL-1 and IL-6 or IL-1 and NF-κβ, respectively. The transcription factors C/EBP and NF-κβ upregulate the production of IL-6 which will activate the transcription factor STAT3 (signal transducer and activator of transcription) which, in

turn, will bind to IL-6 response elements (IL-6 Res) on the promoter of the Type II APP genes. Fibrinogen is a type II positive acute phase reactant protein and its expression is increased 2-10-fold throughout acute phase response. All three promoter regions from the three fibrinogen genes *FGA*, *FGB* and *FGG* contain IL-6 responsive elements, a C/EBP site and a HNF1 site [64] [65] [66]. Moreover, it is known that glucocorticoids synergistically enhance IL-6 response, and IL-6 at the same time has a synergistic effect upon glucocorticoid signaling [67]. Consequently, both IL-6 and glucocorticoids up-regulate fibrinogen expression (transcription of the three genes) during the acute phase response (Figure 6) [68] [69].

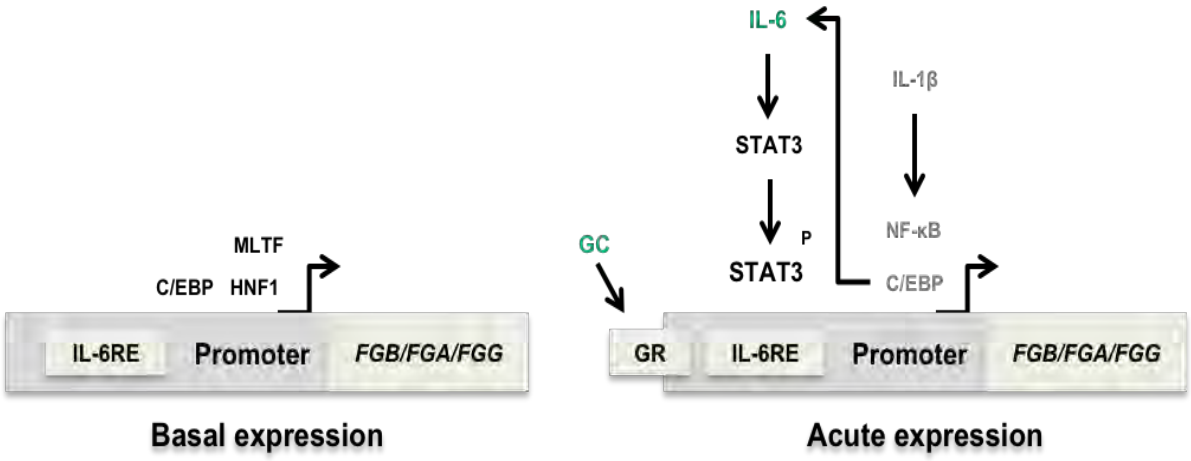

Figure 6. *Fibrinogen gene expression regulation. Left: fibrinogen gene regulation under basal conditions. Represented are the three fibrinogen genes (FGA, FGB, FGG) under the control of a promoter region, and the transcription factors i.e., HNF1, C/EBP and MLTF which bind to the promoter near the TSS (arrow). Right: fibrinogen gene expression under the acute phase inflammatory response. Glucocorticoids (GC), IL-1β and IL-6 stimulus lead to the final up-regulation of fibrinogen expression through different activation pathways: (I) GC binds to GC receptor (GR) enhancing IL-6 responses, (II) IL-6 leads to STAT3 activation (through phosphorylation) which then interacts with IL-6RE, (III) IL-1β activates the*

transcription factor (NF)-κB, which together with C/EBP up-regulates fibrinogen expression. Figure adapted from [46].

Enhancers

To date, four enhancers in the fibrinogen cluster have been identified in our laboratory: CNC12, PFE2, E3 and E4 [70] [71]. This cluster is flanked by CTCF sites, zinc-finger proteins that bind DNA which play a role in insulating genomic regions thus delineating regulatory units along the genome [72] [73]. In this sense, the fibrinogen cluster represents a regulatory unit where regulatory elements interact together and coordinate gene expression. Recently, our group investigated the local chromatin interactions inside the fibrinogen gene cluster which contribute to expression of the fibrinogen genes and protein production. Loss of function and restoration of function experiments using CRISPRcas9 technology to delete and reintroduce CTCF sites at one boundary of the fibrinogen gene cluster were performed in HepG2 cells [74]. This study showed that this cluster has a loop configuration and that local chromatin interactions occurring in the cluster contribute to gene expression and protein output. Furthermore, while the mRNA levels of the three fibrinogen genes were increased after inflammatory stimuli (IL-6, TNF-α), the fibrinogen loop configuration was not affected. Therefore, this cluster represents a regulatory unit where regulatory elements interact and contribute to coordinated expression, but its regulatory architecture is not affected by inflammatory stimuli [74].

III. Fibrinogen biosynthesis

Fibrinogen is composed of six polypeptide chains held together by 29 disulfide bonds making two symmetrical half-molecules which assemble together to form the fibrinogen hexamer (AαBβγ)2. Aα, Bβ and γ chains have 8, 11 and 10 cysteine residues, respectively, and the N-terminals of each of the six chains are held together in the central globule by disulfide bonds (rings). Three inter-chain disulfide bonds link the two half-molecules together and are necessary for assembly [75]. The inter-chain and intra-chain disulfide bonds between the fibrinogen chains play an important role in assembly and secretion, and their disruption will affect either one of the two or both processes [76] [77]. The fibrinogen genes translate into nascent polypeptides of pre-pro-Aα chain (644 aa residues), pre-pro-Bβ chain (491 aa residues) and pre-pro-γ chain (437 aa residues). During translocation through the ER, a signal peptide from each chain is cleaved and the full chain assembly occurs [78]. After translation, processing in ER and Golgi apparatus occurs. During assembly, facilitated by ER chaperones, there is a first step where two chain complexes are formed from single chains (Aα-γ and Bβ-γ), secondly a third chain is added to the two chain complexes, either by combining a Bβ chain with an Aα-γ dimer or an Aα chain with a Bβ-γ dimer, forming trimeric half molecules (AαBβγ). The final assembly step is where the two trimers (half-molecules) dimerize in the central nodule at their N-termini to form the hexamer [79] [80] [81] [82]. Then, before secretion, fibrinogen undergoes co-translational and post-translational modifications. N-glycosylation begins in the ER and finishes in the Golgi apparatus while phosphorylation and sulfation occur at a posterior ER stage (Figure 7) [83] [84]. All these modifications lead to a total molecular mass of about 340 kDa.

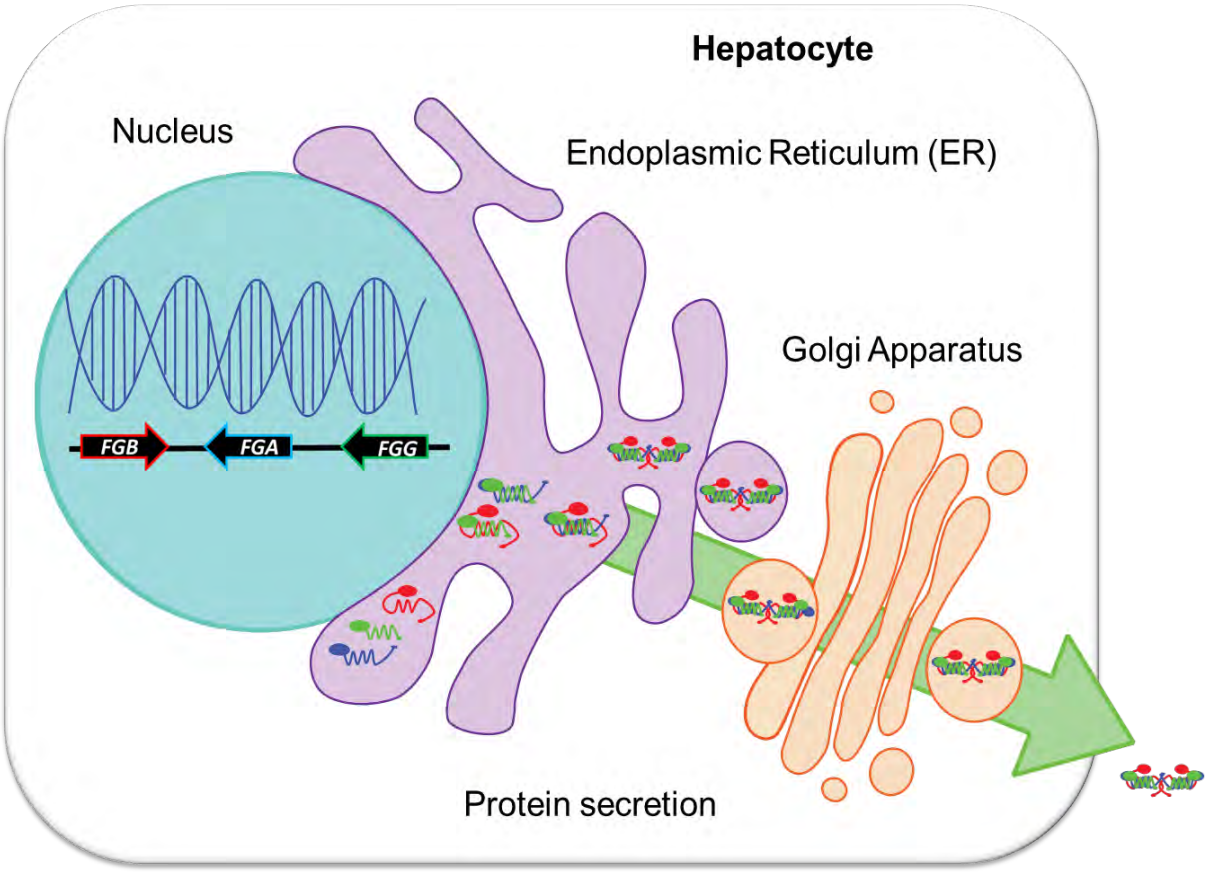

Figure 7. *Fibrinogen synthesis and expression. Fibrinogen is composed by two copies of three polypeptide chains (Aα, Bβand γ) which are encoded by FGA, FGB and FGG genes, respectively, which are clustered on human chromosome 4. After translation, fibrinogen chains are assembled in a stepwise manner in the endoplasmic reticulum (ER). Single chains assemble into Aα-γ or Bβ-γ precursors then into half molecules and finally into hexameric complexes. After fibrinogen is processed in the ER and the Golgi Apparatus, fibrinogen is released into the bloodstream by hepatocytes. Aα chains are showed in blue. Bβchains are showed in red and γ chains are showed in green.*

The fibrinogen molecule contains one central E region, two terminal D regions, two αC regions and two Bβ N regions. In the E region, there are four domains: (I) two coiled-coil domains, each one comprising a triple α-helical structure composed by the C-terminal of the Aα, Bβ and γ chains; (II) one funnel-shape domain formed by the N-terminal portions of two Aα and two Bβ chains; (III) and one γ N-domain, formed by the N-terminal part of both γ chains. Each D region is formed by seven domains: (I) a coiled-coil domain comprising a triple alpha-helical structure which is formed by the N-terminal parts of the Aα, Bβ and γ chains, (II) a β nodule (which has an A, B and P domain) and (III) a γ nodule (which has an A, B and P domain) formed by the C-terminal parts of the Bβ and γ chains, respectively. Furthermore, each fibrinogen half molecule has two other regions: an αC region and a Bβ N region. Although the structure of these regions is not present in the crystal structure, it is known from NMR studies. In turn, the αC region is formed by structurally distinct portions: (I) an α connector which is a flexible tether which contains the N terminal part (residues Aa221–391) and connects the αC region to the rest of the molecule, and (II) an αC folded compact domain which contains the C-terminal part (residues Aa392–610). The Bβ N region (functional BβN-domain) is composed by the N-terminal portion of the Bβ chain. Disruption of the αC region affects the hydrodynamic behavior of fibrinogen (Figure 8) [85] [86] [87] [88].

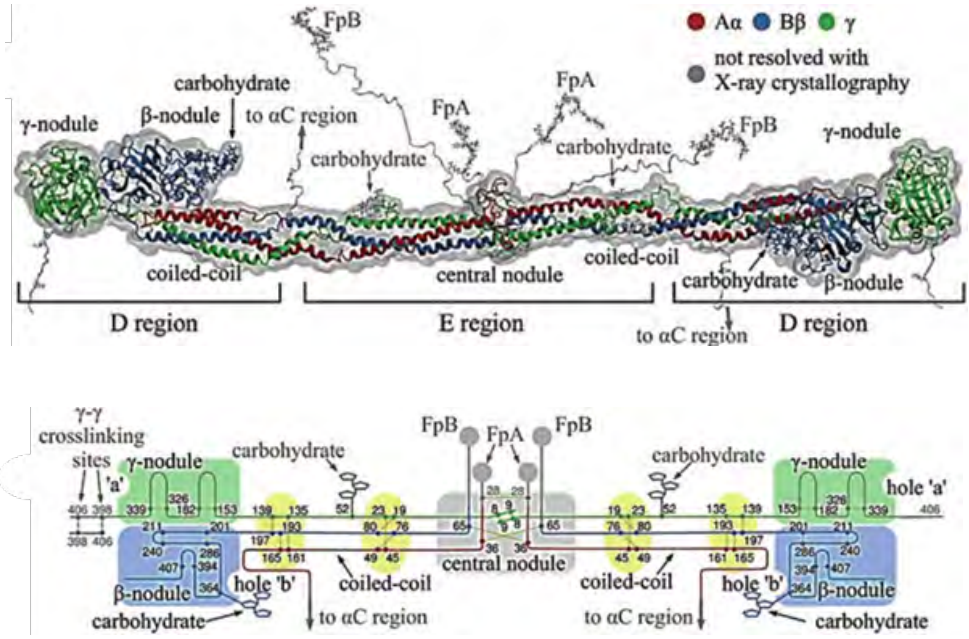

Figure 8. *Upper figure. X-ray crystallography structure of the fibrinogen molecule (PDB Entry: 3GHG) is shown and the portions missing from the crystal structure were reconstructed computationally (the N-terminal parts of Aα and Bβ chains with FpA and FpB in the central nodule and the beginning of the αC regions). Bottom figure. Diagram of the three fibrinogen polypeptide chains Aα, Bβ and γ chains are represented by lines and several structural regions of the fibrinogen molecule are labeled. (Weisel JW, 2017 [86].*

IV. Fibrinogen conversion to fibrin

The ultimate goal of the coagulation cascade is to stop bleeding with the conversion of the soluble form fibrinogen into the insoluble form fibrin. This conversion occurs through two steps: the enzymatic step and the non-enzymatic step.

In the enzymatic step, activated thrombin cleaves fibrinopeptides A and B from the N-terminal ends of Aα and Bβ fibrinogen chains [89] [90] [91], respectively, yielding α and β chains without the fibrinopeptides and exposing knobs “a” and knobs “b”, respectively. Fibrinopeptide A (FpA) is cleaved at the peptide bond AαArg16-Gly17 (without signal peptide), and Fibrinopeptide B (FpB) is cleaved at the peptide bond BβArg14-Gly15 (without signal peptide) [86]. Thus, the polypeptide composition of fibrinogen molecule after the fibrinopeptide thrombin cleavage is designated as (αβγ)2. No peptides are cleaved from γ chains; thus, the polypeptide composition of fibrin is (αβγ)2 and the conversion of fibrinogen to fibrin can be described as: (AαBβγ)2 → (αβγ)2 + 2FpA + 2FpB [92]. Depending on the order of fibrinopeptides removal from fibrinogen, there are different types of fibrin monomers. Normally, the fibrinopeptide A is first removed from fibrinogen to produce desA fibrin (αBβγ)2 and then fibrinopeptide B is removed to produce desAB fibrin (αβγ)2 [87]. More rarely, fibrinopeptide B is removed first to produce desB fibrin (Aαβγ)2. This distinction is due to spatial restrictions of the thrombin binding to fibrinogen where the N-terminal of the Aα chain is more accessible to thrombin active site than the one of Bβ chain [93].

The non-enzymatic step consists of spontaneous fibrin self-assembly. The cleavage of fibrinopeptides A and B from fibrinogen exposes new N-terminal sequences Gly-Pro-Arg (GPRV) called knobs (binding sites) “A” and Gly-His-Arg-Pro (GHRP)

sequences called knobs (binding sites) “B” in α and β chains, respectively, in the E region of fibrinogen [94] [86]. These knobs are complementary to pockets/holes “a” and “b”, respectively. Knobs “A” interact with pockets or holes “a” that are located in the γ nodule of D region; this is called an A-a interaction. The residues in the γ nodule involved in the A-a binding are γTrp315-Trp330, γTrp335-Asn365 and γPhe295-Thr305 [95]. Knobs “B” interact with pockets or holes “b” that are located in the β nodule of the D region, leading to a B-b interaction [96]. In this sense, knob-hole interactions lead to spontaneous fibrin self-assembly. The polymerization of fibrin starts after fibrinopeptide cleavage. Initially fibrinopeptide A is cleaved off more rapidly than fibrinopeptide B. After fibrinopeptide A is released from a fibrinogen molecule, monomers self-assemble to form a half-staggered dimer where the A-a binding is holding together that interaction [97]. In this sense, fibrin monomers add longitudinally to form two-stranded fibrin oligomers which lengthen to form two-strand protofibrils. Then protofibrils self-interact and aggregate laterally and longitudinally forming thicker fibers that branch to create the 3D structure network of fibrin polymer [87]. The lateral aggregation between two fibrin strands is mediated by the central E region of one fibrin strand and two D regions from two other fibrin strands. There are two different types of branch junctions in fibrin networks: (I) tetramolecular or bilateral junctions which occur when two double-stranded fibrils interact laterally to form a four-stranded fibril; and (II) trimolecular or equilateral junctions are formed by interaction of three double stranded fibrils (Figure 9) [98].

Despite the evidence for physical B-b interaction, their physiological role remains unclear. It seems B-b interactions are involved in lateral aggregation of proto-fibrils, although lateral aggregation can occur without the cleavage of FpB [99]. It appears

that B-b interactions can occur when A-a interactions are compromised, but their role is still unknown.

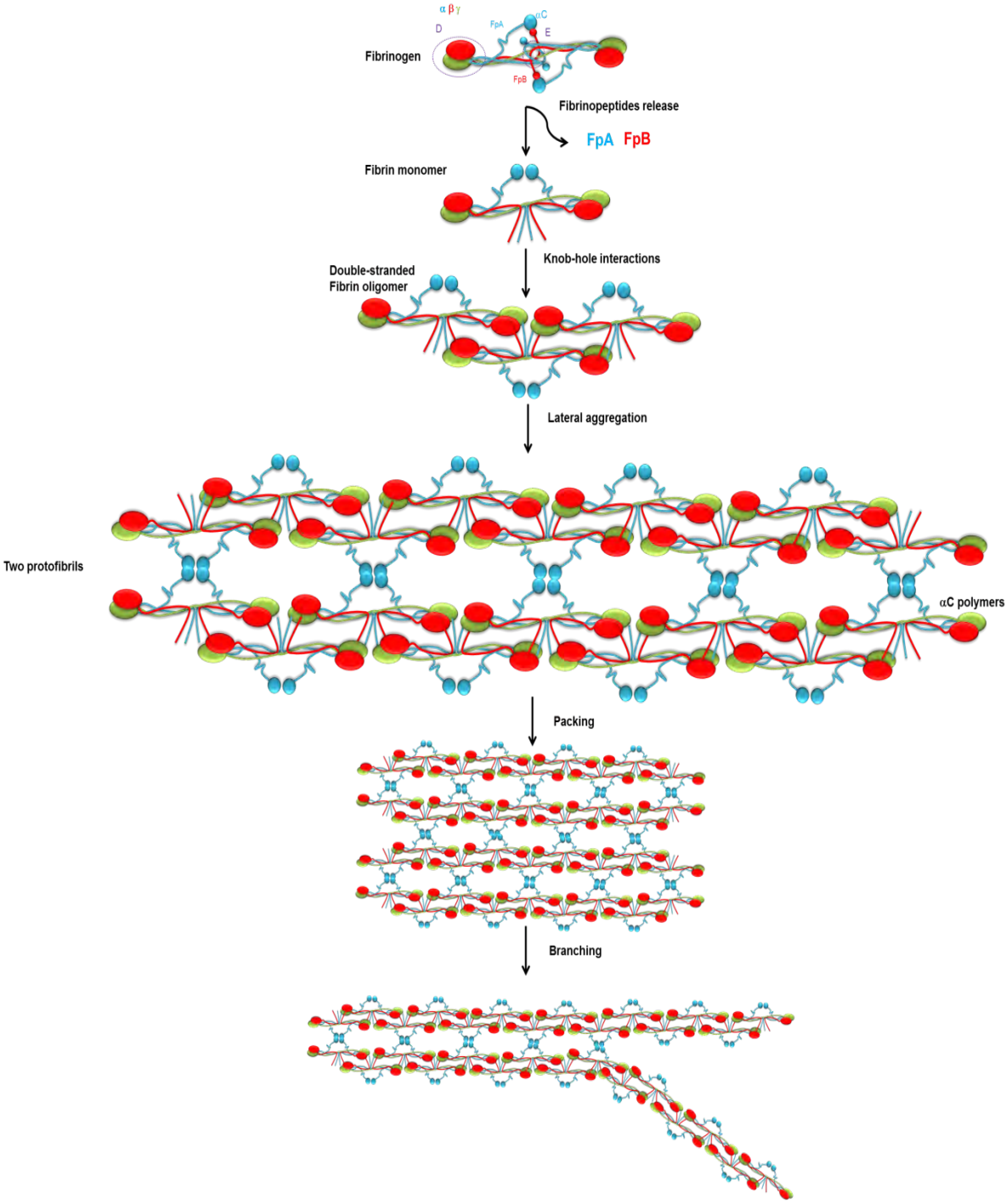

Figure 9. *Fibrin polymerization. Thrombin removes fibrinopeptides A and B from Aα and Bβ chains respectively, then knob-hole interaction occurs forming oligomers*

which elongate producing two-stranded proto-fibrils. Finally, proto-fibrils aggregate laterally to form fibrin fibers. Figure adapted from [99].

Fibrin crosslinking

When a fibrin clot is formed, it incorporates RBCs, white blood cells and platelets inside [100]. To stabilize the clot structurally and mechanically, fibrin is covalently crosslinked by the transglutaminase-activated FXIII [88] which is activated by thrombin in the presence of Ca2+. FXIIIa crosslinks fibrin by creating intra- and inter-fiber ε –amino(γ–glutamyl) lysine isopeptide bonds between lysine and glutamic acid residues [101]. The C-terminal region of each γ fibrinogen or fibrin chain contains one crosslinking site at which FXIIIa catalyzes the formation of γ dimers by introducing the intermolecular isopeptide covalent bond between the lysine 406 of one γ chain, and a glutamine 398 or 399 of another γ chain [102] [103] [104] [105] [106]. Other isopeptide bonds formed but at a lower rate are the intermolecular bonds between α C regions creating α oligomers and α polymers [107] [108]. Intermolecular crosslinking also occurs between α and γ chains [109]. Before crosslinking, fibrin polymerization is reversible, but, after crosslinking, it becomes irreversible, and the clot is more stable and resistant to fibrinolysis.

Fibrin clot structure

Fibrin is cross-linked by factor XIIIa and forms fibrillar aggregates, which, in combination with red blood cell and platelets, provide structural integrity to the

growing thrombus [36]. A fibrin clot is a gel where the fibrin fibers branch together, filling the space and creating a 3D network.

The structure of the clot can be characterized by the diameter of the fiber, its density, the number of branch points, the distance between the branch points and the pore size. The structure, formation and stability of the clot are influenced by the conditions present during fibrin generation [36] [37]. Factors affecting the formation and structure of fibrin can be divided into four categories. (I) Variations in the fibrinogen structure (hereditary and acquired): different clot structures are formed from different fibrinogen variants, polymorphisms and posttranslational modifications; for example, nearly all dysfibrinogenemia mutations have an impact on fibrin polymerization [110]. (II) Environmental conditions: the pH, endogenous or exogenous compounds (polyphosphates), medications, and plasma proteins affect polymerization. For instance, it has been observed that the local thrombin concentrations affect clot structure; at higher thrombin concentrations, the clot formed is more stable [25] [111] [112]. (III) Cellular effects: clot formation and architecture can be affected by direct contact with different cell types or by the active compounds and micro particles released from those cells. For instance, platelets affect clot contraction, stiffness, stability and resistance to fibrinolysis [113]. (IV) Hydrodynamic flow: blood flow, flow direction and shear forces of the bloodstream affect fibrin network and its structure and properties.

The structure of the fibrin clot can be directly related to clinical conditions [94] [114] [99]. While fragile clots are more susceptible to fibrinolysis and are likely to cause bleeding, firm clots are more resistant, but may promote thrombosis. Studies of thrombus formation and structure *in vitro* [115] [116] and *in vivo* [117] [118] [119] have been useful in explaining the mechanisms of several diseases related to

thrombotic complications. This information provides potential targets for modulating coagulation *in vivo* and new approaches to treat thrombotic or bleeding complications. Therefore, fibrin clot architecture is key in determining the efficiency of coagulation and fibrinolysis processes [120] [121].

V. Fibrin(ogen) biological functions

Fibrinogen is important in hemostasis because it acts as a bridge linking activated platelets together and favoring the formation of a platelet plug (primary hemostasis) but also because its conversion into fibrin generates a blood clot which stops hemorrhage (secondary hemostasis). During primary hemostasis, fibrinogen binds the integrin platelet receptor $\alpha_{IIb}\beta_3$, supporting platelet adhesion and aggregation [122] [123]. It has been reported that this fibrinogen- $\alpha_{IIb}\beta_3$ binding is mediated through three potential integrin binding sites found in fibrinogen: two RGD sequences in fibrinogen Aα-chain and one AGD amino acid sequence in γ-chain [124] [125] [126]. Moreover, it has been reported that AGD amino acid sequence serve as a competitive ligand for the $\alpha_{IIb}\beta_3$ integrin receptor in platelets [127], showing this receptor has a relaxed specificity for RGD and AGD ligands. Red blood cells (RBCs) play also an important role in hemostasis [128] [129]. The binding of fibrinogen to the RBC membrane is believed to be mediated by an integrin-like receptor (not $\alpha_{IIb}\beta_3$ but a related integrin with a β3 subunit) [130] or an integrin-associated protein (CD47) [130] [131] [132]. Recently, it was suggested that the integrin involved in this binding was the $\alpha_V\beta_3$ integrin through RGD motifs present in the fibrinogen Aα-chain [133] [134].

Apart from its role in hemostasis, the blood clot also functions as a temporary ECM matrix for wound healing, tissue repair, leukocyte cell adhesion and EC migration in angiogenesis [135]. The multifaceted role of the blood clot is due to fibrinogen and fibrin present multiple peptide motifs that can bind integrin and non-integrin receptors (ICAM-1, VE-Cadherin1) which are present on a variety of cells and molecules. Therefore, fibrinogen and fibrin act as a bridging molecule for many types of cell-cell

(platelets, RBCs, leukocytes, endothelial cells) and cell-molecule interactions (clotting factors, ECM proteins, growth factors, cytokines) [25], thus playing a pivotal role in coagulation and fibrinolysis (platelets, RBCs and clotting factors), inflammation (leukocytes, clotting factors and cytokines) [134] [136], angiogenesis and wound healing [135] [137] [138] [139] [140] (endothelial cells, clotting factors, ECM proteins, growth factors i.e., PDGF, TGF-β, FGF2, bFGF, VEGF and cytokines i.e., TNF-α, IL-1β). Integrin receptors can be classified into four categories based on their ligand specificity: RGD receptors ($\alpha_5\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_1$,$\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, and $\alpha_{IIb}\beta_3$), leukocyte specific receptors ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, and $\alpha_D\beta_2$), laminin receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, and $\alpha_6\beta_4$) and collagen receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$); or in their ability to recognize RGD-sequences in their ligands, LVD-sequences in their ligands, laminin or collagen, respectively [141] [142].

VI. Fibrinogen 420

Fibrinogen 420 structure

In a subclass of fibrinogen molecules that account for the 1-3 % of the total fibrinogen plasma, a globular C-terminal domain elongates the Aα-chain leading to the AαE isoform (Figure 10) [143]. The AαE chain is more than 50% longer than the common Aα chain since it contains an extra C-terminal globular domain encoded by exon 6 which is approximately 40% homologous to the C-terminal globular domain of fibrinogen Bβ and γ chains [143] [144]. Fibrinogen molecules, in which each of the two Aα-chains is replaced by an AαE isoform, are symmetrical molecules (homodimers) of the structure (AαEBβγ)2 and have a molecular mass of 420 kDa. This larger fibrinogen molecule is referred to as Fibrinogen 420. The Fib420 isoform is present throughout the vertebrate kingdom from lampreys to humans [144] [145] [146], and the AαE C-terminal globular domain is the single largest conserved portion of the Aα-chain. Therefore, the preservation of this fibrinogen subclass throughout vertebrate evolution suggests a vital physiological role that remains to be identified.

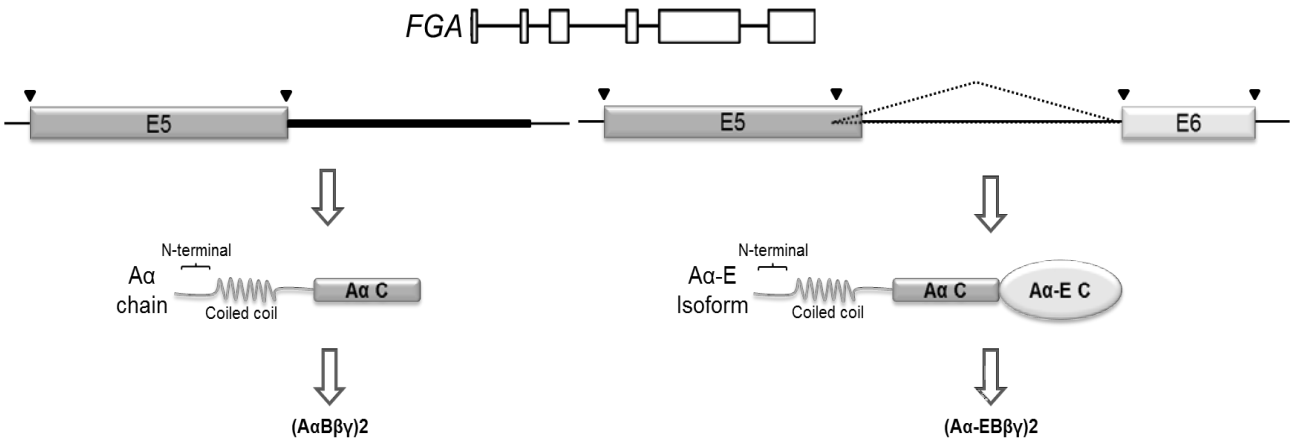

Figure 10. *Fibrinogen 340 and 420 produced from FGA gene. The FGA gene consists of six exons and three different mRNA transcripts are produced. In this figure appear two of them: the transcript encoding for the common Aα chain (on the left) and the transcript encoding for the AαE isoform (on the right) which is produced from alternative splicing. Both Aα C-terminal and AαE C-terminal globular domains are shown in the figure. Fibrinogen molecules formed from two Aα common chains are designed as (AαBβγ)2, while fibrinogen molecules formed from two AαE isoforms are designed as (AαEBβγ)2.*

Role of Fibrinogen 420

The AαE isoform has some distinguishable features which can be attributed to its possible functions. Because the AαE C-terminal domain contains a calcium binding site [147], it could be involved in the maintenance of a compact native structure. Because this domain is the only region of any Aα-chain to be glycosylated [148], it could play a role in the folding and stability of the protein, as well as in cell to cell adhesion processes via sugar binding. Because the AαE C-terminal domain supports strong adhesion and migration of leukocytes such as monocytes, neutrophils, and activated lymphocytes expressing $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins [134] [136], it could have a specific role in inflammation. Finally, because the AαE C-terminal domain enjoys more degrees of spatial freedom, it could be involved in the binding to other macromolecules or cells [146] [149] [150] with functions in coagulation, inflammation or healing.

Regarding clot formation (through polymerization and crosslinking) and the proteolytic susceptibility of Fib420 in comparison to fibrinogen 340 (Fib340), contradictory data is reported in the literature. Some studies have shown that both species have similar behaviors [147], while others have concluded that the AαE isoform is less susceptible to proteolytic degradation due to the inaccessibility of AαE chains to proteases [143] [149]. When it comes to the ultrastructure of both species, findings appear to be different. Studies have shown that Fib420 presented thinner and more highly branched fibers compared to Fib340 [149], likely due to the fact that AαE C-terminal domains are occupying the fiber surface and are slowing down the lateral fibrin assembly process. Fib420 and Fib340 are different in protein features and structure, which suggests that Fib420 could be involved in other biological functions. However, to date there is no suggestion that the presence of Fib420 may enhance or deteriorate clot lysis or formation (in structure or strength). Similarly, there is no indication whether it impacts coagulation, inflammation or healing in a positive or negative manner.

Newborn and adult levels of Fibrinogen 420

The development of coagulation is characterized by differential expression patterns and functions of certain coagulation factors from neonatal life throughout adult life [151] [152]. The hemostatic mechanism in newborns is immature and does not fully develop until the age of six months [92]. Factors I, V and VIII are within the normal adult range from conception until the third trimester and on. All other factors are variably reduced at birth, are lower in pre-term infants, and dependent on gestational age. Other characteristics of coagulation factors during fetal and neonatal

life include unique structures and functions. For instance, the transition of the fetal/neonatal fibrinogen form to the adult fibrinogen form is shown to have occurred by three weeks after birth [153], the significance of which is still under debate.

As previously mentioned, Fib420 is suspected to have a vital physiological function based on its preservation throughout vertebrate evolution. Analysis of plasma samples from adults and newborns, revealed a statistically significant difference between the levels of Fib420 in neonates (100ug/ml) compared to adults (34ug/ml). This is 3 times higher in newborns than adults [154]. On average, 1 out of 100 fibrinogen molecules in adult plasma belongs to the Fib420 subclass. Fib420 levels drop around six months after birth when other components of the coagulation system mature [92]. This suggests a change in the expression of the Fib420 subclass during life, which could be explained by an important but still unknown embryonic function of Fib420 in newborns. The mechanism by which this pattern of low Fib420 expression is maintained among healthy adult individuals but not among newborns is not fully understood, however it cannot be related to the initiation of fibrinogen transcription due to the fact that both FGA transcripts derive from the same *FGA* gene and have the same promoter. Thus, it is believed that it could be due to the regulation of the termination of transcription or the splicing efficiency [154]. It is also important to mention that Fib420, in contrast, is not a fetal form of fibrinogen since it persists through adult life, although in a lower concentration.

FIBRINOGEN DEFICIENCIES

Abnormal levels of proteins, enzymes or co-factors in the coagulation cascade can lead to various forms of coagulation disorders. Disorders affecting fibrinogen can be acquired or inherited. Acquired fibrinogen disorders can be caused by clinical conditions such as inflammation, tissue injury, liver disease, disseminated intravascular coagulation (DIC), sepsis with DIC, and cancers such as renal carcinoma and multiple myeloma [155] [156] [157], or induced by certain drugs and therapies such as tPA therapy, isotretinoin, valproic acid, thrombin inhibitors i.e., heparin, dabigatran, bivalirudin and argatroban, drug induced antifibrinogen antibodies i.e., isoniazid; and therapy induced antifibrinogenic antibodies i.e., fibrin sealant from bovine sources [158] [159] [160]. Hereditary disorders are due to mutations in the fibrinogen genes (*FGA*, *FGB* and *FGG*) (Figure 11) and lead to the deficiency of fibrinogen through various mechanisms: at the level of DNA by affecting transcription, at the level of RNA by altering mRNA splicing, stability or processing, or at the level of protein affecting translation, protein synthesis, assembly, stability, degradation, or secretion. Fibrinogen mutations have been described in all three encoding genes, but the largest number of mutations has been found in the *FGA* gene [161]. Congenital fibrinogen disorders (CFDs) are rare and can be classified into Type I (qualitative) and Type II (quantitative) disorders [111], affecting quantity (afibrinogenemia and hypofibrinogenemia), quality (dysfibrinogenemia), or both aspects (hypodysfibrinogenemia) of the fibrinogen molecule (Table 1) [162] [45] [163] [164]. While quantitative disorders may result from mutations affecting the synthesis, assembly, intracellular processing, and secretion of fibrinogen, qualitative disorders may be caused by mutations affecting fibrin polymerization, crosslinking, or

fibrinolysis processes [165]. According to annual global survey of the World Federation of Hemophilia (WFH), and the European Network of Rare Bleeding Disorders (EN-RBD), it has recently been established that congenital fibrinogen disorders represent around 8% of rare bleeding disorders [166] and that their prevalence is similar among men and women.

I. Hereditary fibrinogen disorders

Type I fibrinogen disorders:

Afibrinogenemia

Congenital afibrinogenemia is an autosomal recessive disease. It was first reported in 1920 in a 9-year-old boy [167], but the genetic cause of the disease was only identified in our laboratory in 1999 [11]. Homozygosity or compound heterozygosity for null mutations such as large deletions, frame shift, early-truncating nonsense, or splice-site mutations, as well as missense mutations, in one of the three fibrinogen genes, can cause afibrinogenemia.

The most frequent mutations found in afibrinogenemic patients are localized in the *FGA* gene, and are the 11 kb deletion [168], which is predicted to lead to the absence of Aα-chain; and the IVS4+1G>T splicesite mutation [169], which leads to a splicing defect. Along with large deletions, nonsense genetic variants linked with afibrinogenemia are among the most frequently found in *FGA* sequences [170] [171]. The incidence of afibrinogenemia is estimated to be 1 in 1 million (although is probably an underestimation), and is higher in areas where consanguinity is common. Afibrinogenemia is characterized by the absence of fibrinogen in plasma [168] [172] and may be associated with spontaneous bleeding, including bleeding in the umbilical cord after birth, skin, oral cavity, joint, gastrointestinal tract, genitourinary tract, or the central nervous system during intracranial hemorrhage, bleeding after trauma or surgical intervention, obstetric complications such as menorrhagia, recurrent spontaneous abortions, first-trimester miscarriages,

antepartum or postpartum hemorrhage, thrombosis (arterial and venous thromboembolic complications), musculoskeletal damage, or impaired wound healing. The predisposition to thrombosis episodes may be due to the fact that antithrombin activity of fibrin [173] [174] is absent in afibrinogenemic patients. These patients have increased prothrombin activation and thrombin generation [175] which stimulates platelet aggregation and consequently may increase the likelihood of thrombotic events [176] [177].

AFIBRINOGENEMIA Autosomal recessive disorder in homozygosity or compound heterozygosity	Complete absence of fibrinogen	Bleeding or asymptomatic phenotype Thrombotic phenotype
HYPOFIBRINOGENEMIA Autosomal recessive disorder mainly in heterozygosity	Proportional decrease of functional and antigenic fibrinogen levels	Mainly asymptomatic
DYSFIBRINOGENEMIA Autosomal dominant disorder mainly in heterozygosity	Decreased functional and normal antigenic fibrinogen levels	Bleeding or thrombotic phenotype or asymptomatic
HYPODYSFIBRINOGENEMIA Autosomal dominant disorder mainly in heterozygosity	Discrepant decrease of functional and antigenic fibrinogen levels	Bleeding or thrombotic phenotype

Table 1. *Congenital fibrinogen disorders. Classification of quantitative (afibrinogenemia and hypofibrinogenemia) and qualitative (dysfibrinogenemia and hypodysfibrinogenemia) congenital fibrinogen disorders.*

Hypofibrinogenemia

Hypofibrinogenemia was first described in 1935 [178] [111] and is characterized by reduced levels of functional fibrinogen in plasma. Patients with this disorder are often heterozygous carriers of mutations accounting for afibrinogenemia

[179]. Although the prevalence of this disorder is unknown, it is more frequent than that of afibrinogenemia. The majority of patients are asymptomatic because the reduced levels of fibrinogen in plasma are sufficient to avoid bleeding episodes, however, some experience bleeding episodes after trauma or if they present another bleeding disorder.

Thrombotic phenotypes in hypofibrinogenemic patients have been associated with fibrinogen β-chain variants. For example, the fibrinogen Bβ-chain Gly472Val missense change identified in a woman who had experienced thrombosis episodes and miscarriages [180], and the fibrinogen Bβ-chain Tyr368His variant identified in a man with venous thrombosis episodes [181]. Furthermore, a subgroup of hypofibrinogenemic patients present liver disease caused by hepatic inclusions due to the misfolding of a mutant form of fibrinogen that aggregates and accumulates in the endoplasmic reticulum from hepatocytes; the so-called fibrinogen storage disease (FSD) [182]. Only six mutations leading to FSD in hypofibrinogenemic patients have been found, and they are all clustered in exons 8 and 9 of *FGG*. Some examples include fibrinogen Brescia γGly284Arg (*FGG* c928G>C/A) [183] which was the first discovered, and fibrinogen Aguadilla γArg375Trp (*FGG* c1201C>T) [184].

Type II fibrinogen disorders:

Dysfibrinogenemia

Dysfibrinogenemia is an autosomal dominant disorder, mainly caused by heterozygosity for missense mutations in one of the three fibrinogen genes. In 1958 the first case of dysfibrinogenemia was described [185], while the first molecular

cause of this disorder was identified in 1968 (fibrinogen Detroit) [186]. The patient with this mutation showed excessive menstrual bleeding and was found to carry the AαArg38Ser mutation change.

Most of the variants identified in dysfibrinogenemic patients are found within the *FGA* exon 2 or the *FGG* exon 8 sequences, close to the N-terminal end of the fibrinogen Aα-chain and the C-terminal end of the fibrinogen γ-chain, respectively [162]. Overall, dysfibrinogenemia causing mutations lead to the production of a fibrinogen protein that is abnormal in structure and function, which has an impact on fibrinopeptide cleavage and release, fibrin polymerization, fibrin cross-linking, platelet aggregation [187] and fibrinolysis [162] [188].

As for hypofibrinogenemia, its prevalence in the population is higher than that of afibrinogenemia. Dysfibrinogenemias are characterized by normal antigen levels associated with disproportionately low functional activity. Patients are frequently asymptomatic, but some can experience bleeding (mostly after trauma, surgery or postpartum), and thromboembolic, mainly venous, complications, or both. Furthermore, women with dysfibrinogenemia may present miscarriages.

One of the most common hotspot dysfibrinogenemia mutations is the fibrinogen mutation AαArg16Cys. This mutation prevents thrombin cleavage of FpA, leading to impaired FpA release, delayed and/or impaired fibrinolysis and thus an aberrant and disorganized clot structure [189]. Another exampe is the heterozygous dysfibrinogenemia mutation fibrinogen Sumida (AαCys472Ser), which leads to a fibrinogen variant that impairs lateral aggregation of protofibrils [190]. Moreover, dysfibrinogenemia causing mutations can also affect the fibrinolytic process. For instance, the fibrinogen Dusart mutation (γArg554Cys), which can impair the binding

of tPA to fibrin, resulting in reduced plasminogen activation, impaired fibrinolysis, and an increased tendency for thrombosis [191] [192]. Additionally, some dysfibrinogenemic patients may experience renal amyloidosis [193] [188] [162]. In renal amyloidosis, the aberrant form of fibrinogen (e.g., aberrant form of fibrinogen due to the Indianapolis mutation, which is a frame shift mutation occurring in the C-terminal end of fibrinogen Aα chain [194] deposits extracellularly as amyloid fibrils in the kidney, ultimately leading to renal failure [195].

The possible causes explaining thrombotic events in patients with dysfibrinogenemia are that the aberrant fibrinogen molecule does not bind properly thrombin, leading to an increase in circulating thrombin, which can increase the activation of platelets and vascular endothelium, increasing the autoreactivity of both, or that the aberrant fibrinogen molecule forms an aberrant clot that is resistant to fibrinolysis [193].

It is often difficult to predict the genotype-phenotype relationship, making it difficult to anticipate whether a certain mutation will lead to bleeding, thrombosis, both or neither. Thus, in some cases structural analysis of fibrinogen is used to correlate the mutation with alterations in the function of the protein [188] [162].

Hypodysfibrinogenemia

Hypodysfibrinogenemia is an autosomal dominant disorder, mainly caused by missense mutations in one of the three fibrinogen genes [196], that leads to the production of an abnormal fibrinogen protein [162] [188]. It is associated with reduced levels of fibrinogen (<0.5 – 1 g/L) with low functional activity. The majority of the missense variants identified in hypodyfibrinogenemia occur within the C-terminal end

of the fibrinogen Aα and γ chains [197]. Patients with hypodysfibrinogenemia can experience bleeding and thrombosis but the incidence of these complications is not well estimated. The molecular mechanism causing hypodysfibrinogenemia can be traced to mutations affecting the processes of assembly, secretion and clearance [198] [199].

Abnormalities in the fibrinogen molecule have an impact on its protein structure, the physical and biological properties affecting fibrinopeptide release, fibrin polymerization, fibrin cross-linking and fibrinolysis processes. For instance, the hypodysfibrinogenemia fibrinogen Montpellier II (IVS2+3insCAT), which is an insertion of three nucleotides close to the donor splice site after exon 2 in the *FGA* gene. This mutation in heterozygosity leads to a mRNA missing exon 2 and therefore lacking the amino acids of the FpA and the knobs 'A'. This has detrimental effects on fibrin polymerization which impacts clot formation, leading to an abnormal clot characterized by non-uniform clusters of thinner fibers and large pores [200] [197].

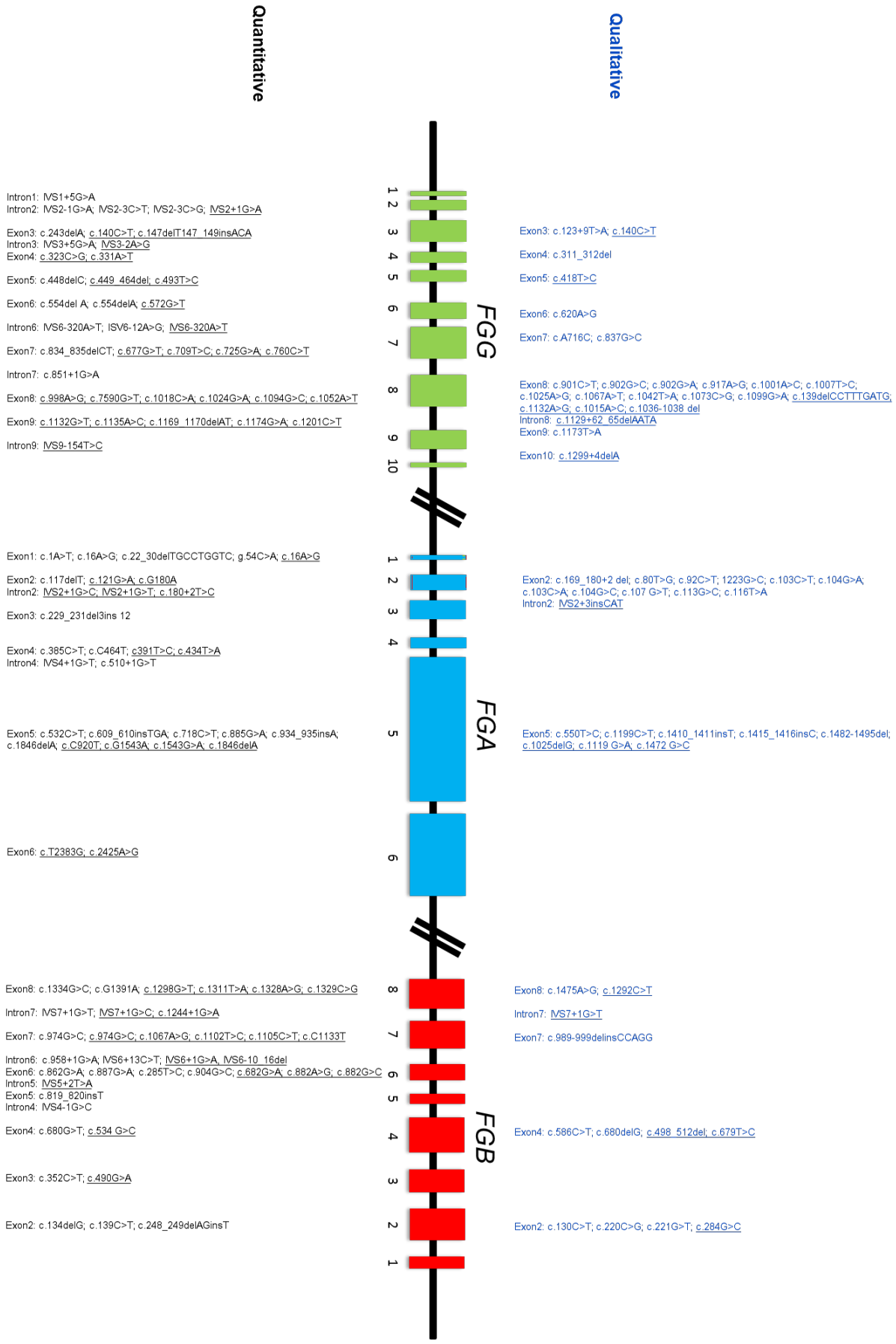
Quantitative
Qualitative
Intron1: IVS1+5G>A
Intron2: IVS2-1G>A; IVS2-3C>T; IVS2-3C>G; IVS2+1G>A
Exon3: c.243delA; c.140C>T; c.147delT147_149insACA
Intron3: IVS3+5G>A; IVS3-2A>G
Exon4: c.323C>G; c.331A>T
Exon5: c.448delC; c.449_464del; c.493T>C
Exon6: c.554del A; c.554delA; c.572G>T
Intron6: IVS6-320A>T; ISV6-12A>G; IVS6-320A>T
Exon7: c.834_835delCT; c.677G>T; c.709T>C; c.725G>A; c.760C>T
Intron7: c.851+1G>A
Exon8: c.998A>G; c.7590G>T; c.1018C>A; c.1024G>A; c.1094G>C; c.1052A>T
Exon9: c.1132G>T; c.1135A>C; c.1169_1170delAT; c.1174G>A; c.1201C>T
Intron9: IVS9-154T>C
1 2 3 4 5 6 7 8 9 10
FGG
Exon3: c.123+9T>A; c.140C>T
Exon4: c.311_312del
Exon5: c.418T>C
Exon6: c.620A>G
Exon7: c.A716C; c.837G>C
Exon8: c.901C>T; c.902G>C; c.902G>A; c.917A>G; c.1001A>C; c.1007T>C; c.1025A>G; c.1067A>T; c.1042T>A; c.1073C>G; c.1099G>A; c.139delCCTTTGATG; c.1132A>G; c.1015A>C; c.1036-1038 del
Intron8: c.1129+62_65delAATA
Exon9: c.1173T>A
Exon10: c.1299+4delA
Exon1: c.1A>T; c.16A>G; c.22_30delTGCCTGGTC; g.54C>A; c.16A>G
Exon2: c.117delT; c.121G>A; c.G180A
Intron2: IVS2+1G>C; IVS2+1G>T; c.180+2T>C
Exon3: c.229_231del3ins 12
Exon4: c.385C>T; c.C464T; c391T>C; c.434T>A
Intron4: IVS4+1G>T; c.510+1G>T
Exon5: c.532C>T; c.609_610insTGA; c.718C>T; c.885G>A; c.934_935insA; c.1846delA; c.C920T; c.G1543A; c.1543G>A; c.1846delA
Exon6: c.T2383G; c.2425A>G
1 2 3 4 5 6
FGA
Exon2: c.169_180+2 del; c.80T>G; c.92C>T; 1223G>C; c.103C>T; c.104G>A; c.103C>A; c.104G>C; c.107 G>T; c.113G>C; c.116T>A
Intron2: IVS2+3insCAT
Exon5: c.550T>C; c.1199C>T; c.1410_1411insT; c.1415_1416insC; c.1482-1495del; c.1025delG; c.1119 G>A; c.1472 G>C
Exon8: c.1334G>C; c.G1391A; c.1298G>T; c.1311T>A; c.1328A>G; c.1329C>G
Intron7: IVS7+1G>T; IVS7+1G>C; c.1244+1G>A
Exon7: c.974G>C; c.974G>C; c.1067A>G; c.1102T>C; c.1105C>T; c.C1133T
Intron6: c.958+1G>A; IVS6+13C>T; IVS6+1G>A, IVS6-10_16del
Exon6: c.862G>A; c.887G>A; c.285T>C; c.904G>C; c.682G>A; c.882A>G; c.882G>C
Intron5: IVS5+2T>A
Exon5: c.819_820insT
Intron4: IVS4-1G>C
Exon4: c.680G>T; c.534 G>C
Exon3: c.352C>T; c.490G>A
Exon2: c.134delG; c.139C>T; c.248_249delAGinsT
8 7 6 5 4 3 2 1
FGB
Exon8: c.1475A>G; c.1292C>T
Intron7: IVS7+1G>T
Exon7: c.989-999delinsCCAGG
Exon4: c.586C>T; c.680delG; c.498_512del; c.679T>C
Exon2: c.130C>T; c.220C>G; c.221G>T; c.284G>C

Figure 11. *Fibrinogen gene mutations accounting for afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia and hypodysfibrinogenemia. In black quantitative: afibrinogenemia (normal font), hypofibrinogenemia (underlined), in blue: qualitative: dysfibrinogenemia (normal font), hypodysfibrinogenemia (underlined). References for the original publications are found in www.geht.org. Figure adapted from [163].*

II. Diagnostic testing

The clinical impact of fibrinogen disorders is hard to predict, as it can vary from acute bleeding to thrombotic complications. Diagnosis is based on routine laboratory tests and confirmed by genotype although additional functional analysis can be performed to better define the patient's phenotype. The routine laboratory tests for the coagulation cascade, such as *in vitro* plasma-based laboratory tests, are used to identify deficiencies in the extrinsic (prothrombin time, PT), intrinsic (activated partial thromboplastin time, aPTT), and common pathways (thrombin time, TT) [201]. These three tests measure the time passed from the activation of coagulation until fibrin formation at different time points. The aPTT measures the time needed to form fibrin, using the initiation of intrinsic pathway as a starting point. To activate the intrinsic pathway two substances are used: (I) kaolin which directly activates FXII and (II) cephalin which provides the phospholipid surface needed for normal functioning of the coagulation cascade. The aPTT test is abnormal when XII, IX, XI, VIII, X, V, fibrinogen and prothrombin are present in small amounts (intrinsic and common pathways) or due to the presence of inhibitors to these reactions. Therefore, this test is a good method to screen hemophilia A (FVIII deficiency) and hemophilia B (FIX deficiency) deficiencies, in which this test is prolonged. The PT measures the time needed to form fibrin using the activation of FVII as starting point. In this test, thromboplastin is used as an activating agent. A PT test is more sensitive than an aPTT test, and it can identify a small decrease in FVII levels, this makes it a good test to detect FVII deficiency. The TT measures the time needed for fibrinogen to fibrin conversion reaction, so it is sensitive to abnormal levels of fibrinogen. Abnormalities can be due to a decrease in fibrinogen (afibrinogenemias and/or

hypofibrinogenemias), aberrant fibrinogen (dysfibrinogenemias and/or hypodysfibrinogenemias), or the presence of an inhibitor to the reaction (heparin and/or fibrin degradation products (FDP)). Thrombin is needed in this test to activate conversion of fibrinogen into fibrin [202] [203] [204].

III. Clinical management

The clinical management of patients with congenital fibrinogen disorders is challenging. Current therapeutic strategies for the treatment and prevention of bleeding in patients with fibrinogen related disorders (FRD) include fibrinogen replacement therapy: fibrinogen concentrates, cryo-precipitates and fresh frozen plasma (FFP) [193]. Between them, fibrinogen concentrates (Clottafact/Clottagen, Fibrinogen HT, Haemocomplettan/RiaSTAP) represent the best alternative because they carry a lower risk of transfusion reactions. Conversely, FRD patients with thrombotic complications should be treated with anticoagulants (Warfarin, low MW heparins) alone or in combination with fibrinogen replacement therapy [163] [193]. Treatments for bleeding and thrombosis will ultimately depend on the type and severity of symptoms as well as the genotype and familial history of the patients.

IV. Fibrinogen in disease

In healthy individuals, fibrinogen levels vary between 2 to 4 mg/ml. The variability in the levels of plasma fibrinogen between individuals is due to genetic and environmental factors such as sex, race and age. Genetic factors accounting for gene mutations, and environmental factors accounting for lifestyle (diet, alcohol consumption, smoking history, and physical activity), hormone and cholesterol levels, obesity, blood pressure, and medications [205].

As previously discussed, activation of the coagulation pathway is essential to stop bleeding after a vascular injury. Fibrin will seal damaged tissue but will also serve as a matrix for wound healing and tissue repair. Deregulation of these tightly controlled processes is associated with hemorrhagic or thrombotic incidents and tissue fibrosis and inflammation.

Elevated fibrin(ogen) and/or D-dimers (fibrin degradation products) in the blood are considered markers for inflammation, increased coagulation activity and thrombosis. The association of fibrin(ogen) with a risk of cardiovascular diseases (CVDs) and mortality has been proven already through different meta-analysis studies (The Fibrinogen Studies Collaboration [206], The Emerging Risk Factors Collaboration) [207], showing that an increase in plasma fibrinogen concentration was linked to atherogenesis, thrombogenesis, ischemia, coronary heart disease, stroke and cerebrovascular and peripheral vascular disease [208] [209].

The fibrinogen variant γA/γ′ has been shown to be an etiologic risk factor for CVDs. Several studies have associated elevated circulating levels of the γA/γ′ fibrinogen isoform with coronary artery disease [210] [206], myocardial infarction [211], and

ischemic stroke [212]. Furthermore, the abnormal fibrin clot structure formed from the γA/γ′ fibrinogen variants, was shown to be associated with CVDs risk [213] [214]. However to date, there are no studies associating the other fibrinogen variant, the AαE isoform, with CVDs.

Apart from bleeding after a physical vascular injury, there are several vascular, neurologic, infectious, inflammatory, autoimmune, or cancerous diseases that involve vascular rupture resulting in consequent leakage of plasma proteins in the perivascular space [215] [216] [217]. The interaction of plasma factors with the perivascular interstitial fluid ultimately determines the development and extent of these diseases [218] [216]. Fibrin(ogen) and D-dimers pro-inflammatory role has been described in autoimmune and inflammatory diseases (rheumatoid arthritis, bacterial infection, colitis, and lung and kidney fibrosis) [219], neurologic diseases with BBB disruption (multiple sclerosis, Alzheimer's disease) [220], and several types of cancer [221]. Their pro-inflammatory function is associated with their ability to bind different immune cells through different ligand-receptor interactions; i.e., the fibrinogen-leukocyte binding through the $\alpha_M\beta_2$ leukocyte receptor [216] [215].

Overall, the multifaceted role of fibrinogen, fibrin and D-dimers as pro-coagulant and pro-inflammatory molecules in both the intravascular and the perivascular space has recently become evident [217] [216].

V. Animal models for fibrinogen disorders

The contribution of fibrinogen to coagulation has been studied in several mice models [222]. Loss of fibrinogen in circulation has been studied thanks to the generation of the afibrinogenemic mice model, which had disrupted the fibrinogen Aα chain gene (*Fga*$^{-/-}$) [223]. These mice showed complete loss of fibrinogen in plasma, loss of clotting and platelet aggregation, spontaneous hemorrhagic episodes in neonates and adults, wound healing defects [224] and termination of pregnancy in females [223]. The symptoms seen in these mice recapitulate the phenotype seen in afibrinogenemia patients, which make them a good model to study afibrinogenemia disorder.

The role of fibrinogen versus fibrin has been studied thanks to the generation of a transgenic mouse model called *Fib*^{AEK}. The *Fib*^{AEK} mutant mice have a fibrinogen form that cannot be cleaved by thrombin (so cannot support fibrin formation) but that can support platelet aggregation [225]. These mice showed bleeding complications, indicating that not only fibrinogen-platelet interaction but also fibrin formation is critical for hemostasis. Furthermore, a mouse model called *Fbg*$^{\gamma\Delta 5}$, was genertaed to identify the critical role of the C-terminal end of γ-chain in platelet aggregation (platelet-fibrinogen-platelet interaction). This mouse model has a five-residue truncation in the C-terminal of the γ-chain that leads to the elimination of the motif QAGDV, which is recognized by the platelet integrin receptor $\alpha_{IIb}\beta_3$ [226]. Homozygous mice showed indefinite bleeding at injury sites in large vessels; however, homozygous females were able to support pregnancy.

Another mouse model called *Fbg*$^{\gamma\Delta 319,320}$ has been generated to mimic the human heterozygous dysfibrinogenemia mutation, the fibrinogen Vlissingen/Frankfurt IV

[227]. This mutation is characterized as a deletion of the amino acids Asn319 and Asp320 from the C-terminal end of the fibrinogen γ-chain. These mice had a fibrinogen molecule (similar to humans) defective in platelet aggregation, fibrin polymerization and calcium binding [228]. Homozygous mice with this mutation showed severe bleeding, and most died afterbirth due to abdominal hemorrhage, while heterozygous mice appeared normal.

Moreover, hyperfibrinogenemia has been studied thanks to the generation of a transgenic mouse model accounting for elevated fibrinogen in plasma [229] by over-expressing all three fibrinogen genes. No sign of mortality, morbidity, increase in organ size or histological differences (lung, liver, spleen, and kidney) was found between normal and hyperfibrinogenemia transgenic mice. Nevertheless, high fibrinogen plasma levels are still considered as a marker for atherothrombotic and/or cardiovascular diseases in humans.

Besides coagulation, the contribution of fibrinogen to inflammation has also been studied in several mice models (Table 2) [216]. The role of fibrinogen-leukocyte binding during inflammation was studied thanks to the generation of the *Fibγ*$^{390-396A}$ mouse model, which carries a mutation in γ-chain that eliminates the fibrinogen binding motif to $\alpha_M\beta_2$ receptor from leukocytes. This mouse model showed compromised antimicrobial host defense function [230] and a protective role against inflammatory diseases like arthritis [231], neuroinflammatory disease [232], and musculoskeletal disease [233].

Fibrinogen-deficient mice with a targeted deletion of the fibrinogen γ-chain gene (*Fgg*$^{-/-}$) were generated to study pulmonary fibrosis. Although wild type and fibrinogen deficient mice (*Fgg*$^{-/-}$) showed similar lung collagen deposition after inducing

pulmonary fibrosis (with bleomycin drug). Wild type mice showed a significant increase in neutrophil infiltration in the early days during inflammation, suggesting a possible role of fibrinogen during the early acute inflammatory response [234].

Furthermore, fibrinogen contribution in host defense mechanisms and infection has been studied thanks to the generation of several mice models. For instance, a fibrinogen deficient (*Fga*$^{-/-}$) mouse model [223] showed compromised pathogen clearance after Staphylococcus aureus infection [235]; a similar outcome to that observed in the *Fib*^{AEK} mutant mouse [225]. Thanks to these two last mice models we can also unravel differences in the function of fibrinogen versus fibrin during microbial infection, with fibrin, rather than fibrinogen, being most effective for antimicrobial host defense.

	Mice models	Mouse line	Murine phenotype	References
Coagulation				
	Fib$^{-/-}$	Afibrinogenemia	Loss of clotting and platelet aggregation, spontaneous hemorrhagic episodes, absorptions, wound healing defects	[223] [224]
	Fib^{AEK}	Mice with fibrinogen form that cannot support fibrin formation	Bleeding complications	[225]
	Fbg$^{\gamma\Delta5}$	Mice with fibrinogen without $\alpha_{IIb}\beta_3$ binding motif in γ-chain	Impaired platelet aggregation and increased bleeding time	[226]
	Fbg$^{\gamma\Delta319,320}$	Dysfibrinogenemia mutation Fibrinogen Vlissingen/Frankfurt IV	Fibrinogen molecule defective in calcium binding, fibrin polymerization and platelet aggregation	[227] [228]
	Fibrinogen transgene (FT) mice	Hyperfibrinogenemia	Normal phenotype compared to wild type mice	[229]
Inflammation	***Fib$^{-/-}$***	Pulmonary fibrosis	Increase in neutrophil infiltration at early days during inflammation	[234].
		Host defense	Compromised pathogen clearance after microbial infection	[235]
	Fib^{AEK}	Mice with fibrinogen form that cannot support fibrin formation	Compromised pathogen clearance after microbial infection	[225]
	Fibγ $^{390–396A}$	Mice with fibrinogen without $\alpha_M\beta_2$ binding motif in γ-chain	Compromised antimicrobial host defense function, protective function against inflammatory diseases (arthritis, neuroinflammatory and musculoskeletal disease)	[230] [231] [232] [233]

Table 2. *Summary of fibrinogen mice models in coagulation and inflammation studies and their related pathologic phenotype.*

Mice are the mammalian species most commonly used as a research model in thrombosis and hemostasis [236] [237] [238] [239]. They possess a mammalian system with physiological similarities to humans, they are easy to manipulate genetically and they are easy to house and breed [240]. However, a new animal model to study several human disorders was proposed in 1980 by Streisinger [241] and has since become a useful tool for the study of thrombosis and hemostasis: This animal model is zebrafish.

ZEBRAFISH

I. Zebrafish as a model to study hemostasis and thrombosis

In vitro studies using [242] [243] [244] [245] transfected cell models have been useful to study and characterize several coagulation factors and their mutations at different levels: mRNA expression and splicing, protein synthesis, assembly, and secretion. Nevertheless, these *in vitro* models show limitations because they cannot explain most of the physiological functions of coagulation factors and the pathophysiology of their mutations.

Mouse models have been used [222] [239], but these are expensive and time consuming. The zebrafish (*Danio rerio*), is a vertebrate organism broadly used to model several human disorders [246], including blood bleeding disorders [247] [248]. This model shows several advantages in thrombosis and hemostasis research. First, the transparent embryogenesis of zebrafish embryos, where blood vessels are visible between 48h to 5dpf, make them useful for *in vivo* studies in vascular biology (e.g., laser mediated vascular injury). Second, hemostatic processes are conserved between vertebrates [249] [250]. Indeed, the zebrafish has orthologs for the majority of human coagulation factors [251] (with the exception of FXI, FXII and prekallikrein) [252] [253], it possesses a vascular endothelium that resembles the mammalian one [254], and nucleated thrombocytes, which are the functional cellular equivalents to human platelets [255]. Third, straightforward genetic manipulation is possible because the zebrafish genome is available [256], which makes feasible the use of

genetic engineering systems (i.e., ZFN technology, TALEN system [257] and/or CRISPR-Cas9 [258] for generating knock-outs; antisense morpholinos for generating knock-downs [171]; and single-stranded oligodeoxynucleotides ssODN and/or CRISPR-Cas9 for generating knock-ins [259] to generate zebrafish models for different human disorders.

Utilizing *in vivo* morpholino technology to knock-down genes responsible for bleeding disorders or deficiencies makes it possible to further study their mechanism of action in the context of hemostasis and thrombosis (i.e., bleeding/hemorrhagic phenotype, thrombocyte function and thrombotic phenotype). Although morpholino technology was used to gene knock-down several coagulation factors (fibrinogen, vWF, prothrombin, factor VII) [252], this technique shows an important limitation: the transient and incomplete silencing of the gene, which leads to variability in the observed phenotype as well as misinterpretation. To avoid this limitation, knock-out (ko) zebrafish models for several clotting factors, and for mutations that cause bleeding disorders have been generated over the past years. The first stable zebrafish model for a human bleeding disorder was the afibrinogenemia zebrafish, which was generated in our lab using zinc-finger nuclease technology (ZFN) [260]. Since then, other ko zebrafish models for known clotting factors responsible for human bleeding disorders were generated; i.e., the antithrombin III deficiency zebrafish model using ZFN technology [261], the FX deficiency zebrafish model using genome editing with transcription activator-like effector nucleases (TALEN) [262], and the FV deficiency zebrafish model using CRISPR-mediated genome editing [263].

Furthermore, transgenic animals are powerful tools to study gene and cellular functions *in vivo.* There are several transgenic zebrafish lines that were generated to study cellular functions [264]*:* (I) the *Tg(itga2b:EGFP)* zebrafish, which expresses the

green fluorescent protein GFP under the control of the *itga2b* gene promoter. This line has green fluorescent thrombocytes visible in the bloodstream by microscopy around 48h post fertilization (hpf) [265]; and (II) the *Tg(gata1:dsRed)*^{sd2} zebrafish, which expresses the dsRed cDNA under the control of the *gata1* promoter. This line has red erythrocytes in the bloodstream, after the onset of blood circulation, at 24 hpf [266]. The *Tg(itga2b:EGFP)* and *Tg(gata1:dsRed)*^{sd2} zebrafish lines have been used to study hematopoiesis but also to study the roles of thrombocytes and erythrocytes, respectively, in hemostasis [267] [268] [269] [270] (i.e., characterizing thrombocyte/erythrocyte function) and in thrombosis [271] [272] (i.e., studying thrombosis by measuring the fluorescence intensity of thrombocyte/erythrocyte aggregates).

Overall, knock-out and fluorescent reporter transgenic zebrafish lines, combined with *in vivo* microscopy, are useful tools to understand and decipher the pathophysiology of bleeding-related disorders.

II. Functional tests to study hemostasis and thrombosis

Apart from genetic screenings to study the hemostatic function of coagulation factors and cells in physiological and pathophysiological (bleeding and/or thrombosis) conditions in zebrafish embryos and adults, there are other functional tests that, used in combination with genetic screening methods, help us to better understand the pathophysiology of specific disorders.

First, some considerations must be made before selecting a good model of thrombosis and/or hemostasis. A good model for both clinical phenomena, should: (I)

trigger the shortcoming which leads to a thrombotic/bleeding phenotype, (II) be studied in a vessel that resembles anatomically, the one in which thrombosis/bleeding occurs, (III) the outcome should be easily translated into data and (IV) this data should provide as much information as possible about thrombosis/bleeding (i.e., blood flow rate, dynamics, blood clot formation and dissolution and platelet behavior) as possible [273].

There are several vascular injury methods used to study hemostasis and thrombosis *in vivo* in murine and non-murine animal models (i.e., electrical and mechanical injuries); these involve severe surgical intervention that can lead to non-specific activation of the coagulation cascade. To avoid this problem, a laser-induced injury model was proposed; whereby the energy of the laser is focused at the site of interest, and the intensity of the laser beam is adjusted to control the injury depth in the tissue [274].

In recent years, the laser-mediated endothelial injury has been used as a useful functional test to assess bleeding and thrombus formation under several experimental conditions [275] [276]. Applied to zebrafish [277] [278] [279], this method is used to make a laser-mediated vascular injury in the posterior caudal vein (PCV) and/or in the dorsal aorta (DA) of zebrafish larvae, in order to study different parameters (i.e., clot formation, stability and dissolution) (Figure 12). The time to occlusion (TTO) is the time at which blood flow is completely stopped due to the formation of a blood clot (venous/arterial occlusion). The time to adherence (TTA) is the time taken for the first cell to adhere in the injured endothelium after laser injury. The time to dissolution (TTD) is the time at which the blood clot (thrombus) begins to dissolve [277] [239]. Gene knock-downs, gene knock-outs and gene knock-ins zebrafish, when combined with the laser-induced thrombosis method, represent a

powerful combination to study complex processes such as hemostasis and thrombosis in a living animal.

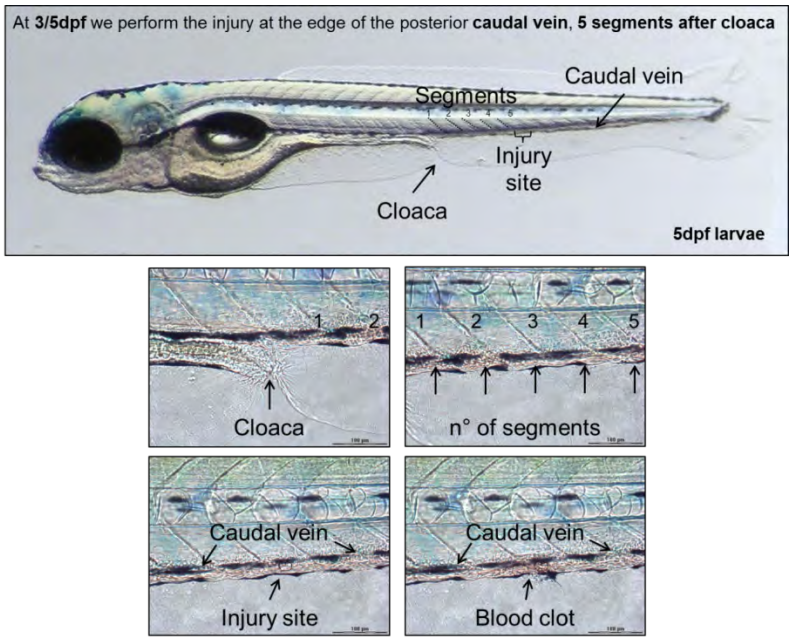

Figure 12. *Laser induced vessel injury in zebrafish larvae. 5 days post fertilization (dpf) larvae bright field images (20X) taken from the microscope Leica Laser Microdissection (LMD). Larvae cloaca, n° of segments, caudal vein, site of injury and blood clot formation images are shown.*

RESULTS

PART I: Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders

Abstract

Congenital fibrinogen disorders can be divided into quantitative (i.e. afibrinogenemia) and qualitative disorders (i.e. dysfibrinogenemia). Afibrinogenemia is characterized by the complete absence of fibrinogen in plasma, while dysfibrinogenemia is characterized by normal levels of a low functional fibrinogen molecule. Both disorders are associated with bleeding and thrombotic phenotypes. Our goal was to characterize venous thrombosis in larval zebrafish models of afibrinogenemia and dysfibrinogenemia to distinguish the pathophysiological differences in these disorders. To do this, we used the afibrinogenemia (*fga*$^{-/-}$) zebrafish model, previously generated in our laboratory; and attempted to generate a dysfibrinogenemia (*fga* R28C) zebrafish model using CRISPR/Cas9 genome editing technology. The *fga* R28C mutant zebrafish have a mutation in the fibrinogen Aα thrombin cleavage site equivalent to the *FGA* R35C mutation found in dysfibrinogenemic patients. The *fga* R28C mutant zebrafish unexpectedly skipped *fga* exon 2, leading to a mutant zebrafish (*fga*$^{+/\Delta 19\text{-}56}$) that resembled the human mutation Fibrinogen Montpellier II, which causes hypodysfibrinogenemia. We therefore modeled the human *FGA* R35C dysfibrinogenemia mutation in zebrafish by transgenic expression of *fga* R28C cDNA in *fga* knock-out (*fga*$^{-/-}$) and/or *fga* knock-down zebrafish embryos. To characterize the thrombotic phenotype in these zebrafish models, we measured venous thrombosis via time to occlusion (TTO) and thrombocyte recruitment (adhesion and accumulation) assays, after performing vascular endothelial laser injuries in the zebrafish larvae. We observed that *fga*$^{-/-}$ larvae failed to support venous thrombosis and showed a decrease in thrombocyte recruitment, in contrast to wild-type larvae. Additionally, dysfibrinogenemic (larvae expressing *fga* R28C cDNA) and hypodysfibrinogenemic (*fga*$^{+/\Delta 19\text{-}56}$) larvae showed similar dominant effects on TTO

and thrombocyte recruitment assays. We concluded that thrombosis assays performed in larval zebrafish models are good methods to distinguish phenotype differences between zebrafish models of quantitative and qualitative congenital fibrinogen disorders.

Aims

Congenital fibrinogen disorders are caused by mutations in the three fibrinogen genes *(FGA, FGB, FGG*), these include afibrinogenemia, where there is no detectable plasma fibrinogen; and dysfibrinogenemia where the concentration of plasma fibrinogen is discordant with its functional activity. Both disorders can lead to bleeding and thrombotic complications.

In the first part of the thesis, our first aim was to study the pathophysiology of afibrinogenemia and dysfibrinogenemia human disorders using zebrafish as a model organism. For this purpose we have generated zebrafish models of afibrinogenemia (*fga*$^{-/-}$) and dysfibrinogenemia dyfibrinogenemia (*fga*$^{+/R28C}$) by genome editing. The *fga*$^{-/-}$ zebrafish lack plasma fibrinogen, while the *fga*$^{+/R28C}$ zebrafish have a mutation in the fibrinogen Aα thrombin cleavage site which prevents normal fibrin formation, as seen in dysfibrinogenemic patients with the Aα R35C mutation.

Since fibrinogen and fibrin are known to play overlapping roles in coagulation, fibrinolysis, inflammation and wound healing; our second aim was to unravel the differences in the roles of fibrinogen versus fibrin in the context of hemostasis and thrombosis.

Manuscript to submit

Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders*

C. Freire[1], R.J. Fish[1], Corinne Di Sanza[1], Marguerite Neerman-Arbez[1]

[1]Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva.

My contribution: Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, Figure 7B and Figure 8B. Supplementary Figures 1, 2, 3, 4 and 5.

*Manuscript to submit

Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, 1, rue Michel Servet, 1211, Geneva Switzerland

Tel: +41223795655

Fax: +41223795707

Email: Marguerite.Neerman-Arbez@unige.ch

Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders

Cristina Freire[1], Richard J. Fish[1], Corinne Di Sanza[1], Marguerite Neerman-Arbez[1]

[1] Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Geneva, Switzerland.

*Manuscript to submit

Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, 1, rue Michel Servet, 1211, Geneva Switzerland

Tel: +41223795655

Fax: +41223795707

Email: Marguerite.Neerman-Arbez@unige.ch

Essentials

- Venous thrombosis occurs in quantitative and qualitative congenital fibrinogen disorders
- Venous thrombosis was measured in zebrafish models of afib-, dysfib- and hypodysfibrinogenemia
- Vessel occlusion and thrombocyte binding assays provided a thrombosis profile for each model
- The experimental thrombosis phenotype in zebrafish can distinguish certain fibrinogen disorders

Summary

Background: Venous thrombosis occurs in patients with quantitative and qualitative fibrinogen disorders. Injury-induced thrombosis in zebrafish can be used to model aspects of human coagulopathies.

Objectives: To characterize venous thrombosis in zebrafish models of afibrinogenemia and dysfibrinogenemia and determine whether their thrombotic phenotypes differ.

Methods: Larval venous laser injuries were used to measure thrombosis via the time-to-occlusion (TTO) and the binding and aggregation of fluorescent *Tg(itga2b:EGFP)* thrombocytes. These assays were applied to models of afibrinogenemia (*fga*$^{-/-}$), dysfibrinogenemia with transgenic expression of a mutated fibrinogen Aα chain that mimics a causative human mutation, and in a hypodysfibrinogenemia model fortuitously produced by CRISPR-Cas9 genome editing.

Results and Conclusions: *fga*$^{-/-}$ larvae failed to support occlusive venous thrombosis and showed reduced thrombocyte binding and aggregation at injury sites compared to controls. *fga*$^{+/-}$ larvae were largely unaffected. When genome editing zebrafish to produce fibrinogen Aα R28C, equivalent to the human R35C dysfibrinogenemia mutation, we detected in-frame skipping of exon 2 in the fga mRNA, thereby encoding Aα$^{\Delta 19\text{-}56}$. This mutation is similar to Fibrinogen Montpellier II which causes hypodysfibrinogenemia. Aα$^{+/\Delta 19\text{-}56}$ fish had prolonged TTO and reduced thrombocyte activity, a dominant effect of the mutation. Finally, we used transgenic expression of *fga* R28C cDNA in *fga* knock-down or *fga-/-* mutants to model thrombosis in dysfibrinogenemia. Aα R28C expression had similar dominant effects on TTO and thrombocyte activity as Aα$^{+/\Delta 19\text{-}56}$. We conclude that thrombosis assays in larval

zebrafish can distinguish between quantitative and quantitative fibrinogen disorder models and may assist in anticipating a thrombotic phenotype of novel mutations.

Keywords: Fibrinogen, Fibrin, Thrombocytes, Thrombosis, Zebrafish.

Introduction

Mutations in the three fibrinogen genes give rise to congenital fibrinogen disorders [1]. Fibrinogen has a crucial role as the soluble fibrin precursor in blood clotting, and its deficiency or dysfunction manifests in both bleeding and thrombotic clinical events in these disorders [2, 3]. Two copies of three protein chains make up the soluble fibrinogen hexamer, $(A\alpha B\beta\gamma)_2$, with each chain encoded by its respective gene (*FGA*, *FGB* or *FGG*) that is expressed in the liver. Mutations in any of the genes can lead to a quantitative or qualitative fibrinogen disorder. Quantitative disorders include afibrinogenemia, where plasma fibrinogen antigen is undetectable, and hypofibrinogenemia where fibrinogen is below the typical healthy range (1.5-4g/L). The qualitative disorders are dysfibrinogenemia, where a normal antigen level is accompanied by a lower functional level of fibrinogen when compared to control plasma in clotting assays, and hypodysfibrinogenemia where an abnormally low antigen level is discordant with an even lower functional activity [4].

Afibrinogenemia shows recessive inheritance, two mutated alleles of a fibrinogen gene are required for its appearance, and hypofibrinogenemia can be detected in heterozygous carriers of alleles that would cause afibrinogenemia in homozygosity [5]. Dysfibrinogenemia and hypodysfibrinogenemia show dominant inheritance and are typically caused by heterozygous missense mutations [6]. Quantitative and

qualitative fibrinogen disorders are rare [7] and patients can suffer from both bleeding and thrombosis. Put together, this creates a particular therapeutic difficulty where maintenance of hemostasis and prevention of thrombosis may need to be balanced in a patient group for which reports of optimal treatments are scarce [6, 8].

While diagnosis can usually be achieved by laboratory tests and genetic studies of the fibrinogen genes, prediction of the clinical phenotype of fibrinogen disorders beyond a bleeding tendency is challenging [2]. Familial histories can be informative, but different family members with the same causative mutation may display bleeding or thrombosis (for example [9]). Therefore, a complement of additional tests, including global hemostasis assays and fibrin clot analysis, can offer a more complete predictive picture of the propensity of a given mutation to lead to an adverse clinical outcome [10, 11].

Bleeding events linked to low plasma fibrinogen, or a dysfunctional fibrinogen molecule, can be explained by a deficiency in the quantity or quality of the major physiological substrate for coagulation-based clotting. While thrombosis in afibrinogenemia and dysfibrinogenemia may seem paradoxical, several mechanisms have been proposed. The binding of thrombin to fibrin is the basis of fibrin's antithrombin I activity [12, 13]. Fibrin sequesters thrombin, but if fibrin is absent [14], or thrombin binding is affected [15], this potentially releases thrombin activity. This may increase activation of platelets and their aggregation mediated by von Willebrand Factor [16]. Fibrinogen can also inhibit platelet binding to surface-bound fibrinogen [17], suggesting that platelet adhesion to a vessel wall could be enhanced by a reduction of soluble fibrinogen. Dysfibrinogenemia mutations which cause altered clot structure and affect plasminogen binding can lead to impaired fibrinolysis [18, 19], a further possible contributor to thrombosis. This panel of possibilities helps

explain the prevalence of thrombosis in fibrinogen disorders but also implies that it is fibrin(ogen) quantity and quality that influence it.

In order to study the pathophysiology of coagulation disorders, animal models have been extensively employed, especially mice [20]. This spans from rare bleeding disorders through a spectrum of thrombosis models [21]. The zebrafish, *Danio rerio*, has emerged as an alternative animal model in thrombosis and hemostasis research. Initially it was proposed due to its conserved vertebrate fibrin-based clotting mechanism [22] and amenability to genetic screening that could unveil novel factors in hemostasis [23]. The model's major strength is a combination of high fecundity, accessibility of near transparent larval blood vessels and tissues that can be manipulated and observed in real time, and robust tools to make genetic modifications [24]. This has been demonstrated in a number of recent models of hemostasis and coagulation factor deficiencies that recapitulate phenotypes found in patients and enable screening of potential pathogenic variants [25-31].

Rather than platelets, like all teleosts, zebrafish have thrombocytes [32]. These leukocytes arise about two days into embryonic zebrafish development but are only circulating in sufficient numbers to contribute to hemostasis and thrombosis after about four days. Thrombocytes bear many features of mammalian platelets, and are considered their functional equivalents despite being considerably larger and retaining a nucleus [33]. The developmental time period when transparent larval zebrafish are particularly useful for observing and challenging the hemostatic system, at 3 to 5 days post-fertilization, spans a shift from a circulatory system that is almost devoid of thrombocytes, to one where thrombocytes are present and play an active role in hemostasis and thrombosis.

In the present study, our goal was to assess the experimental thrombotic response to a venous laser injury [34] in larval zebrafish models of afibrinogenemia and dysfibrinogenemia. We aimed to understand whether assays in zebrafish larvae could distinguish between models phenotypically as a possible future functional *in vivo* read-out to novel mutations identified in patients with fibrinogen disorders.

Afibrinogenemic larvae (*fga*$^{-/-}$) failed to support venous thrombosis, as described previously [27], and had markedly reduced thrombocyte binding and accumulation at sites of injury, with aggregates showing a tendency to embolize. In preparing a mutant zebrafish line by genome editing, designed to mimic the dysfibrinogenemia mutation found in fibrinogen Metz I (Aα R28C in zebrafish), we detected fga transcripts with exon 2 skipping and an mRNA encoding an Aα chain lacking amino acids 19 to 56 (Aα$^{\Delta 19\text{-}56}$). This resembles fibrinogen Montpellier II, found in hypodysfibrinogenemia. Venous thrombosis occurred almost normally in Aα$^{+/\Delta 19\text{-}56}$ larvae but thrombocyte accumulation at laser injury sites was reduced, a dominant effect of the mutation. With no possibility for Aα chain fibrinopeptide A cleavage, Aα$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae had prolonged or absent thrombosis but produced embolic clumps of cells after laser injury. In order to model thrombosis in dysfibrinogenemia, we used morpholino knock-down of fga mRNA, or *fga* mutants, with transgenic expression of Aα R28C cDNA. Fibrinogen with the R28C mutation gave a similar thrombotic phenotype to Aα$^{\Delta 19\text{-}56}$, with prolonged venous thrombosis times and reduced thrombocyte activity in models of a heterozygous state. We conclude that laser-induced thrombosis in zebrafish can distinguish between models of quantitative and qualitative fibrinogen disorders, particularly with respect to thrombocyte activity, the inheritance mode of the disorder and demonstrate the thromboembolic nature of both disease types.

Methods

Zebrafish lines and maintenance

Adult zebrafish were maintained at 26°C, pH 7.5, and 500 µS conductivity. Embryos from natural matings were raised at 28.5°C. *fga* mutant zebrafish, a model of afibrinogenemia, were described previously [25]. Genome edited zebrafish expressing fibrinogen $A\alpha^{\Delta 19\text{-}56}$, described below, were generated in the TU strain. Zebrafish with the *itga2b:EGFP* transgene were a gift from Leonard Zon's laboratory, Harvard Medical School. Experimentation was authorized by local veterinary authorities.

Production of zebrafish fibrinogen in transfected cells and thrombin cleavage

The human fibrinogen Aα R35C mutation prevents FpA cleavage by thrombin. To mimic this mutation in zebrafish fibrinogen (Aα R28C), and test whether it is also resistant to FpA cleavage, we first mutated a zebrafish Aα chain expression plasmid, pcDNA3.1-ZF-Aα, using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) and the oligonucleotides fgaR28C-F (5'GGACACAGTGGTGAACCCTTGCGGTGCTCGTCCTATTGAGC3') and fgaR28C-R (5'GCTCAATAGGACGAGCACCGCAAGGGTTCACCACTGTGTCC3'). The mutation was confirmed by DNA sequencing. The wild-type or mutated Aα plasmids, were co-transfected into HEK-293T cells in 10 cm cell culture dishes with plasmids for the expression of zebrafish fibrinogen Bβ and γ chains, using Lipofectamine 2000 (Invitrogen). A non-transfected control sample was also prepared. Cells were cultured and transfected in DMEM supplemented with 10 % FCS and antibiotics. 24h post-transfection the culture medium was removed, cells washed with PBS and then cultured for a further 24h in OptiMEM (Invitrogen) without serum. Conditioned media

were recovered, and cell lysates prepared in RIPA buffer (with protease inhibitors). Samples of each were incubated with or without 0.5 U/mL human thrombin (Sigma) for 1h at 37°C and subjected to western blotting using rabbit anti-zebrafish Aα or Bβ antibodies, as described previously [25].

Generation of zebrafish producing fibrinogen Aα$^{\Delta 19\text{-}56}$

We aimed to produce a zebrafish line expressing fibrinogen Aα R28C. A CRISPR-Cas9 strategy was used. Early embryos of the zebrafish TU strain were microinjected with a 1-2nL mixture containing 0.5ng/nL recombinant Cas9 nuclease (PNABio), 250pg/nL of a single guide RNA (sgRNA) with complementarity to zebrafish *fga* exon 2, 0.44 ng/nL of a single stranded oligonucleotide (ssODN) for template-mediated homology-directed repair (HDR), Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH 7.6) and phenol red as a tracer. The sgRNA was produced by in vitro transcription using the MEGAshortscript Kit (Thermofisher) of a linearized plasmid based on pDR274 [35] that contains the template for *fga* exon 2 targeting introduced by cloning of annealed oligonucleotides (R28sg1F, 5'TAGGACACAGTGGTGAACCCTAG3' and R28sg1R, 5'AAACCTAGGGTTCACCACTGTGT3'). pDR274 was a gift from Keith Joung (Addgene plasmid 42250; http://n2t.net/addgene:42250; RRID:Addgene_42250). The ssODN repair template was designed to mutate the zebrafish fibrinogen Aα chain Arginine 28 codon to Cysteine. It also introduced 3 codon usage changes to avoid re-targeting of the edited sequence by sgRNA-guided Cas9. One of these also introduced an HpaI restriction site, useful for PCR-genotyping. The full ssODN sequence is given below with the HpaI site underlined, the Cysteine codon boxed and the codon usage changes in bold. The anti-sense sequence with respect to *fga* transcription was used:

5’CCATACCCAGTCATCATCGGTACACCCTGGCCATTCTTTTGTCTGGCAGGTGT

CTTGTGCCTTGAAGCCGTGCTCAATAGGACGAGCGCCGCAAGGGTT**A**ACCACC**G**

GT**A**TCCTCCTCGGCCTAAAAAGTAAGTACTTTATAAC3’

Microinjected F0 embryos were raised to adulthood and crossed with wild-type fish to identify a founder animal. F1 offspring were raised and genomic DNA from F1 embryos extracted and assayed for the R28C mutation by PCR genotyping and HpaI digestion and DNA sequencing to confirm the mutation.

RNA was isolated from embryos with the mutation, or from adult liver samples, with Trizol (Life technologies), reverse transcribed (Superscript II, Invitrogen), DNAse treated (Turbo DNAse, Ambion) and amplified by PCR targeting the fga transcript. PCR products were cloned in pCRII TOPO (Invitrogen) and sequenced. This demonstrated that *fga* exon 2 skipping occurred in transcripts where the Aα R28C codon was introduced, and encoded $A\alpha^{\Delta 19\text{-}56}$.

Tol2 plasmid constructions

A plasmid for expression of the zebrafish fibrinogen AαE chain, under the control of a ubiquitin (*ubb*) promoter (subsequently labelled *ubi*), was prepared by Gateway cloning (Invitrogen). Middle entry clones for AαE cDNAs were made using attB1- and attB2-tagged oligonucleotides to PCR amplify cDNAs and pDONOR221 (Invitrogen) as a vector for recombination with PCR products, yielding pME-AαE. These were used in 4-way Gateway recombination reactions with pENTR5'_ubi, (Addgene plasmid #27320) [36], pDestTol2CG2-U6:gRNA (Addgene plasmid #63156) [37], both gifts from Leonard Zon, and p3E polyA from the Tol2kit [38]. The final plasmid for transgenesis was named pTol2ubi-AαE. A pTol2ubi-AαE R28C clone was prepared by site-directed mutagenesis, as described above for pcDNA 3.1 Aα R28C.

Microinjections for fga mRNA knockdowns and fibrinogen alpha chain overexpression

1 to 2 cell zebrafish embryos were microinjected with approximately 1 nL of injection mixes. These contained Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES pH7.6), phenol red, and where described 2 ng of an *fga* exon1-intron1 splice site-targeting antisense morpholino (5'GCATTATATCACTCACCAATGCAGA3', Genetools Inc.). For transgenic expression of AαE or AαE R28C, injection mixes included 25 ng of pTol2ubi-AαE, pTol2ubi-AαE R28C and approximately 35 ng of 5'capped, in vitro-polyadenylated Tol2 transposase mRNA. Where noted injection included 12.5 ng pTol2ubi-AαE or a mixture of 12.5 ng pTol2ubi-AαE + 12.5 ng pTol2ubi-AαE R28C.

Genotyping

Mutations were detected by PCR-genotyping of adult tail fin clips or embryo lysates using *fga* oligonucleotides for PCR (fgaset1F, 5'AATGGCCTATGTTGGCAGAC3' and fgaset1R, 5'CAGTGGTTATCAGCTGACAG3') and restriction disgests with BceAI for the previously-described *fga* mutants [25] or HpaI to detect $A\alpha^{\Delta 19\text{-}56}$.

Plasma collection and western blots

Dilute plasma was prepared from wild-type adult fish and those with 1 or 2 copies of the mutated *fga* allele leading to expression of $A\alpha^{\Delta 19\text{-}56}$ ($fga^{+/+}$, $fga^{+/\,\Delta 19\text{-}56}$, $fga^{\,\Delta 19\text{-}56/\,\Delta 19\text{-}56}$), as described previously [25], a protocol adapted from [39]. Reduced sample preparations of dilute plasma were subjected to western blotting with anti-zebrafish fibrinogen Aα antibodies, as above for transfected cell-conditioned media, stripped and re-probed with anti-zebrafish ceruloplasmin.

Laser-mediated thrombosis and thrombocyte accumulation in zebrafish larvae

Venous thrombosis in zebrafish larvae was assessed with two assays. The time to occlusion (TTO) was measured in seconds after laser injury of the posterior cardinal vein (PCV) in 3 day post-fertilization (3dpf) larvae [40]. In the second test, older, 5dpf *Tg(itga2b:EGFP)* larvae with circulating fluorescent thrombocytes were subjected to a similar PCV injury and the thrombocyte fluorescence accumulation around the injury site recorded and measured. For both assays larvae were anesthetized in Tricaine (170 µg/mL), placed on 0.22% low gelling point agarose on glass cover slips and visualized with a Leica LMD microscope. The microscope used an HCX PL FLUOTAR L 20x/0.40 corr objective and a cryslas laser (max. pulse energy: 50 µJ, pulse frequency: 80 Hz, wavelength: 355 nm). Bright field images were acquired with a Leica LMD CC7000 camera and fluorescence with a Leica DFC 360 FX, using LMD and LAS-AF software, at room temperature. Thrombocyte activity image series were analyzed with MetaMorph Offline software, v.7.10.1.161. Thrombocyte numbers in 5dpf larvae with the *fga*$^{+/+}$, *fga*$^{+/\ \Delta 19\text{-}56}$, *fga* $^{\Delta 19\text{-}56/\ \Delta 19\text{-}56}$ genotypes were compared by counting *itga2b:EGFP*-positive cells flowing through the PCV over a 30 second period.

Statistical analysis and graphics

Statistical analysis and graphical representations were made using Prism (GraphPad Software). Figure 7A, 8A and part of Figure 1A were made with BioRender (https://app.biorender.com/).

Results

Afibrinogenemic zebrafish larvae fail to support venous thrombosis or sustain thrombocyte adhesion and aggregation at injury sites

Laser injury of the posterior cardinal vein in 3-day post fertilization (3dpf) zebrafish larvae can lead to occlusive venous thrombosis and when monitored gives the time to occlusion (TTO, Figure 1A). We used this technique in fibrinogen mutants to test whether the absence of fibrinogen alters the TTO. As reported previously in similar mutants [27], injury in *fga*$^{-/-}$ larvae did not lead to occlusion (Figure 1B, 1C). Of the larvae supporting occlusion the mean TTO was 72 seconds in *fga*$^{+/+}$ and 114 seconds in *fga*$^{+/-}$ animals, respectively (Figure 1B). Examples of laser injuries in *fga*$^{+/+}$ and *fga*$^{-/-}$ are shown in supplementary video files 1 and 2.

The adhesion and aggregation of fluorescent thrombocytes was assessed after laser injury of the PCV in 5dpf *Tg(itga2b:EGFP)* *fga*$^{+/+}$, *fga+/-*, and *fga-/-* larvae. Thrombocyte binding and accumulation was hindered in *fga*$^{-/-}$ larvae compared to *fga*$^{+/+}$or *fga*$^{+/-}$. Fluorescence curves for *fga*$^{+/+}$ and *fga*$^{+/-}$ superimposed (Figure 2A). Two minutes after laser injury, *fga*$^{-/-}$ larvae typically had few bound thrombocytes at the injury site and showed a clear tendency for embolism of clumps of fluorescent cells after initial adhesion at the injury site (Figure 2B). This thrombocyte activity after PCV laser injuries is illustrated in Supplementary video file 3 (*fga*$^{+/+}$) and 4 (*fga*$^{-/-}$).

Genome editing of *fga* exon 2

A frequently detected missense mutation found in dysfibrinogenemia patients is the *FGA* c.103C→T variant [41], leading to the fibrinogen Aα chain R35C amino acid change. Aα R35 (R16 in the mature protein after signal peptide cleavage) is part of

the Aα chain thrombin cleavage site. When present with the non-mutated Aα chain, Aα R35C leads to prolonged fibrin polymerization, delayed fibrinopeptide-A release, decreased binding to platelets, fibrinolytic resistance, and disordered fibrin networks [42].

We aimed to produce zebrafish with a mutation equivalent to human Aα R35C. This corresponds to R28C in the zebrafish Aα (Figure 3A). Thrombin catalyzed fibrinopeptide A (FpA) release does not occur in the human Aα R35C chain. To verify that zebrafish Aα R28C also prevents thrombin cleavage, we transfected HEK-293T cells with plasmids for expression of wild-type or Aα R28C zebrafish fibrinogen. Cell lysates and conditioned medium were treated with and without human thrombin and FpA and fibrinopeptide B (FpB) cleavage verified by immunoblotting with anti-zebrafish fibrinogen Aα and Bβ chain antibodies, respectively. Size shifts were seen in the zebrafish Aα and Bβ chain with thrombin treatment but not in the Aα R28C-containing fibrinogen, confirming its resistance to thrombin cleavage and FpA release (Supplementary Figure 1).

With the TU zebrafish laboratory strain, we used CRISPR-Cas9-based genome editing to induce double-stranded genomic DNA breaks near the R28 codon in exon 2 of the zebrafish *fga* gene. By microinjection of early zebrafish embryos with recombinant Cas9 nuclease, a single guide RNA targeting the region, and an oligonucleotide (ssODN) with the desired codon change as a template for homology-directed repair (HDR), in subsequently raised fish we identified a founder animal that transmitted the expected mutated *fga* allele to its offspring. The HDR oligonucleotide also contained 3 nucleotide changes to avoid re-targeting of the mutated allele with the CRISPR-Cas9 reagents (Figure 3B). These changes conserved the Aα chain amino acid sequence and introduced an endonuclease restriction site to facilitate

PCR-based genotyping (Supplementary Figure 2A). Confirmation of the mutated (mut) allele DNA sequence is shown in Figure 3C.

Exon 2 skipping in fga mRNA after genome editing

To confirm the expected mutation at the RNA level, we generated cDNA from liver RNA extracted from adult *fga*$^{+/+}$, *fga*$^{+/mut}$, and *fga*$^{mut/mut}$ fish. Shorter than expected *fga* amplicon RT-PCR products were detected in *fga*$^{+/mut}$, and *fga*$^{mut/mut}$ cDNA, corresponding to shortened Aα and AαE transcripts (Supplementary Figure 2). Analysis of the *fga* cDNA sequences, by Sanger sequencing, revealed exon 2 skipping in mRNA from the *fga*^{mut} allele (Figure 4A). We did not detect exon 2 sequences in RNA transcribed from the mutated allele when RT-PCR products were cloned and multiple clones analyzed (49 clones in total, data not shown). Instead of Aα R28C, the predicted Aα chain translated from this exon 2-skipped transcript lacks amino acids 19 to 56 but retains the natural open reading frame, encoding Aα Δ19-56.

To understand why the genome edited sequence led to exon 2 skipping in fga mRNA, we tested the hypothesis that it was affecting an exon splicing enhancer sequence (ESES) using prediction software (RESCUE-ESE Web Server - genes.mit.edu). The software predicted disruption of several ESESs in the mutated sequence (Supplementary Figure 3) providing a possible source for changes in fga mRNA splicing.

We named the *fga*^{mut} allele *fga*$^{\Delta 19\text{-}56}$, for clarity. To assess whether mutant fibrinogen containing the Aα Δ19-56 chain was present in the zebrafish circulation, plasma from adult *fga*$^{+/+}$, *fga*$^{+/\Delta 19\text{-}56}$, and *fga* $^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ fish was subjected to immunoblotting, with ceruloplasmin as a plasma protein control. Fibrinogen Aα was present in *fga* $^{\Delta 19\text{-}56/\Delta 19\text{-}}$

^{56} plasma but, when compared with ceruloplasmin, may be at lower levels compared to *fga*$^{+/+}$and *fga*$^{+/\Delta 19\text{-}56}$ (Figure 4B). We did not detect a size shift in the Aα Δ19-56 immunoblot compared to wild-type animals, despite a predicted loss of approximately 4.3kDa. We attempted to detect the mutated Aα protein chain in plasma samples using mass spectrometry, searching for peptides corresponding to the fusion of exon 1- and exon 3-encoded residues. We did not detect such peptide species, but an exon 2-encoded peptide (EWPGCTDDDWGSK) was detected in wild-type and not *fga* $^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ plasma (Supplementary Figure 4).

The change in the zebrafish Aα Δ19-56 protein sequence, compared to wild-type, resembles closely the Aα chain which is expressed as a result of a human splice-site mutation detected in a family with hypodysfibrinogenemia [9]. Fibrinogen Montpellier II, caused by the *FGA* IVS2+3insCAT mutation, lacks human Aα chain residues encoded by *FGA* exon 2. This Aα-chain is also devoid of a thrombin cleavage site and leads to detrimental effects on clot structure with thinner fibers and increased fiber ends, presumably due to the abnormal unpolymerized chain ends formed in the protofibril. An alignment of the amino-termini of the Aα chain in Fibrinogen Montpellier II (Aα Δ19-60) and zebrafish Aα Δ19-56 is shown in Figure 4C. With this similarity, we decided to characterize the venous thrombosis phenotype in zebrafish expressing Aα Δ19-56, as a potential model of hypodysfibrinogenemia.

Venous thrombosis Aα Δ19-56-expressing zebrafish larvae

We compared laser-induced TTO in the PCV of 3dpf *fga*$^{+/+}$, *fga*$^{+/\Delta 19\text{-}56}$ and *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae. In contrast to *fga*$^{+/+}$ larvae from the AB strain (Figure 1B) used for generating *fga*$^{-/-}$ animals, we recorded a relatively short TTO for all *fga*$^{+/+}$ fish with the TU genetic background (mean 23sec., Figure 5A). This difference has been

investigated in other work and relates to the ability to express the predominant larval fibrinogen alpha chain isoform (AαE) in larvae (CF, RJF, MNA, submitted). All *fga*$^{+/\Delta 19\text{-}56}$ larvae also supported vessel occlusion after injury with a mean TTO of 29 sec. A two-tailed unpaired t-test between TTOs measured for *fga*$^{+/+}$ (n=17) versus *fga*$^{+/\Delta 19\text{-}56}$ (n=42) gave a *P*-value of 0.361. No occlusion was measured in 11 of 16 *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae. The mean TTO in the remaining 5 *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae that supported occlusion was 77 sec. t-tests comparing the TTO from this sub-group of 5 *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae and the *fga*$^{+/+}$ or *fga*$^{+/\Delta 19\text{-}56}$ larvae gave *P*-values of <0.0001 for both analyses. We conclude that the presence of a single *fga*$^{+/\Delta 19\text{-}56}$ allele prolongs TTO, compared to wild-type, but homozygous *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae either fail to show vessel occlusion or display a further prolongation. Figure 5B shows vessel occlusion recorded 30 sec post injury in *fga*$^{+/+}$ larvae. A typical *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ result is shown in Figure 5C where occlusion was not reached after 70 sec.

In addition to differences in TTO, we observed qualitative differences in the clots formed in *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae, compared to *fga*$^{+/+}$ controls. When occlusion occurred in *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae it was short-lived and accompanied by visible clumping of erythrocytes in the vasculature. This difference can be seen by comparing Supplementary video 5 (*fga*$^{+/+}$) and Supplementary video 6 (*fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$). Even in *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae where a TTO could not be recorded (no occlusion), cellular clumping arose and then resolved in the minutes after injury (Supplementary video 7).

We evaluated *Tg(itga2b:EGFP)* thrombocyte responses to venous laser injury in 5dpf *fga*$^{+/+}$, *fga*$^{+/\Delta 19\text{-}56}$ and *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae. Initially we verified the number of circulating thrombocytes was equivalent in 5dpf larvae for each *fga* genotype. No significant differences were measured (Supplementary Figure 5). As for *fga*$^{-/-}$ larvae

(Figure 2), in *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ homozygotes thrombocytes largely failed to adhere or aggregate after PCV laser injury (Figure 6A and 6B). However, in contrast to *fga*$^{+/-}$ larvae which showed equivalent thrombocyte accumulation to *fga*$^{+/+}$, *fga*$^{+/\Delta 19\text{-}56}$ heterozygotes gave an intermediate thrombocyte binding and accumulation phenotype, with the fluorescence curve measured over time clearly intermediate between *fga*$^{+/+}$ and *fga*$^{+/\Delta 19\text{-}56}$ (Figure 6A). This demonstrates a dominant effect on thrombocyte activity for the mutated allele in *fga*$^{+/\Delta 19\text{-}56}$. Examples for fluorescent thrombocyte activity in *fga*$^{+/+}$ and *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ are given in Supplementary videos 8 and 9, respectively.

A zebrafish model of dysfibrinogenemia

As our initial aim was to assess venous thrombosis in a model of dysfibrinogenemia, but targeted genome editing gave fga mRNA exon 2 skipping instead of a missense mutation, we used an alternative approach (Figure 7A). TU strain zebrafish embryos were microinjected with an antisense morpholino (MO) that targets splicing of the fga pre-mRNA and leads to >90% depletion in fga mRNA (CF, RJF, MNA, submitted). In the TTO assay with 3dpf larvae, 18/20 MO-injected larvae gave no occlusion 3 minutes after laser injury, whereas all non-injected larvae gave occlusion (mean TTO 19.5 sec, n=21) (Figure 7B). In separate injection mixes, the MO was complemented with Tol2 transposon plasmid DNA and transposase mRNA for transgenic expression of the fibrinogen AαE chain cDNA or AαE R28C. The MO cannot target the cDNA sequence. The AαE chain is the predominant fibrinogen alpha chain isoform expressed in larval TU zebrafish (CF, RJF, MNA, submitted) and was therefore chosen as the most relevant chain in the TTO assay.

Transgenic expression of AαE complemented MO knock-down, reversing the MO TTO phenotype with all larvae supporting venous occlusion and a mean TTO of 27 sec (n=19) (Figure 7B). Expression of AαE R28C permitted occlusion in 16/22 larvae but the TTO was prolonged compared to non-injected or AαE-injected larvae (88 sec, n=16). To mimic the heterozygous state of patients with dysfibrinogenemia and the R35C mutation, we injected the MO with a 1:1 mix of AαE and AαE R28C plasmids. Venous occlusion was measured in 20/21 larvae, with shorter TTO than AαE R28C alone, but markedly prolonged TTO compared to AαE alone (mean TTO 62 sec, n=20). To control for the half quantity of AαE plasmid injected in the AαE + AαE R28C condition we also injected ½ quantity of AαE plasmid with the MO and measured TTO at 3dpf. All larvae supported occlusion with a mean TTO considerably shorter than the AαE + AαE R28C mix and marginally longer than the initial MO + AαE condition. This demonstrates the dominant effect of AαE R28C expression, seen when comparing the TTO in AαE + AαE R28C versus AαE alone, is not due to lower quantities of injected AαE plasmid in the mixed condition.

To monitor the effect of the fibrinogen AαE R28C mutation on laser-induced thrombocyte adhesion and aggregation we used transgenic expression of AαE and AαE R28C in *fga*$^{-/-}$ larvae with the *itga2b:EGFP* transgene and fluorescent thrombocytes at 5dpf (Figure 8A). The MO-based fga mRNA knock-down could not be used as it is transient and does not persist effectively to 5dpf (RJF, unpublished). Expression of AαE increased markedly the initial adhesion and accumulation of thrombocytes to sites of venous laser injury compared to non-injected *fga*$^{-/-}$ larvae (Figure 8B). Fluorescence curves for thrombocyte activity in larvae with transgenic expression of AαE R28C, or a mix of AαE + AαE R28C to mimic a heterozygous state, were largely similar to non-injected *fga*$^{-/-}$ controls, demonstrating a failure of

AαE R28C to complement fibrinogen deficiency and a dominant effect of AαE R28C in the mixed condition. Injecting half the quantity of AαE to control for the amount of AαE in the AαE + AαE R28C condition gave a similar result to AαE, reiterating the dominant effect of AαE R28C on thrombocyte binding and accumulation at sites of venous laser injury.

Discussion

In this study we assessed the phenotype of larval zebrafish models of congenital fibrinogen disorders using laser-induced venous thrombosis and fluorescent thrombocyte adhesion and accumulation as the functional read-outs. We aimed to determine whether the experimental venous thrombosis phenotype of afibrinogenemia, a quantitative disorder, differs from that of dysfibrinogenemia – a disorder of fibrinogen quality.

The model we used for afibrinogenemia has been reported previously [25] and our TTO assay data are similar to those reported elsewhere [27]. The TTO in 3dpf zebrafish larvae measures the propensity to thrombose after injury but is also a measure of hemostasis potential, as *fga*$^{-/-}$ animals fail to support clotting and subsequent thrombosis. In 5dpf *fga*$^{-/-}$ larvae we observed a hint of the thromboembolic phenotype seen in afibrinogenemic patients [8]. Thrombocyte aggregates form, but can be mobile, partly explaining the flattened accumulation curves for fluorescent thrombocytes described. In the future it will be interesting to monitor the destination and fate of these aggregates, as a potential source of distant vessel occlusion.

We initially encountered difficulties in modelling the R35C dysfibrinogenemia mutation. CRISPR-Cas9 based genome edits of *fga* lead to a splicing defect in fga mRNA despite incorporation of the desired transmissible sequence changes. Engineering codon usage changes and an enzyme recognition sequence alongside the missense mutation (R28C) appears to have disrupted the regulation of the usual fga mRNA splicing mechanism. While we were able to use the resulting mutant line as a model of hypodysfibrinogenemia ($fga^{+/\Delta 19\text{-}56}$), we would not have seen the final outcome of genome editing had we not assayed the fga transcripts in our mutants. Caution should therefore be taken when basing mutant phenotype assignment based on genomic sequence change alone.

The zebrafish has the key attribute of accessible larval blood vessels which can be readily targeted with a laser to induce hemostasis and thrombosis. For the present study an obvious limitation is that the disorders we model are diagnosed in part by the concordance of plasma fibrinogen levels and activity. At present, to our knowledge, plasma fibrinogen cannot be measured accurately in larval zebrafish blood due to low blood volumes and a lack of methodology. The larval injury models can therefore suggest the phenotypic effects of a given disorders mutation and its inheritance mode, and detect detrimental functional effects of a mutation, but cannot be used to project a precise correlation between fibrinogen quantity, quality and a clinical phenotype.

The mutated fibrinogen alpha chains produced in our proposed models of dysfibrinogenemia (R28C) and hypodysfibrinogenemia ($fga^{+/\Delta 19\text{-}56}$) lack the Aα amino terminal sequence for effective thrombin-mediated cleavage and FpA release. While clots are still likely to occur via B:b kno-hole interactions [43], retention of FpA is known to affect fibrin polymerization, change clot structure [42] and susceptibility to

fibrinolysis [44]. The prolonged thrombosis phenotype we observed in both models is consistent with changes in fibrin polymerisation, and altered thrombocyte binding to injury sites may reflect changes in cellular interactions with an altered clot structure.

Thus far, the differences between the distinct model phenotypes we report are subtle. Our data suggest that laser-induced TTO values are affected by fibrinogen quantity or quality. This can be seen by the prolongation of TTO in *fga*$^{+/-}$ or *fga*$^{+/\Delta 19\text{-}56}$, compared to *fga*$^{+/+}$ controls, and the longer TTO measured in fga-targeted morpholino knock-down larvae with AαE + AαE R28C expression, compared to AαE expression alone. The phenotype of binding and accumulation of fluorescent thrombocytes after laser injury is possibly more discriminatory between the models. Curves of fluorescence used to represent thrombocyte activity after laser injury were unaffected in *fga*$^{+/-}$ larvae compared to *fga*$^{+/+}$, whereas thrombocyte accumulation was lowered in models of the heterozygous state in dysfibrinogenemia or hypodysfibrinogenemia.

This leads us to propose an expected zebrafish phenotype profile for models of quantitative versus qualitative disorders in our two laser injury assays. Venous TTO at 3dpf is expected to be prolonged in models of both fibrinogen disorder classes in the heterozygous state. Afibrinogenemia models fail to support venous occlusion, whereas models of qualitative disorder mutations in homozygosity (*fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ or expression of AαE R28C), that are detected in heterozygosity in patients, give some larvae where TTO is measurable and many others with no occlusion. In afibrinogenemia models, 5dpf thrombocytes adhere and aggregate poorly to laser injury sites and have a propensity for embolism. This is a recessive trait; in heterozygous carriers of alleles which confer afibrinogenemia in homozygosity, thrombocyte activity is similar to wild-type controls. Models of qualitative congenital

fibrinogen disorders are expected to demonstrate dominant negative effects on thrombocyte activity. Neither of the functional read-outs at their present resolution distinguish between the dysfibrinogenemia and hypodysfibrinogenemia models presented.

In the future we aim to use this guide to assess the phenotype of newly uncovered mutations linked to congenital fibrinogen disorders and take steps towards correlating the larval zebrafish model phenotypes with clinical indicators in patients.

Authorship Contributions

C. F. performed the experiments, analyzed and interpreted the data and wrote the draft of the manuscript. R. J. Fish designed and performed the experiments, analyzed and interpreted the data and corrected and wrote the manuscript. C.D.S. performed experiments and analyzed and interpreted data. M. Neerman-Arbez designed and directed the research and contributed to writing.

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Figure Legends

Figure 1 – Laser induced venous thrombosis in an afibrinogenemia model. (A) Diagram of time to occlusion (TTO) assay. The Posterior Caudal Vein (PCV) is targeted for laser injury at the 5th somite distal to the cloaca and the time to PCV occlusion measured. A 3 minute cut-off is used to determine no occlusion. **(B)** TTO in 3dpf *fga*$^{+/+}$, *fga*$^{+/-}$ and *fga*$^{-/-}$ larvae. Each circle represents an individual larva. **(C)** Time lapse images of 3dpf *fga*$^{+/+}$ and *fga*$^{-/-}$ larvae before and after laser injury at the position marked (LI). DA is the dorsal aorta.

Figure 2 – Thrombocyte adhesion and aggregation in afibrinogenemic zebrafish.

(A) Laser-induced vessel injury to measure thrombocyte binding and accumulation in 5dpf *Tg(itga2b:EGFP)* embryos with fluorescent thrombocytes. The thrombocyte-associated fluorescence accumulation after laser injury within a defined region was measured over time for individual larvae. Each line represents the mean fluorescence (+/- SEM) for each group. The number of larvae per group is indicated in brackets. **(B)** Images from *fga*$^{+/+}$ and *fga*$^{-/-}$ larvae upon (0sec) and after laser injury, times as indicated.

Figure 3 – Genome editing of *fga* exon 2. (A) Aligned N-terminal region of human and zebrafish fibrinogen Aα chains. Aα R35 and Aα R28 are indicated. **(B)** CRISPR/Cas9 strategy for *fga* exon 2 editing through Homology Directed Repair (HDR). In red is part of the sgRNA targeting the edited region by complementarity, in bold is the PAM sequence. Part of the ssODN for HDR is shown to highlight edited nucleotides (blue), including codon usage changes and the Arg to Cys codon change. **(C)** Sanger sequencing to confirms genome edited sequence in genomic

DNA from homozygous mutant fish (*fga*$^{mut/mut}$), compared to wild-type (*fga*$^{+/+}$). Changed nuclotides are highlighted with arrows.

Figure 4 – *fga* exon 2 skipping. (A) A scheme to highlight changes in *fga* exon 2 genomic DNA in red (gDNA) lead to exon 2 skipping after transcription and splicing. A partial cDNA sequence to show the exon 1 to exon 3 splicing is shown. **(B)** Immunoblotting of plasma samples from *fga*$^{+/+}$, *fga*$^{+/\Delta 19\text{-}56}$ and *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ fish with anti-ceruloplasmin and anti-fibrinogen Aα chain antibodies. (**C)** Amino acid alignment of the predicted fibrinogen Aα chains resulting from the human *FGA* IVS2+3insCAT mutation and the zebrafish *fga*$^{\Delta 19\text{-}56}$, mRNA. The junction of exon 1- and exon 3-encoded amino acids are in bold and underlined for each chain.

Figure 5 – Laser induced venous thrombosis in a hypodysfibrinogenemia model. (A) TTO for 3dpf *fga*$^{+/+}$, *fga*$^{+/\Delta 19\text{-}56}$, and *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ zebrafish larvae. Each circle represents one larva. The number of larvae tested in each group is shown in brackets. **(B), (C)** Time lapse images of *fga*$^{+/+}$ or *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae upon (0sec) or 30sec after injury.

Figure 6 – Thrombocyte adhesion and aggregation in a hypodysfibrinogenemia model. (A) Fluorescent thrombocyte binding and adhesion in 5dpf *Tg(itga2b:EGFP)* embryos after laser injury of the PCV over time in *fga*$^{+/+}$, *fga*$^{+/\Delta 19\text{-}56}$ and *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ genotypes. Each line represents the mean (+/-SEM) of thrombocyte fluorescence reaching a threshold fluorescence within a defined area. **(B)** Time lapse images of *fga*$^{+/+}$ and *fga*$^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae upon (0sec) and 180sec after laser injury.

Figure 7 – TTO measurements in fga morpholino knockdown embryos with transgenic expression of AαE, AαE R28C or both, to model dysfibrinogenemia. (A) A scheme representing the experimental set-up. Early TU zebrafish embryos

were injected with and without an fga mRNA-targeting morpholino (MO) in the absence or presence of Tol2 plasmids for expression of fibrinogen AαE, AαE R28C, AαE + AαE R28C or a half quantity of AαE (1/2 AαE). Tol2 transposase mRNA was also present within injection mixes (not shown). At 3dpf the TTO assay was performed to measure venous thrombosis time. The MO inhibits endogenous fga mRNA splicing but cannot target the transgenically expressed AαE and AαE R28C mRNAs. **(B)** TTO data for each experimental group, each circle represents one larva (n=19 to 22).

Figure 8 – Fluorescent thrombocyte binding and aggregation after PCV laser injury in *fga*$^{-/-}$ larvae with transgenic AαE or AαE R28C expression: a model of dysfibrinogenemia. (A) A scheme representing the experimental setup. Early *Tg(itga2b:EGFP), fga*$^{-/-}$ embryos were microinjected with Tol2 transgenesis plasmids for expression of fibrinogen AαE or AαE R28C, AαE + AαE R28C or a half quantity of AαE (AαE ½). Tol2 transposase mRNA was also present in injection mixes (not shown). **(B)** Fluorescent thrombocyte binding and adhesion at 5dpf were monitored for each experimental group over time after PCV laser injury. Each line represents the mean fluorescence (+/-SEM). The number of larvae per group is shown in brackets.

Figures

Figure 1

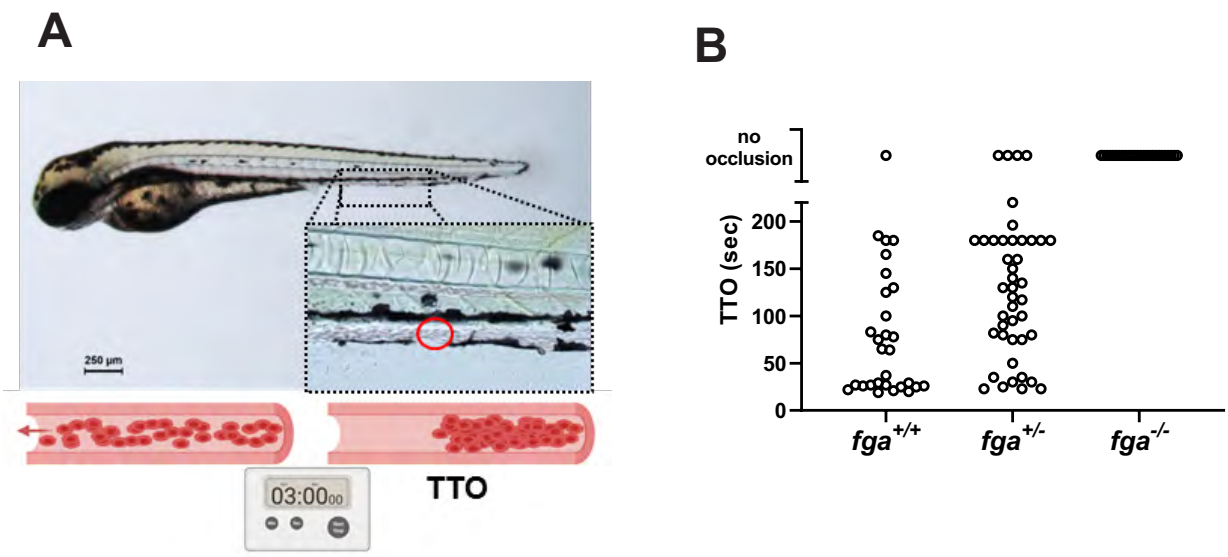


Figure 2

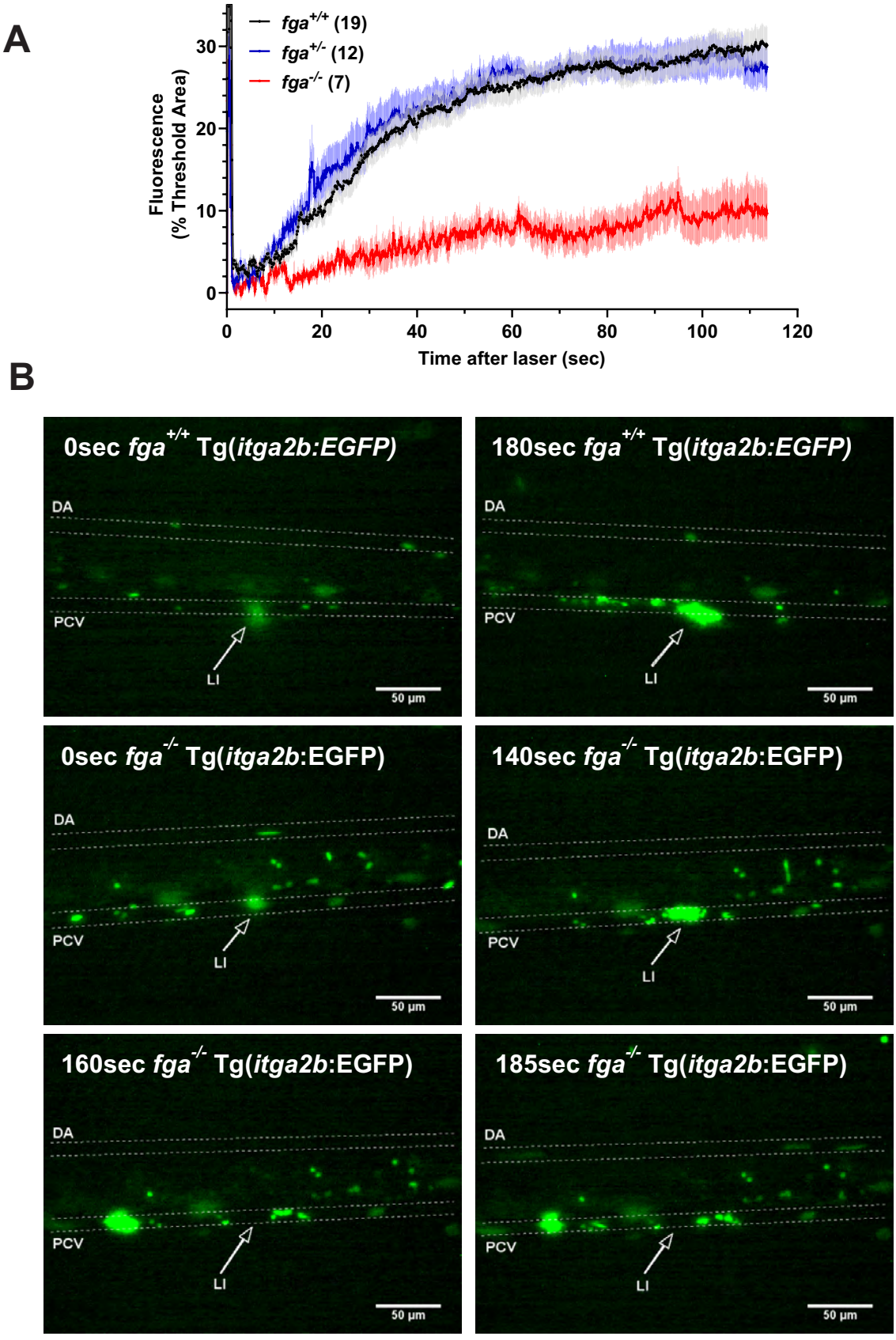
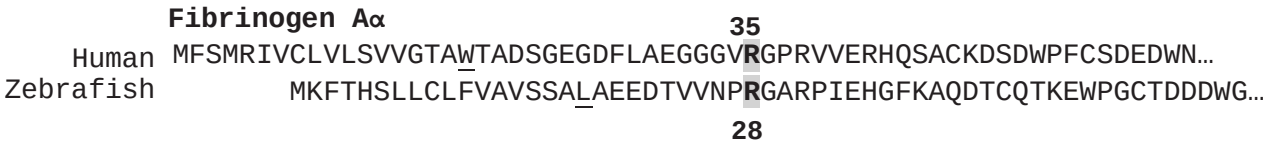
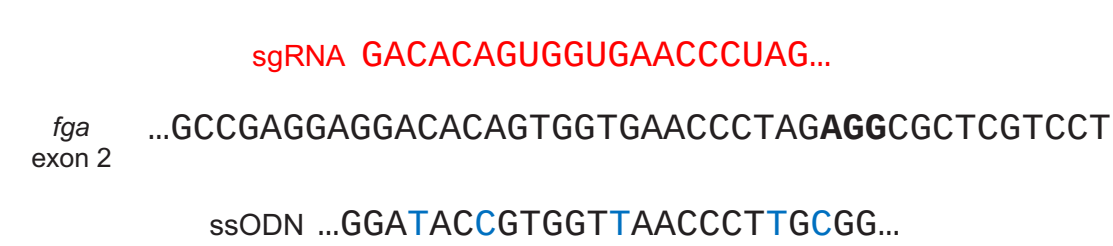

A
fga+/+ (19)
fga+/- (12)
fga-/- (7)
Fluorescence (% Threshold Area)
Time after laser (sec)
B
0sec fga+/+ Tg(itga2b:EGFP)
180sec fga+/+ Tg(itga2b:EGFP)
0sec fga-/- Tg(itga2b:EGFP)
140sec fga-/- Tg(itga2b:EGFP)
160sec fga-/- Tg(itga2b:EGFP)
185sec fga-/- Tg(itga2b:EGFP)
DA
PCV
LI
50 µm

Figure 3

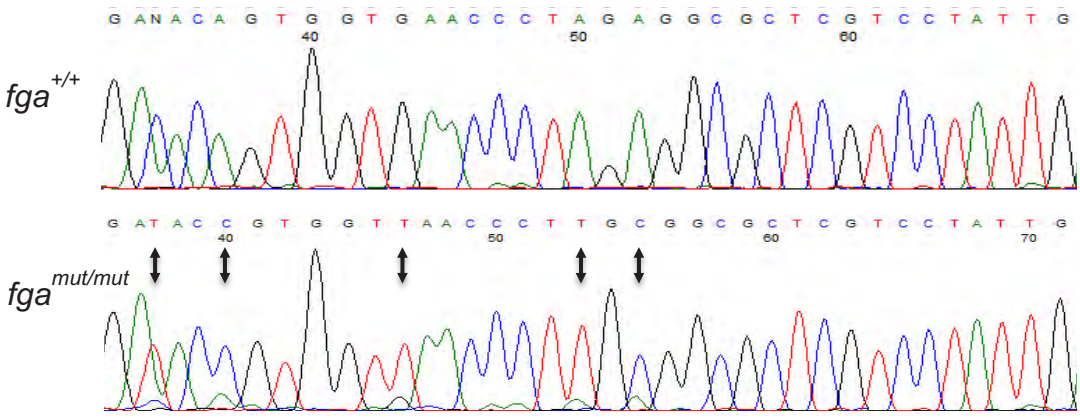
A



B



C



fga exon 2

Figure 4

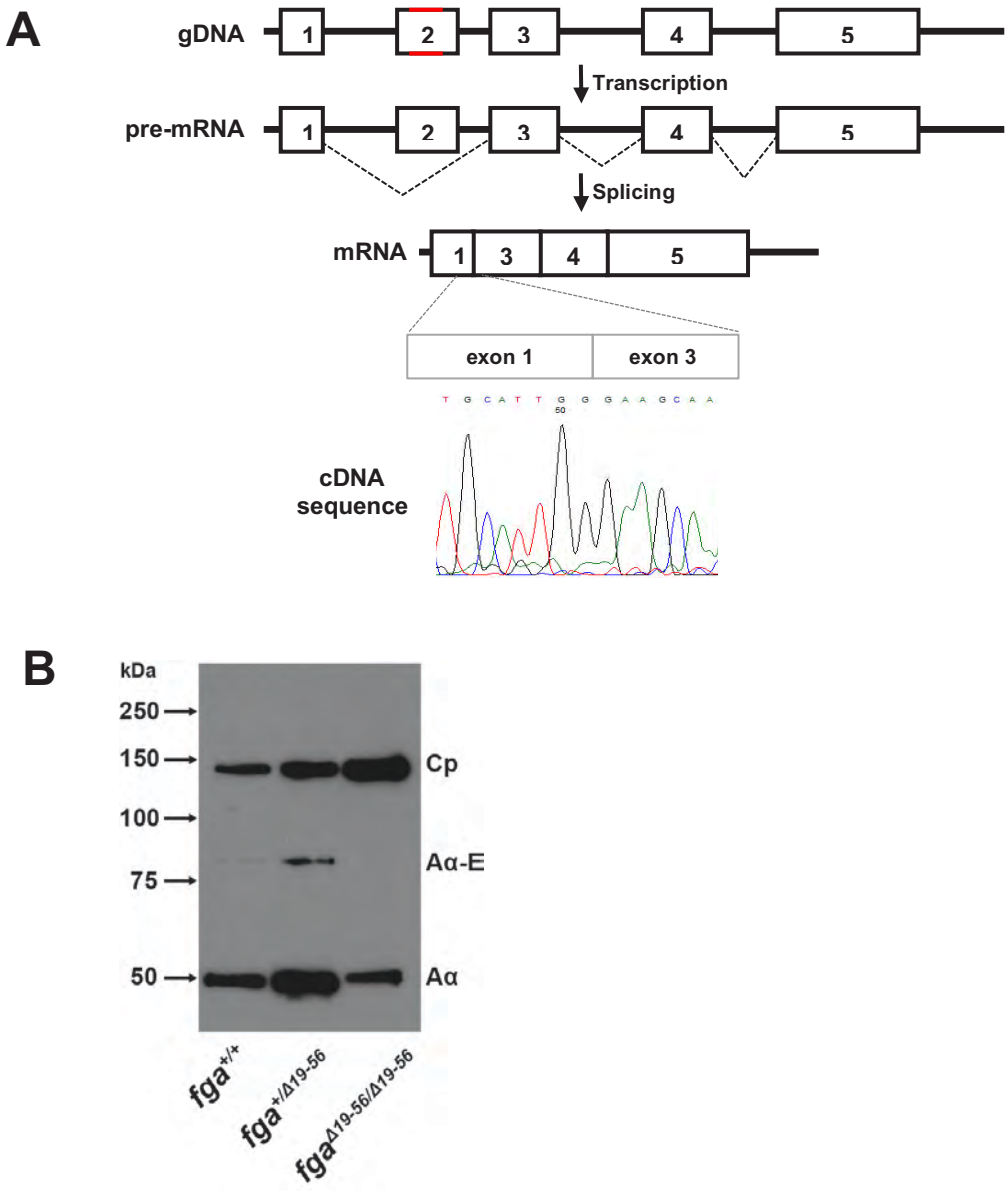

A
gDNA
1
2
3
4
5
Transcription
pre-mRNA
Splicing
mRNA
exon 1
exon 3
cDNA sequence
B
kDa
250
150
100
75
50
Cp
Aα-E
Aα
fga+/+
fga+/Δ19-56
fgaΔ19-56/Δ19-56
C
Human Aα Δ19-60 MFSMRIVCLVLSVVGTAWNYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHS…
Zebrafish Aα Δ19-56 MKFTHSLLCLFVAVSSALGSKCPSGCRVQGLMDKADHDLLKKIEKIRRLLVEGQKLYRSADE…

Figure 5

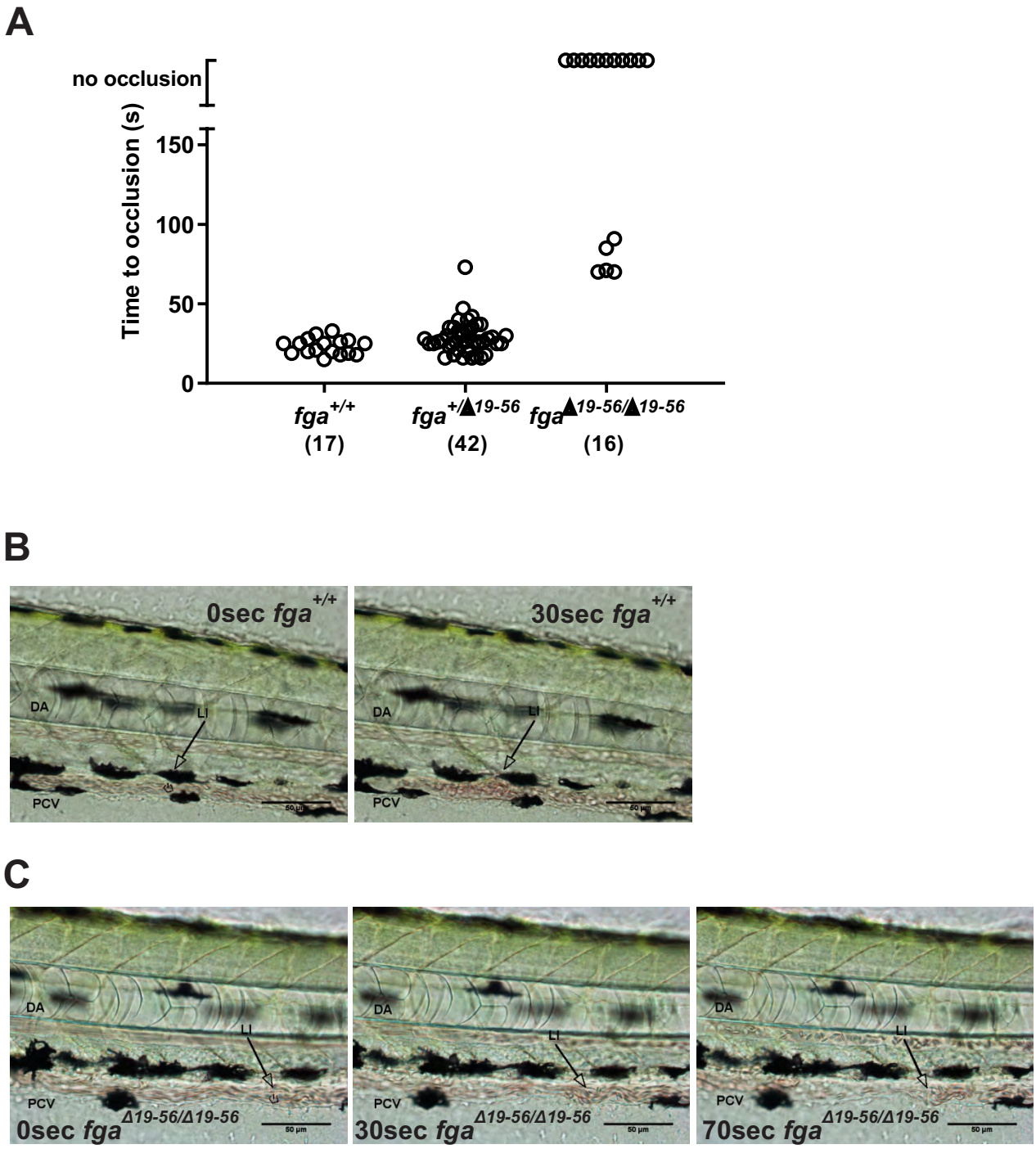

A
no occlusion
Time to occlusion (s)
150
100
50
0
fga+/+
(17)
fga+/Δ19-56
(42)
fgaΔ19-56/Δ19-56
(16)
B
0sec fga+/+
30sec fga+/+
DA
LI
PCV
C
0sec fgaΔ19-56/Δ19-56
30sec fgaΔ19-56/Δ19-56
70sec fgaΔ19-56/Δ19-56

Figure 6

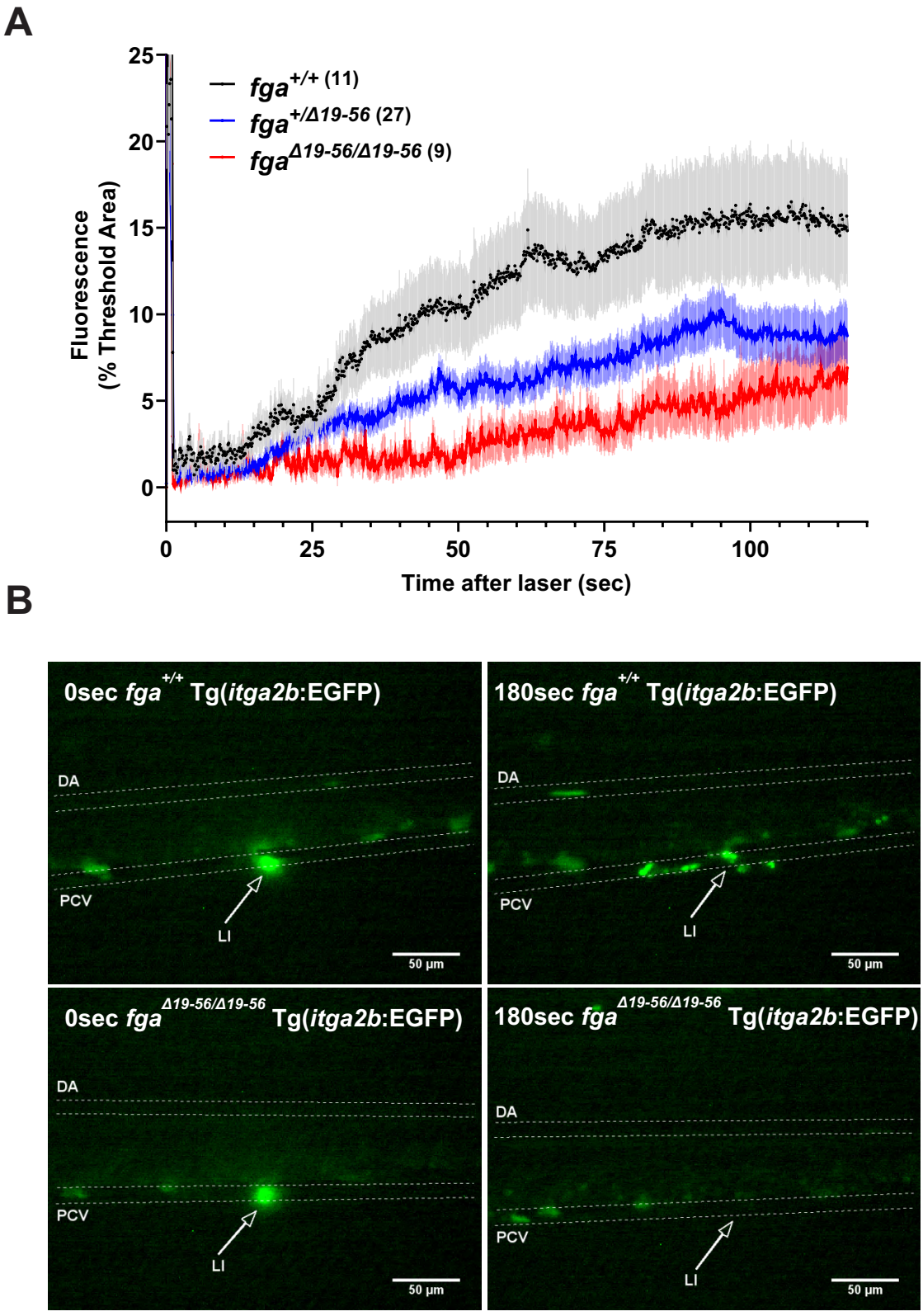

A
fga+/+ (11)
fga+/Δ19-56 (27)
fgaΔ19-56/Δ19-56 (9)
Fluorescence (% Threshold Area)
Time after laser (sec)
B
0sec fga+/+ Tg(itga2b:EGFP)
180sec fga+/+ Tg(itga2b:EGFP)
0sec fgaΔ19-56/Δ19-56 Tg(itga2b:EGFP)
180sec fgaΔ19-56/Δ19-56 Tg(itga2b:EGFP)
DA
PCV
LI
50 µm

Figure 7

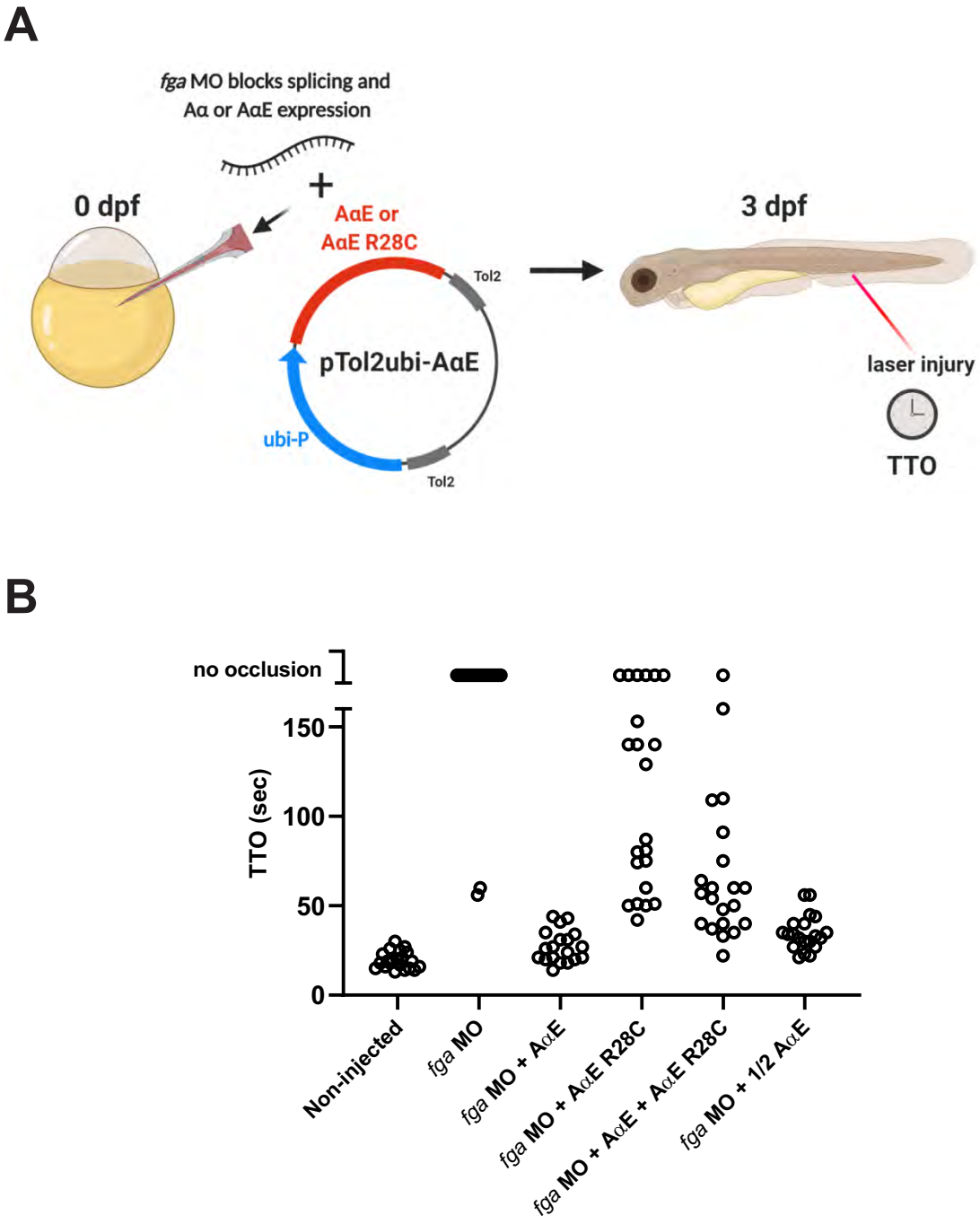
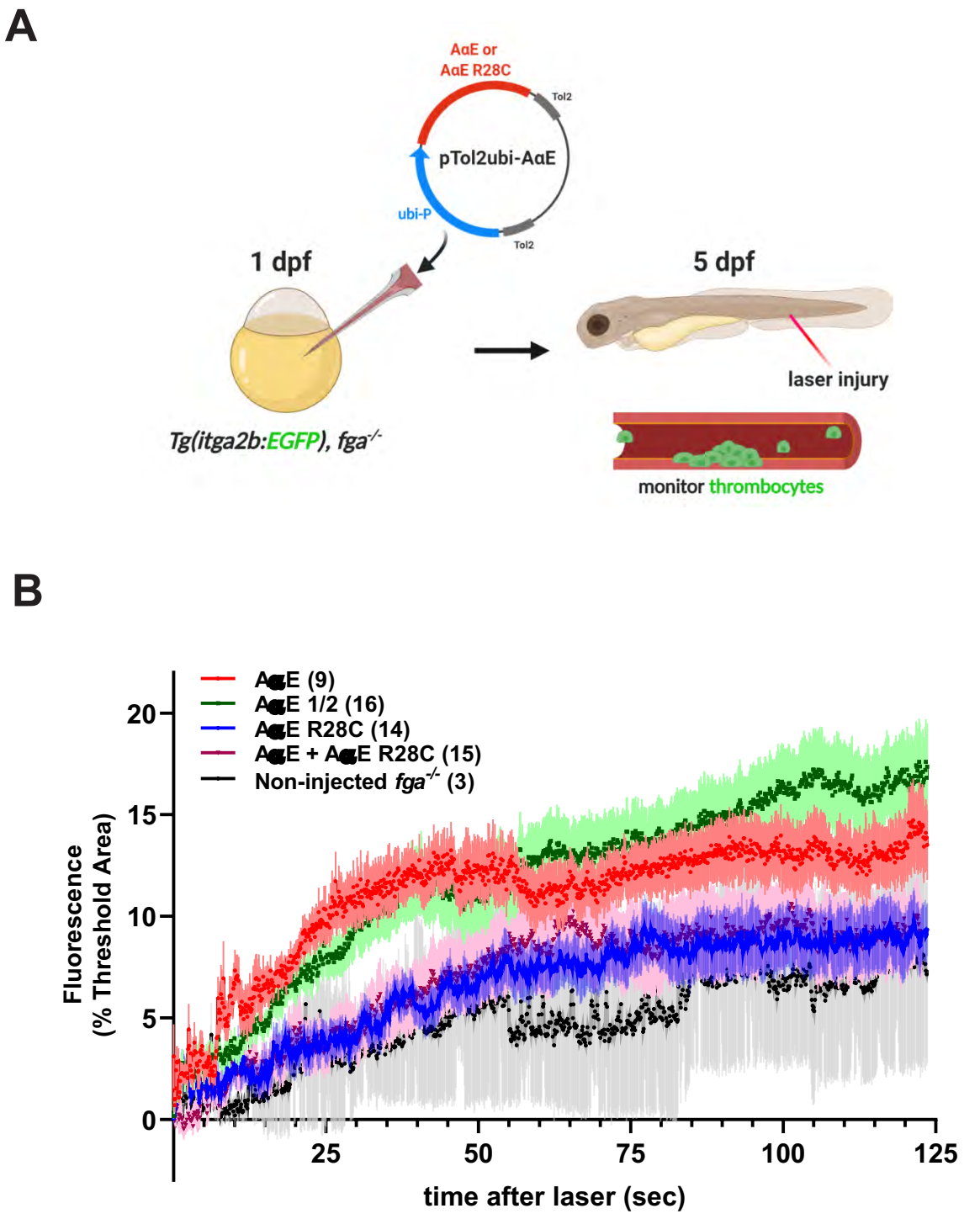
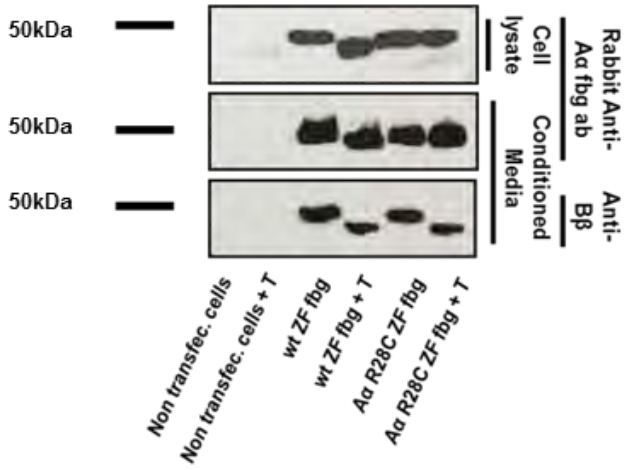

A
fga MO blocks splicing and
Aα or AαE expression
+
0 dpf
AαE or
AαE R28C
Tol2
pTol2ubi-AαE
ubi-P
Tol2
3 dpf
laser injury
TTO
B
no occlusion
150
100
50
0
TTO (sec)
Non-injected
fga MO
fga MO + AαE
fga MO + AαE R28C
fga MO + AαE + AαE R28C
fga MO + 1/2 AαE

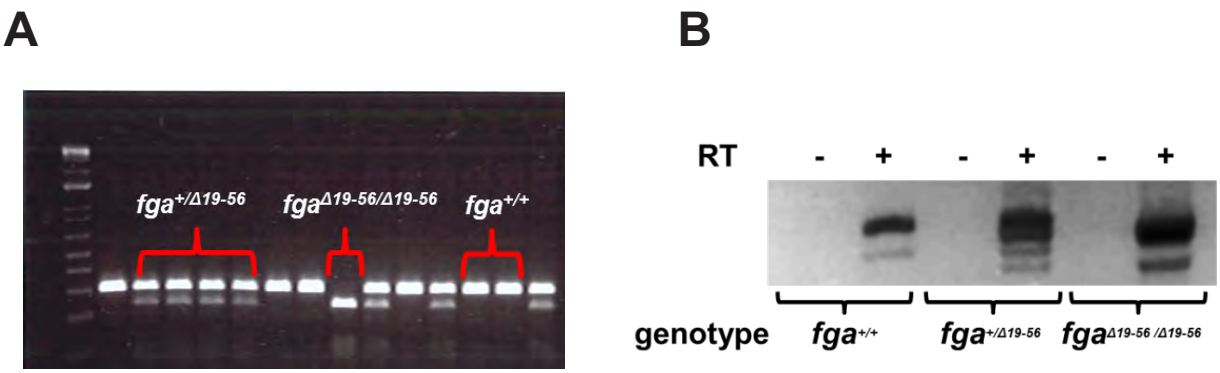
Figure 8


A
AαE or
AαE R28C
Tol2
pTol2ubi-AαE
ubi-P
Tol2
1 dpf
5 dpf
laser injury
Tg(itga2b:EGFP), fga-/-
monitor thrombocytes
B
AαE (9)
AαE 1/2 (16)
AαE R28C (14)
AαE + AαE R28C (15)
Non-injected fga-/- (3)
Fluorescence
(% Threshold Area)
0
5
10
15
20
25
50
75
100
125
time after laser (sec)

Supplementary Data



Supplementary Figure 1 –Thrombin cleavage of zebrafish fibrinogen and fibrinogen Aα R28C Immunoblot of zebrafish fibrinogen in cell lysates and conditioned media from transfected HEK-293T cells with and without thrombin treatment. Anti-Aα and anti-Bβ fibrinogen antibodies are used. Controls were non-transfected HEK-293T cells.

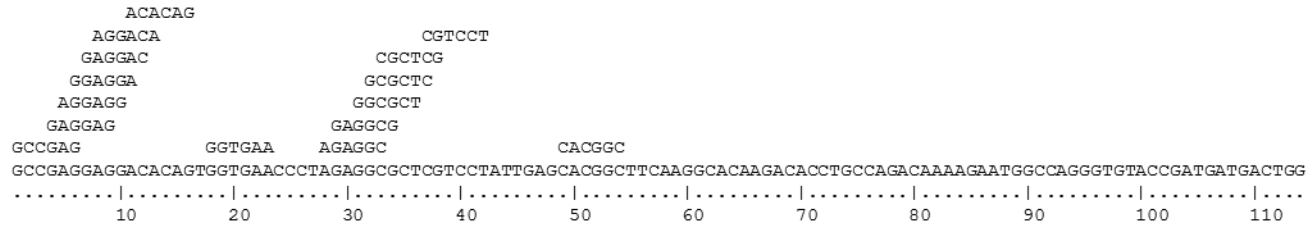


Supplementary Figure 2 – *fga*Δ19-56 genotyping and RT-PCR from liver samples (A) PCR-genotyping individual fish using the restriction enzyme HpaI. After HpaI cut, $fga^{+/+}$: 301 nt; $fga^{+/\Delta 19\text{-}56}$: 301 nt + 230 nt + 71 nt bands and $fga^{\Delta 19\text{-}56/\Delta 19\text{-}56}$: 230 nt + 71 nt bands. **(B)** RT-PCR of RNA from $fga^{+/+}$, $fga^{+/\Delta 19\text{-}56}$, and $fga^{\Delta 19\text{-}56/\Delta 19\text{-}56}$

liver samples. fga transcripts encoding fibrinogen Aα-chain (upper) and Aα-E isoform (lower) are shown.

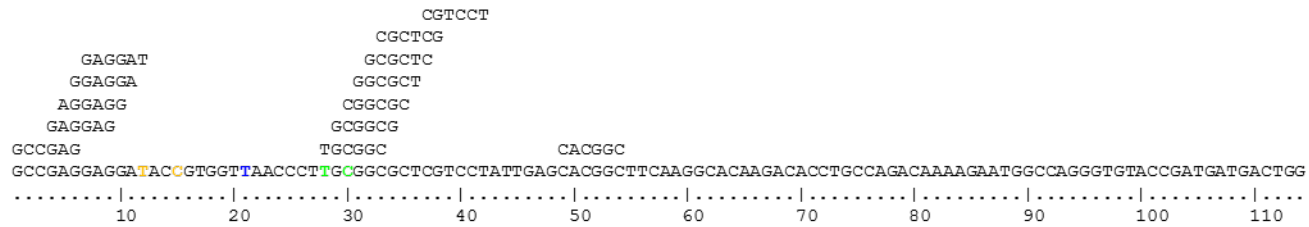
A

***fga* exon 2 – Predicted ESEs:**

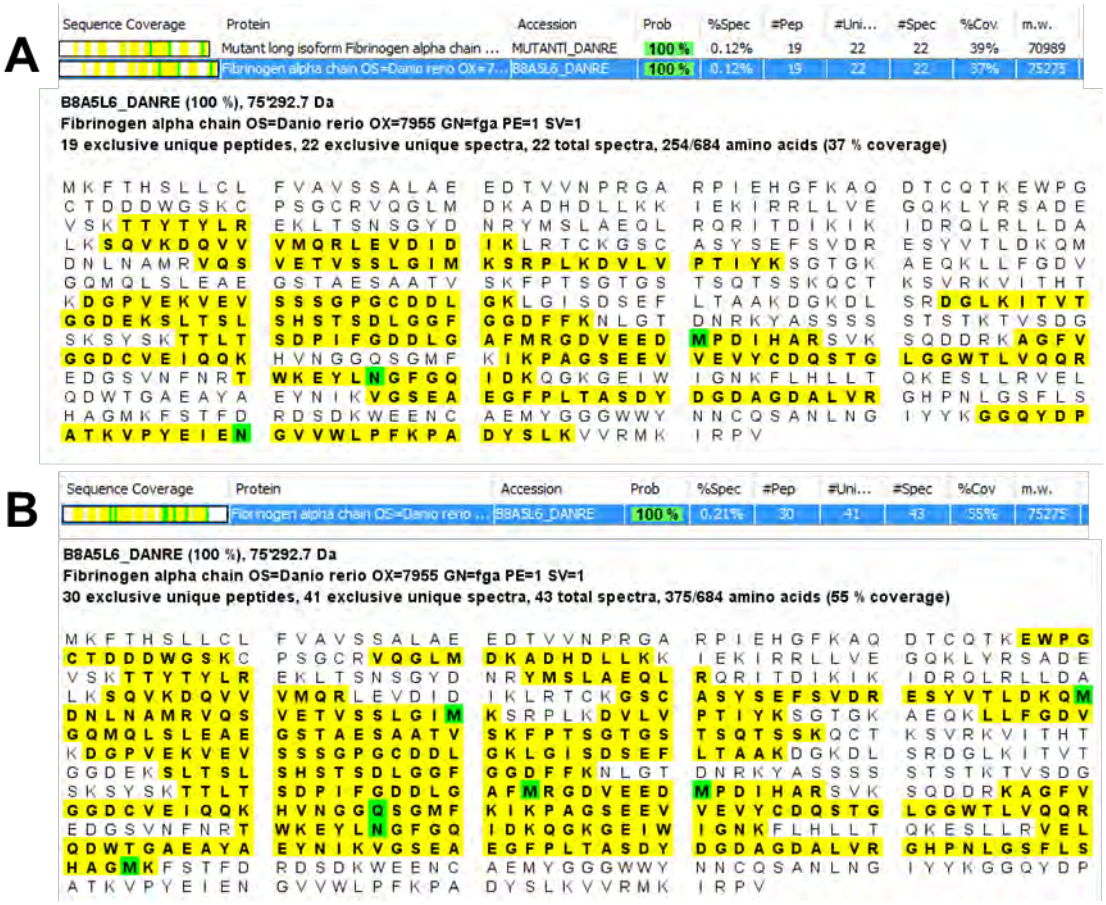


B

Edited *fga* exon 2 – Predicted ESEs:

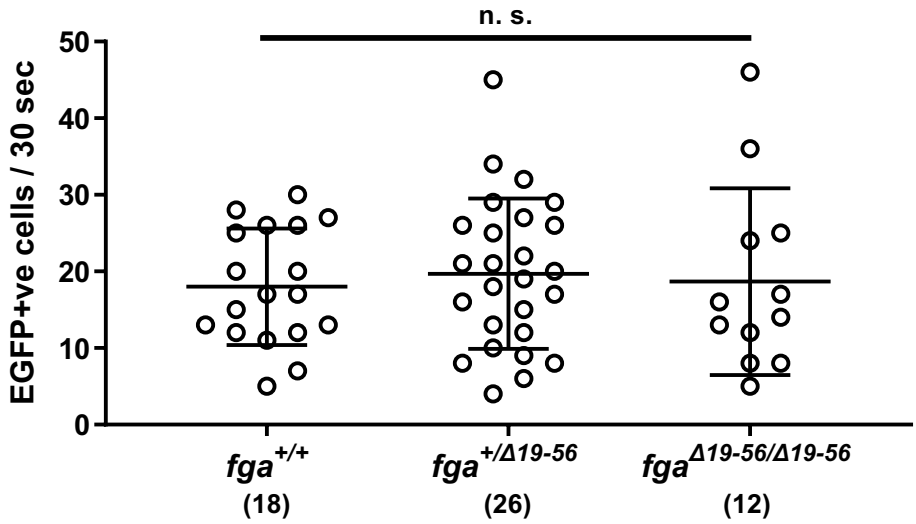


Supplementary Figure 3 –Exon Splicing Enhancer Sequence prediction (ESES) in *fga* exon 2 from wild-type and genome edited. A splicing element annotation tool (RESCUE-ESE Web Server - genes.mit.edu) was used to predict the Exonic Splicing Enhancer (ESE) sequences in *fga* exon 2 and in *fga* exon 2 with targeted genome editing. In *fga* exon 2, 15 ESE sequences were predicted. In the edited *fga* exon 2, 13 sequences were predicted. 5 ESE sequences that were present in the *fga* exon 2 are absent in the edited *fga* exon 2, and 3 new ESE sequences have appeared in the edited sequence. A frequent phenotype seen after mutation of an ESE site is exon skipping [1]. In yellow are nucleotide changes for codon usage, in blue is the nucleotide change to generate an HpaI cleavage site for genotyping, and in green is the codon change designed for the R28C mutation.



Supplementary Figure 4 – Protein identification by ESI-LC-MSMS. Protein identification by ESI-LC-MSMS on two zebrafish plasma samples to distinguish fibrinogen alpha chain from wild-type control and fibrinogen alpha chain from a genome edited mutant sample. After sample concentration, proteins were digested and peptides were analysed by nanoLC-MSMS using an easynLC1000 (Thermo Fisher) coupled with a Qexactive Plus mass spectrometer (Thermo Fisher). Database searches were performed with Mascot (Matrix Science) using Zebrafish Reference Proteome database (uniprot.org) supplemented with fibrinogen mutant sequences. Data were analysed and validated with Scaffold (Proteome Software) with 1% of protein FDR and at least 2 unique peptides per protein with a 0.1% of peptide FDR. **(A)** For the mutant: 323 proteins were identified. Fibrinogen alpha was identified with

19 exclusive unique peptides covering 37% of the protein sequence (yellow). **(B)** For the control sample: 388 proteins were identified. Fibrinogen alpha was detected with 30 exclusive unique peptides covering 55% of the protein sequence (yellow). However, in this sample, a specific peptide unique to the wild type sequence of Fibrinogen alpha was detected (EWPGCTDDDWGSK).



Supplementary Figure 5 – Circulating thrombocytes in 5dpf zebrafish larvae

The number of *Tg(itga2b:EGFP)* thrombocytes passing through the posterior cardinal vein in 30 sec was monitored for $fga^{+/+}$, $fga^{+/\Delta 19\text{-}56}$and $fga^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ larvae at 5dpf. Unpaired t-test: $fga^{+/\Delta 19\text{-}56}$ vs. $fga^{+/+}$ P= 0.5422; $fga^{\Delta 19\text{-}56/\Delta 19\text{-}56}$ vs. $fga^{+/+}$ P= 0.8546; $fga^{+/\Delta 19\text{-}56}$ vs $^{\Delta 19\text{-}56}$ P= 0.7832. The number of assessed larvae is shown in brackets.

Supplementary Data Reference

1. *Berget, S.M., Exon recognition in vertebrate splicing. J Biol Chem, 1995.* ***270****(6): p. 2411-4.*

PART II: A genetic modifier of venous thrombosis in the zebrafish (*Danio rerio*) reveals a functional role for the fibrinogen AαE chain in early hemostasis

Abstract

Fibrinogen is composed of two copies of three types of chains: Aα, Bβ and γ, encoded by *FGA*, *FGB* and *FGG* genes respectively. The *FGA* gene consists of six exons and two different mRNA products are produced: the major mRNA species encoding the common Aα-chain (accounting for the 90% of the transcripts), and the extended AαE isoform which (accounting for the 1–2% of the transcripts). The AαE chain contains an extra C-terminal globular domain homologous to the C-terminal globular domain of fibrinogen Bβ and γ chains by 40%.

In a subclass of fibrinogen molecules, referred to as Fibrinogen 420 (Fib420), each of the two Aα-chains is replaced by an AαE isoform. Since this fibrinogen subclass is preserved throughout the vertebrate kingdom from lampreys to humans, it is thought to have an important physiological function not yet identified. Additionally, an analysis of plasma samples from adults and newborns revealed that the levels of Fib420 in neonates are three times higher than in adults. Since Fib420 levels drop six months after birth when other components of the coagulation system mature, it is believed that they could have an important hemostatic function during developmental hemostasis. Similarly, zebrafish also produce predominantly the AαE containing fibrinogen at larval stages.

We aimed to study the role of the AαE isoform in hemostasis and thrombosis using zebrafish as the model organism. To do this, we performed vascular endothelial mediated laser injuries in larvae from different zebrafish strains (AB, TU and TL). To measure intravascular hemostasis and venous thrombosis after the vascular injuries, we used two different assays: the time to occlusion (TTO) at 3dpf (when clotting is reliant on fibrinogen and erythrocytes), and the thrombocyte recruitment assay at 5dpf (when clotting is dependent on fibrinogen, thrombocytes, and erythrocytes).

When studying intravascular hemostasis, we observed that the AB strain larvae showed prolonged TTO compared to TU and TL strain larvae, and failed to form the AαE isoform due to a mutation in *fga* exon 6. We questioned whether the lack of AαE isoform delayed the TTO in the AB strain larvae. Our results showed that the AαE isoform rescued venous occlusion in fibrinogen deficient mutants or *fga* morpholino knock-down larvae, while the common Aα-chain was less efficient. When studying venous thrombosis at 5dpf, we observed no differences in thrombocyte recruitment at the injury site in larvae expressing the common Aα-chain or the AαE isoform. However, we did observe differences in erythrocyte recruitment at the injury site in larvae expressing the Aα-chain or AαE isoform when studying venous thrombosis at 3dpf.

These observations demonstrate the role of AαE isoform on venous thrombosis during developmental hemostasis, the different contribution of the two isoforms (Aα and AαE) on thrombosis (which seems to be linked to the availability of different cell types for clotting), and that the mutation that prevents AαE formation found in AB strain is a genetic modifier of venous thrombosis in zebrafish larvae.

Aims

Fibrinogen 420 is a subclass of fibrinogen that account for 1 to 3% of total fibrinogen in circulation. The distinctive feature of this molecule is that the two common Aα-chains are replaced by AαE isoforms. The AαE isoforms differs from the common Aα chains in the presence of an extra C-terminal globular domain, which shares homology with the C-terminal domain of Bβ and γ chains. Furthermore fibrinogen 420 molecule is preserved throughout vertebrate evolution and which suggests that it has a vital physiological function.

It has been shown that newborns present three times more fibrinogen 420 compared to adults. The aim of the second part of the thesis is to unravel the functional role of fibrinogen AαE in embryonic hemostasis using different zebrafish strains. Indeed we found that the AB strain has a naturally-occurring single nucleotide deletion in *fga* exon 6, preventing the formation of AαE C-terminus; and we concluded that this mutation is a genetic modifier of venous thrombosis in zebrafish larvae.

Submitted Manuscript

A genetic modifier of venous thrombosis in the zebrafish (*Danio rerio*) reveals a functional role for the fibrinogen AαE chain in early hemostasis*

Cristina Freire*[1], Richard J. Fish*[1], Rui Vilar[1], Corinne Di Sanza[1], Steven J. Grzegorski[2], Catherine E. Richter[2], Jordan A. Shavit[2] and Marguerite Neerman-Arbez[1]

[1]Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Geneva, Switzerland.

[2]Division of Pediatric Hematology/Oncology, Department of Pediatrics, University of Michigan School of Medicine, Ann Arbor, MI.

*equal contribution

My contribution: Figure 1, Figure 3, Figure 4 and Figure 5. Supplementary Figures 1 and 2.

*Manuscript submitted to Blood Advances Journal

Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, 1, rue Michel Servet, 1211, Geneva Switzerland

Tel: +41223795655

Fax: +41223795707

Email: Marguerite.Neerman-Arbez@unige.ch

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[1]Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Geneva, Switzerland.

[2]Division of Pediatric Hematology/Oncology, Department of Pediatrics, University of Michigan School of Medicine, Ann Arbor, MI.

*equal contribution

Short title: A function for larval zebrafish fibrinogen AαE

For correspondence: Marguerite Neerman-Arbez

Department of Genetic Medicine and Development, University of Geneva Medical Centre,

1, rue Michel-Servet, 1211 Geneva 4, Switzerland. Telephone: +41 (0)22 379 56 55, Fax: +41 (0)22 379 57 06, Marguerite.Neerman-Arbez@unige.ch

Text word count: 3989

Abstract word count: 250

Number of figures and tables: 5 figures, 2 supplementary figures

Number of references: 59

Scientific category: Thrombosis and Hemostasis

Key points

- A mutation preventing production of a fibrinogen alpha chain isoform (AαE) is a genetic modifier of venous thrombosis in zebrafish.
- A functional role for the conserved AαE chain in early hemostasis is revealed.

Abstract

Plasma fibrinogen molecules comprise two copies of Aα, Bβ and γ chains folded into a hexameric protein. A minor fibrinogen isoform with an extended Aα chain (AαE) is more abundant in newborn human blood than in adults. Larval zebrafish produce predominantly AαE-containing fibrinogen, but its functional significance is unclear. In 3-day-old zebrafish when hemostasis is reliant on fibrinogen and erythrocyte-rich clotting, but largely thrombocyte-independent, we measured the time-to-occlusion (TTO) in a laser-induced venous thrombosis assay in three zebrafish strains. AB strain larvae showed delayed TTO compared to the rapid occlusion of the TU and TL strains. Mating AB with TU or TL produced larvae with the TU/TL strain phenotype. In contrast to TU, AB larvae failed to produce fibrinogen AαE, due to a mutation in the AαE-specific coding region of *fga*. We investigated whether the lack of AαE explained the delayed AB TTO. Transgenic expression of AαE, but not Aα, shortened the AB TTO to that of TU. AαE rescued venous occlusion in fibrinogen mutants or larvae with morpholino-targeted fibrinogen alpha chain mRNA, but Aα was less effective. In 5-day-old larvae circulating thrombocytes contribute to hemostasis, as visualized in *Tg(itga2b:EGFP)* transgenics. Laser-induced venous thrombocyte adhesion and aggregation is reduced in fibrinogen mutants, but transgenic expression of Aα or AαE restored similar thrombocyte accumulation at the injury site. Our data demonstrate a genetic modifier of venous thrombosis, a role for fibrinogen AαE in early hemostasis,

and suggest a link between differentially expressed fibrinogen isoforms and the cell types available for clotting.

Introduction

The role of fibrinogen as the thrombin substrate is conserved in vertebrate blood clotting.[1] The most abundant soluble human fibrinogen is a hexamer of two Aα, Bβ and γ polypeptides, combining to a molecular weight of about 340 kDa. Variability comes from post-translational modifications[2] and differential mRNA splicing giving rise to γ'[3] and AαE[4,5] chain isoforms. γ' is present in approximately 7% of circulating fibrinogen and can fold into the hexamer with the major γ chain (γA) or γ'. Four C-terminal amino acids of γA are replaced with 20 alternative residues, providing binding sites for Factor XIII[6] and thrombin.[7] The AαE chain arises from splicing of a sixth exon in the Aα chain mRNA. This encodes a C-terminal globular domain (α_EC) with homology to the Bβ and γ chain C-termini.[5] AαE is only present as two copies in human fibrinogen, generating a hexamer of 420 kDa (fibrinogen 420).[4] Despite being represented in only around 1% of circulating fibrinogen, the α_EC domain is highly conserved[8], suggesting functional importance. The proportion of fibrinogen 420 in newborn blood is considerably higher than later in life.[9]

Despite its description over 25 years ago[5], the role of the α_EC domain remains enigmatic, at least when compared to the understanding of the mechanistic[10-12] and clinical implications[13] of the γ/γ' chain isoforms. Studies of the α_EC structure[14], fibrinogen 420-containing fibrin clots[15] and their dissolution[16], have been reported. One proposed function for α_EC is as an additional binding site for leukocyte interactions with fibrinogen or fibrin via integrin receptors.[17] The α_EC domain can be

seen as nodes bulging from fibrin fibers[15], inferring an exposed binding region for cellular interactions. However, it is not clear in which context this interaction is important *in vivo*.

The zebrafish (*D. rerio*) fibrinogen alpha chain gene (*fga*) encodes both Aα and AαE isoforms[18], also due to differential splicing of a sixth exon.[5] The α_EC domain is over 60% identical to human α_EC and both span 236 amino acids. The zebrafish has emerged as a model for thrombosis and hemostasis studies[19] due to the conservation of coagulation proteins[20] and the successful adaptation of *in vivo* hemostasis tests using transparent zebrafish larvae.[21-25] In addition, targeted mutations in a number of coagulation-related proteins, including fibrinogen[26,27], have demonstrated the zebrafish's utility in modeling factor deficiencies and for assessing the impact of patient-derived polymorphisms.[28-31] Zebrafish have nucleated thrombocytes[32], which are considered cellular equivalents to platelets in hemostasis. They participate in blood clotting in a platelet-like manner, rapidly binding and aggregating at injury sites.[33-36]

In previous work we noticed the presence or absence of fibrinogen AαE chains in the plasma of adult zebrafish.[26] This was attributed to a single nucleotide deletion in the sixth exon of *fga* in a laboratory strain, predicted to cause a translational frameshift, preventing α_EC production.

Here we describe how strain-to-strain variability in a laser-induced venous thrombosis assay in zebrafish larvae has a genetic basis that we assign to the *fga* exon 6 variant. Homozygosity of the deletion in the AB strain prevents AαE expression, leads to prolonged venous thrombosis times and low fibrinogen production. Without the deletion, TU strain larvae express predominantly AαE-containing fibrinogen and support rapid thrombosis in response to laser injury. Whole

genome sequencing identified the deletion in an AB x TL hybrid strain. Genetic complementation and expression of Aα or AαE highlighted the importance of the AαE chain in early hemostasis, and we used transgenic reporters to monitor erythrocyte and thrombocyte activity in this setting. We demonstrate the utility of zebrafish for identifying a genetic modifier of hemostasis, an early developmental role for the AαE chain, and differences in the abilities of fibrinogen with Aα or AαE chains to promote thrombosis upon vessel injury. This is discussed in the context of how the highly conserved α_EC domain may affect thrombosis and hemostasis in humans.

Methods

Zebrafish

Adult zebrafish were maintained at 26°C, pH 7.5, and 500 μS conductivity. *fga* mutants, and their genotyping, were described previously.[26,27] Experimentation was authorized by local veterinary authorities. Animals with *itga2b*:EGFP and *gata1*:DsRed transgenes were gifts from Leonard Zon's laboratory, Harvard Medical School. Developing embryos were raised at 28.5°C. Genotyping of *fga* exon6 was by Sanger sequencing of PCR products, amplified using the oligonucleotides fgax6F:CAGATTGCGTTGAAATCCAGCAG and fgax6R:GGTTGGGATGTCCTCTCACTAAC.

Whole Genome Sequencing

Genomic DNA was extracted from adult tails and larval pools of AB x TL hybrid zebrafish using the Qiagen DNeasy Kit. Illumina 150 base pair paired-end high throughput sequencing was made at Novogene (Sacramento, CA). Raw data was

aligned to the GRCz10 reference genome using BWA-MEM[37], and sorted and duplicates marked using Sambamba v0.65 (https://lomereiter.github.io/sambamba/index.html) and biobambam (https://github.com/gt1/biobambam2/releases) to generate analysis-ready BAM files. Variants were called within genes defined by Ensembl (GRCz10 v86) using the GATK 3.7 HaplotypeCaller[38,39] and snpEff v4.3T[40] annotation. Variants within exons and 20 base pair flanking regions with a coverage depth of 7 or more in all samples were extracted for further analysis using SnpSift[41] and custom python and R scripts.

Plasmids

Plasmids for expression of zebrafish fibrinogen Aα or AαE under the control of a ubiquitin gene (*ubb*) promoter (subsequently labelled ubi) were prepared by Gateway cloning (Invitrogen). Middle entry clones for Aα and AαE cDNAs were made using attB1- and attB2-tagged oligonucleotides to PCR amplify cDNAs and pDONOR221 (Invitrogen) as a vector for recombination with PCR products, yielding pME-Aα and pME-AαE. These were used in 4-way Gateway recombination reactions with pENTR5'_ubi, (Addgene plasmid #27320)[42], pDestTol2CG2-U6:gRNA (Addgene plasmid #63156)[43], both gifts from Leonard Zon, and p3E polyA from the Tol2kit.[44] Final plasmids for transgenesis were named pT2ubi:Aα and pT2ubi:AαE. Our Aα cDNA clone encodes the 456 amino acid zebrafish Aα chain, the AαE cDNA the 684 amino acid zebrafish AαE chain. In the shared region of these polypeptides, spanning from methionine 1 to glycine 452, one amino acid differs. Our Aα clone has a serine at position 286 and our AαE clone an asparagine. This polymorphic residue has been described previously in zebrafish alpha chain sequence annotations (Genbank: BC054946.1 and BC075895.1). When aligned to human Aα, and

compared to known human mutations, this residue in zebrafish Aα or AαE is not expected to affect fibrinogen function.

Microinjections

1 to 2 cell zebrafish embryos were microinjected with approximately 1 nL of injection mixes. These contained Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH7.6) and phenol red. Where described, 2 ng of an *fga* exon1-intron1 splice site-targeting antisense morpholino (5'GCATTATATCACTCACCAATGCAGA3') or a 5-nucleotide mis-match control (5'GCTTAATATGACTCACGAATCCAGA3') were included (Genetools Inc.). For transgenic expression of Aα or AαE, injection mixes included 25 ng of pT2ubi:Aα or pT2ubi:AαE and approximately 35 ng of 5'capped, in vitro-polyadenylated Tol2 transposase mRNA.

Laser injury assays and imaging

3- or 5-day post-fertilization (3dpf or 5dpf) zebrafish larvae were anesthetized with 0.17 mg/ml MS-222 (Sigma) and placed on their sides in 0.22% low-gelling temperature agarose (Sigma) on glass microscope slides. Using a Leica LMD microscope, laser injuries were targeted to the posterior cardinal vein (PCV), 5 somites caudal to the cloaca.[24] Constant laser power, amplitude, speed and injury shapes were used for 3dpf and 5dpf larvae, respectively. The system has a Leica DM 6500 microscope with an HCX PL FLUOTAR L 20x/0.40 CORR objective (numerical aperture 0.4) with a Cryslas Laser (maximum pulse energy 50 µJ, frequency 80 Hz, wavelength 355 nm) and images and films were acquired with Leica LMD CC7000 and DFC360 FX cameras using LMD and LAS-AF software, at room temperature.

Fluorescence accumulation over time in a selected 275.25 μm^2 region was assessed using MetaMorph (7.1) software for *Tg(itga2b:EGFP)* or *Tg(gata1:DsRed)* larvae. To evaluate circulating thrombocyte numbers in 5dpf *fga*$^{+/+}$, *fga*$^{+/-}$ and *fga*$^{-/-}$ fish with the *itga2b*:EGFP transgene, larvae were prepared as for laser injuries and fluorescence filmed for 1 minute. GFP-positive cells passing through the PCV in 30 seconds were counted. For laser injuries described in Figure 2, a protocol described formerly was used.[30]

RT-qPCR

RT-qPCR assays for fibrinogen mRNA expression in larval RNA samples were described previously.[26]

Immunoblots

Zebrafish larvae were lysed in T-PER (Invitrogen) with protease inhibitors (Roche) and boiled in SDS-PAGE gel loading buffer (BioRad). Samples were subjected to SDS-PAGE and immunoblotting, as described previously[26], with detection of zebrafish fibrinogen chains using antibodies developed by Covalab and β-actin antibodies from Sigma.

Graphics and Statistics

Graphics were prepared and the statistical tests described made using Prism 8 (GraphPad).

Data sharing statement

For original data, please contact Marguerite.Neerman-Arbez@unige.ch.

Results

Laboratory zebrafish strains reveal a genetic modifier of venous thrombosis

The laser-induced time to occlusion (TTO) of blood in the larval zebrafish posterior caudal vein (PCV) has been used as a general readout for hemostasis.[21,35,45] In 3dpf larvae of the TU zebrafish strain we measured an average TTO of 22.6 seconds (range 8 seconds, n=20). In the AB strain the mean TTO was 89.8 seconds (range 92 seconds, n=14). Four AB embryos failed to support occlusion within 3 minutes of the laser injury. This difference suggested strain-specific variance in embryonic hemostasis. To test for a genetic basis of this difference, TU and AB fish were mated and a mean TTO of 25.1 seconds (range 25 seconds, n=19) measured in their offspring (Figure 1A). This result implies that a genetic modifier of developmental venous thrombosis resides in one of the strains. As the mean TTO from the TU x AB larvae resembles the TU strain, we reasoned that a recessive anti-thrombotic mutation exists in the AB strain or a pro-thrombotic dominant mutation is present in TU.

In previous work[26], we reported a polymorphism in AB zebrafish when developing fibrinogen-deficient animals mutated in *fga*. In some fish a single nucleotide deletion, compared to a TU strain-derived reference sequence, was detected in exon 6 of *fga*. This difference is also present in an AB-derived mRNA sequence (Genbank:BC054946.1). Exon 6 of *fga* encodes the α_EC domain of the extended AαE isoform. The deletion is predicted to frameshift the AαE mRNA. Supporting this, we detected Aα but not AαE in the plasma of animals with the deleted nucleotide.[26] Figure 1B shows the local exon 6 DNA sequence in TU, AB and TU x AB zebrafish, confirming the polymorphism. Figure 1C shows aligned sequences for part of *fga*

exon 6, highlighting the differences detected and their predicted consequences. We subjected protein lysates from 3dpf TU, AB and TU x AB larvae to immunoblotting with anti-zebrafish Aα chain antibodies which were raised to an antigen common to Aα and AαE. At 3dpf, the fibrinogen alpha chain seen in TU larvae was almost exclusively AαE. In AB larvae, the AαE chain was not detected, only a comparatively low amount of the Aα chain. In TU x AB lysates both alpha chains were detected, but predominantly AαE (Figure 1D).

We hypothesized that the *fga* exon 6 polymorphism could lead to effects on fibrin-based coagulation, seen as the genetic modifier of laser-induced TTO in our zebrafish strains and hinting at a role for AαE (and α_EC) in early development.

In a separate colony of AB x TL strain hybrid fish, a similar pattern of delayed TTO was observed. Two adults were crossed and 56 larval offspring underwent laser-induced TTO tests at 3dpf. 42 formed occlusions within 120 seconds and 14 failed to occlude, supporting a recessive inheritance pattern for delayed TTO. Genomic DNA was extracted from both parents and 3 pools of larvae (20 occluding, two distinct pools of 7 non-occluding) and processed for high-throughput sequencing to identify the causative variant. Following sequencing, 881,793 variants were identified within exons and flanking regions with informative coverage across all samples. From the total variants, 393,734 were identified as informative markers for being heterozygous in one parent and non-reference (heterozygous or homozygous) in the other. This enabled the broadest sensitivity in detecting recessive traits. From the informative markers, 5,437 variants were homozygous in both non-occluding pools and heterozygous in the occluding pool. For visualization, the genome was separated into 3 million base pair intervals. In each, the frequency of homozygosity was calculated by dividing the number of homozygous markers by the number of informative

markers. A major peak of homozygosity was seen centered in chromosome 1 from 9-12 million base pairs (Figure 2A). Within this region were 166 missense mutations and 1 frame-shift mutation, the latter was the single base pair G nucleotide deletion in exon 6 of *fga* (Figure 2B). Surprisingly, the entire region was found to be heterozygous in the male parent but homozygous for all variants in the female. Laser injury followed by genotyping revealed that lack or delay of occlusion correlates with the *fga* genotype in a recessive pattern (Figure 2C). Rapid occlusion in four larvae homozygous for the G deletion, with the mapping data, supports the existence of one or more strain-specific suppressor mutations of the non-occluding phenotype.

From these data we concluded: when produced the AαE chain is the major fibrinogen alpha chain in larval zebrafish, that crossing TU or TL strains with AB coincides with increased fibrinogen alpha (Aα or AαE) and rescue of a delayed TTO phenotype, and the single nucleotide deletion in *fga* could be the recessive modifier of larval venous thrombosis.

Fibrinogen AαE, but not Aα, restores hemostasis and thrombosis in *fga* mutants

The delayed TTO phenotype in our AB zebrafish larvae could be due to low fibrinogen production as well as an incapacity to produce the AαE chain. As fibrinogen-deficient zebrafish larvae fail to support laser-induced venous thrombosis[27] we reasoned that by re-introducing alpha chain expression in homozygous *fga* mutant embryos we could compare the relative activity of Aα and AαE isoforms. Using micro-injection of a Tol2 transposon-based expression system[46] we expressed Aα or AαE cDNA under the control of the ubi promoter[42], in embryos from *fga*$^{+/-}$ in-crosses, and measured laser-induced TTO (Figure 3A). Using heterozygous in-

crosses the genotype of larvae could be assessed after TTO analyses, avoiding potential bias. This method has been used to demonstrate complementation of various coagulation factor mutants.[27,30,31] The genetic background of our mutant line was largely AB (mutation 1 in[26]). Non-injected *fga*$^{+/+}$ or *fga*$^{+/-}$ larvae had variable laser-induced TTOs and occlusion could not be measured in *fga*$^{-/-}$ fish (Figure 3B), as described by Hu et al[27] in a similar *fga* mutant. Expression of fibrinogen Aα did not affect TTO in *fga*$^{+/+}$ or *fga*$^{+/-}$ larvae significantly, compared to non-injected larvae, and only rescued venous occlusion in a single *fga*$^{-/-}$ animal (n=9). However, expression of fibrinogen AαE cDNA shortened the laser-induced TTO in all but one of the *fga*$^{+/+}$ (n=29), *fga*$^{+/-}$ (n=44) or *fga*$^{-/-}$ (n=12) larvae (Figure 3B), with TTO values reminiscent of the TU strain (Figure 1A).

AαE fibrinogen facilitates erythrocyte-rich thrombosis

In 3dpf zebrafish larvae, thrombosis relies predominantly on erythrocytes as the cellular component as few thrombocytes have reached the blood circulation at this developmental stage. To assess the role of AαE versus Aα fibrinogen chains in this context, we used an anti-sense morpholino oligonucleotide (MO) to lower fibrinogen expression by targeting splicing of the *fga* mRNA in a transgenic zebrafish line with red fluorescent erythrocytes (*Tg(gata1:DsRed)).* In two control experiments using the TU strain, we injected early embryos with 2 ng of *fga* MO or 2 ng of a control MO, and measured fibrinogen mRNA by RT-qPCR in pools of larvae at 3dpf. Mean *fga* mRNA levels, normalized to 100% in non-injected embryos, were 110% in control MO-injected larvae and 7% with the *fga* MO. The *fga* MO was co-injected with or without transgenesis reagents for expression of fibrinogen Aα and AαE chains in hemizygous *Tg(gata1:DsRed)* embryos from a transgenic parent mated with the TU

strain (Figure 4A). The *fga* MO lowers endogenous Aα or AαE fibrinogen expression but should not prevent expression of cDNA-derived fibrinogen alpha chain mRNA, as it targets unspliced *fga* mRNA. Without exogenous alpha chain expression the *fga* MO knock-down prevents venous occlusion in the TTO assay (Figure 4B). Expression of the Aα chain rescued occlusion in 17/23 larvae with a prolonged TTO compared to non-injected animals (mean TTO, 18 seconds for non-injected, 58 seconds for occluding fga MO + Aα). Expression of the AαE chain rescued occlusion in all *fga* MO + AαE larvae, with an average TTO that was similar to non-injected fish (mean TTO, 21.9 seconds, n=24). The more effective rescue of erythrocyte-rich clotting with AαE versus Aα chain expression, could also be seen when quantifying the accumulation of red fluorescence after the laser injury (Figure 4C). 24 seconds after laser injury, a time point where occlusive thrombosis was indistinguishable in non-injected and *fga* MO + AαE-injected larvae, mean fluorescence had not increased at the injury site after *fga* MO-mediated knock-down and barely changed in *fga* MO + Aα-injected animals.

To approximate fibrinogen protein levels present in the larvae in these analyses, and control for possible differences in expression levels between transgenically expressed Aα and AαE chains (used for data in Figures 3 and 4), we made immunoblots for fibrinogen larval lysates 3 days post-injection. Compared to non-injected larvae, fibrinogen was scarcely detectable after MO injection, but immunoreactive bands of similar molecular weight and intensity to those in non-injected larval lysates were seen after injection of *fga* MO + Aα or *fga* MO + AαE (Supplementary Figure 1).

We conclude that the genetic modifier of venous thrombosis detected is a single nucleotide deletion in exon 6 of *fga* which prevents the production of fibrinogen

containing the AαE isoform. This leads to low embryonic fibrinogen production and variable larval thrombotic responses that can be effectively rescued by fibrinogen AαE, but not Aα, even when Aα is expressed at levels resembling the AαE-containing fibrinogen in larvae lacking the deletion.

Fibrinogen Aα or AαE mediate thrombocyte adhesion and aggregation

In 5dpf zebrafish larvae thrombocytes circulate and participate in clotting in response to laser injury. They are thought to be the functional cellular equivalent to mammalian platelets.[32] We were interested to know if the differences in the ability of fibrinogen Aα and AαE chains to rescue the depletion or absence of fibrinogen in our thrombosis assay at 3dpf were similar at 5dpf in the presence of thrombocytes. Laser injuries were made to the PCV in 5dpf *Tg(itga2b:EGFP)* larvae (Figure 5A), in the presence or absence of fibrinogen (*fga*$^{+/+}$ or *fga*$^{-/-}$) and with transgenic expression of Aα or AαE in *fga*$^{-/-}$ fish. Adhesion and aggregation of GFP-positive thrombocytes post-injury was monitored for 2 minutes by fluorescence microscopy (Figure 5B). This assay does not typically lead to occlusive thrombi, which helps in monitoring the dynamics of thrombocyte interactions at the injured vessel wall. We controlled for effects of *fga* genotype on circulating thrombocyte numbers by counting the GFP-positive cells passing through the PCV in non-injected *fga*$^{+/+}$, *fga*$^{+/-}$ and *fga*$^{-/-}$ fish with the *itga2b*:EGFP transgene. No significant differences were measured (Supplementary Figure 2).

The absence of fibrinogen (*fga*$^{-/-}$) reduced laser-induced thrombocyte accumulation, compared to wild-type larvae (*fga*$^{+/+}$), but did not inhibit initial thrombocyte binding to the injury site or a gradual increase in fluorescence over time. Both Aα and AαE expression in *fga*$^{-/-}$ increased thrombocyte accumulation compared to non-injected

fga$^{-/-}$, with AαE giving faster initial mean thrombocyte binding at the injury site compared to Aα, and Aα facilitating a slower buildup but larger mean thrombocyte aggregate area, compared to AαE (Figure 5B). These data show variability within experimental groups, but overall the role of fibrinogen in larval thrombocyte hemostatic activity, in contrast to developmentally earlier occlusive thrombi, is not markedly dependent on one fibrinogen alpha chain isoform.

Discussion

In this report we demonstrate the molecular basis of a genetic modifier of venous thrombosis in larval zebrafish which in turn assigns a functional role for fibrinogen with the AαE chain isoform, with its α_EC domains, in early hemostasis.

Our study is an example of how disparity between genetic backgrounds can reveal an unambiguous modifying effect on a defined physiological process. Laboratory zebrafish strains are generally not as well defined genetically as other model systems.[47] While this can introduce variability, in this case it helped reveal the basis of differences in venous thrombosis between strains. Our initial results could have been ignored as technical noise, but instead highlight the power of the larval zebrafish model system and the utility of the laser-induced TTO assay for detecting genotype-phenotype correlations in thrombosis and hemostasis. TL and TU zebrafish were derived by researchers in Tübingen, Germany[48], and might be related, while AB originated in Oregon, USA. This demonstrates the importance of genetic background in model organisms, as seen with Gray Platelet Syndrome in mice[49].

The α_EC domain of fibrinogen AαE is conserved across vertebrates[8], suggesting functional importance. It resembles the C-terminal globular domains of the fibrinogen Bβ and γ chains, in agreement with their common ancestry[50,51], but lacks the equivalent residues found in γC and βC involved in fibrin polymerization[15]. The early hemostasis we have studied in zebrafish larvae indicates that AαE is a more effective hemostatic alpha chain. This infers that the AαE chain is better adapted for fibrin formation during early development compared to Aα, perhaps due to the presence or absence of factor(s) in the embryonic coagulation process or the changing nature of circulating cells. A fibrinogen concentrate rich in fibrinogen 420 may have advantageous clotting properties compared to current fibrinogen-based therapies, with altered fibrin formation kinetics or cellular interactions. Further mechanistic studies are warranted to investigate this.

Binding of fibrinogen 420 to leukocyte β_2-type integrins ($\alpha_M\beta_2$, $\alpha_X\beta_2$) has been reported[17], but its importance in vivo is unclear. Our data support the hypothesis that fibrinogen with AαE, rather than Aα, is involved in hemostasis when erythrocytes are the principle cellular component, in the near absence of circulating thrombocytes. This could implicate the fibrinogen α_EC domain in erythrocyte binding or retention. However, rather than β_2-type integrins, the receptors showing experimental evidence of fibrinogen binding on mammalian red blood cells are the $\alpha_V\beta_3$ integrin[52] and CD47[53]. For erythrocyte retention in venous clots, where FXIII activity is important[54], it seems unlikely that α_EC would be critical because of its low abundance. While α-chain cross-linking is important[55], FXIII-mediated erythrocyte retention can be reconstituted with recombinant fibrinogen that presumably lacks α_EC[55]. However, to our knowledge, a role for fibrinogen 420 has not been excluded.

AB strain larvae with the *fga* exon 6 mutation we describe have low fibrinogen levels and variable hemostatic responses. The presence of fibrinogen Aα enabled vascular occlusion after injury in some AB larvae, with an extended average TTO compared to the TU strain. However, additional transgenic production of Aα had little effect on the variable AB TTO while AαE expression gave TTOs resembling the TU strain. It is unclear whether polymorphisms or mutations in *FGA* exon 6 are linked to human pathology. Three *FGA* exon 6 variants have been identified in patients with fibrinogen deficiencies[56-58], but causality has not been proven. As fibrinogen 420 is only present as 1% of circulating adult fibrinogen, and only 3% in newborns[9], we conclude that variation in the α_EC domain is unlikely to lead to a quantitative human fibrinogen disorder[59]. However, if fibrinogen 420 is necessary for leukocyte interactions, its absence or mutation could have clinical consequences.

When a morpholino was used to lower Aα or AαE expression (>90%) in embryos from a cross between transgenic adults and the TU strain, which can produce AαE, Aα cDNA expression rescued laser-induced TTO more effectively than in the AB strain (compare Figure 2B with Figure 3B). We believe this could be due to residual endogenous AαE expression in these larvae, absent in AB larvae described in Figure 2B.

In conclusion, we have demonstrated a genetic modifier of venous thrombosis in the zebrafish which resides in exon 6 of *fga* and impedes fibrinogen AαE chain expression. The differential ability of Aα and AαE chains to complement fibrinogen deficiency in zebrafish larvae supports a role for the α_EC domain in early blood coagulation, and the presence of AαE or Aα chains may be linked to fibrin formation in the context of different cells or factors available for clotting. The zebrafish model is

ideally suited for further investigation of these issues given the accessibility of larvae for studies of early development.

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Authorship Contributions

C.F. and R.J.F. contributed equally. C.F. performed experiments and analyzed and interpreted data. R.J.F. designed and performed experiments, analyzed and interpreted data and drafted the manuscript. R.V., C.D.S., S.J.G. and C.E.R. performed experiments and analyzed and interpreted data. J.A.S. and M.N.-A directed research and contributed to writing. All authors approved the final manuscript.

Conflict of Interest Disclosures

The authors declare no conflicts of interest in relation to this work.

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Figure Legends

Figure 1 - Variability in laser-induced venous occlusion, an *fga* exon 6 polymorphism and changes in fibrinogen quantity and quality in zebrafish strains

In A, the time to vessel occlusion (TTO) after laser injury of the posterior cardinal vein in 3dpf TU (n=20), AB (n=18) or TU x AB larvae (n=19) is represented. The TU strain TTO is significantly shorter than that of AB, but not different (ns) from that measured in TU x AB larvae (Mann-Whitney tests). Each circle represents an individual larva. B shows small stretches of Sanger sequencing chromatograms of part of *fga* exon 6 PCR-amplified from TU, AB and TU x AB larvae. The sequencing is in the reverse direction to the *fga* open reading frame. A single nucleotide deletion can be seen in the AB strain plot, compared to TU, and then heterozygosity at this position in the TU x AB larvae. In C, the consequences of this single nucleotide change for AαE translation are highlighted. In the upper alignment the sequences begin with the proline 474 codon, the nucleotide deleted in the AB strain is underlined in the TU DNA sequence. Three missense residues are encoded after the deletion in the AB strain (LRR, in grey) before a TAG (UAG in mRNA) terminator. The lower alignment shows the AαE sequence from the start of the *fga* exon 6-encoded residues, in TU, AB and human (Hs) AαE. In AB, translation is predicted to terminate after 27 codons instead of 236 in TU or human AαE. Asterisks (*) show identical aligned residues in TU and human AαE. D shows an immunoblot for detection of zebrafish fibrinogen AαE and β-actin in whole larvae lysates from 3dpf TU, AB and TU x AB embryos.

Figure 2 - Whole genome sequencing reveals the *fga* exon 6 polymorphism in the AB x TL genetic background

(A) Frequency of homozygous markers moving across the genome in 3 million base pair intervals identifies a major peak on chromosome 1 in region of the *fga* locus. The circle size represents the number of informative markers in that interval. Circles with the number 250 represent a range from 250-4500, for easier visualization. (B) IGV (Integrative Genomics Viewer) visualization of whole genome sequencing data for *fga* exon 6. The arrow indicates the site of the GGGG/GGG exon 6 polymorphism, male is heterozygous, and female is homozygous GGG. Read coverage is >20 for the entire region. (C) AB x TL fish hybrids that are not homozygous for the exon 6 polymorphism display rapid vessel occlusion of the PCV in response to laser-induced endothelial injury. Offspring produced from group matings of parents with mixed *fga* genotypes were subjected to injury and phenotypic analysis, followed by genotyping. Larvae homozygous for the polymorphism displayed an increased TTO. $fga^{4G/4G}$, $fga^{4G/3G-}$ and $fga^{3G/3G}$ indicate *fga* exon GGGG or GGG alleles, respectively.

Figure 3 - Phenotypic rescue of a venous thrombosis defect and afibrinogenemia with fibrinogen AαE, but not Aα expression in early zebrafish larvae

1 to 2 cell embryos from an $fga^{+/-}$ in cross mating were microinjected with reagents for transgenic expression of fibrinogen Aα or AαE cDNAs under the control of a *ubb* (*ubi* P) promoter sequence. At 3dpf TTO was measured after laser injury of the PCV as illustrated (A). Results are shown in B with *fga* genotype, number of larvae used in brackets and, where appropriate, cDNA expressed on the y-axis. Each shape represents an individual larva. Mann-Whitney tests were made to test for statistical significance of differences between groups (ns, not significant, **** $p<0.0001$).

Figure 4 - More effective rescue of venous thrombosis with fibrinogen AαE than Aα in morpholino-induced fibrinogen knock-downs, monitored in erythrocyte-rich early zebrafish larvae

1 to 2 cell embryos from a *Tg(gata1:DsRed)* x TU mating were microinjected with a morpholino (MO) that inhibits fga mRNA by anti-sense targeting of the exon 1-intron 1 splicing boundary, lowering by over 90% the expression of fibrinogen Aα or AαE. This was done in the presence or absence of Aα or AαE chain cDNA expression, which cannot be targeted by the MO, and the TTO assessed at 3dpf after laser injury in the PCV. Measurements were made by monitoring the red fluorescent erythrocytes in these larvae, a single frame image of which is given to the right in A. The dorsal aorta, posterior cardinal vein and laser injury position are labelled (DA, PCV, LI). B shows the TTO results for the respective conditions with the number of embryos assessed below each in brackets, and statistical test results using a Mann-Whitney test. Each circle represents an individual larva. In C the *gata1*:DsRed-associated fluorescence accumulation in the first 24 seconds post-laser injury are plotted, for each group of larvae from within a defined 75 μm^2 region around the laser injury site. Average fluorescence over time is plotted with error bars representing the standard deviation for each grouping. The number of larvae per group is shown in brackets next to the group color indicators.

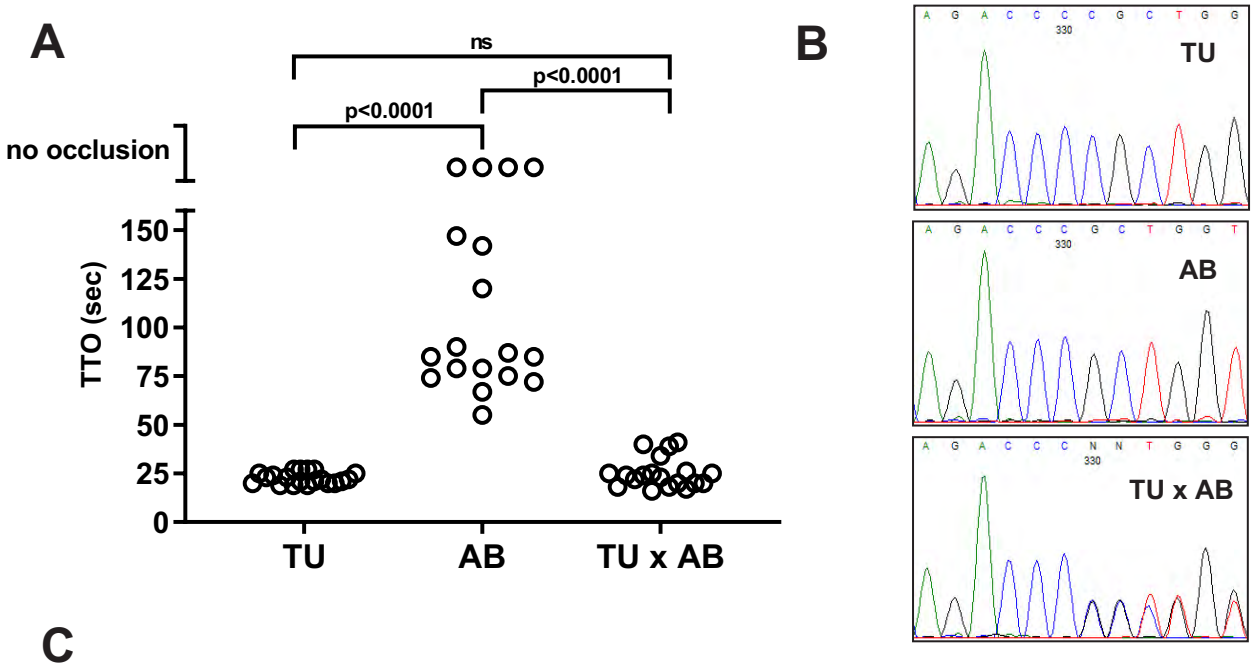
Figure 5 - Fibrinogen Aα or AαE chains rescue laser-induced thrombocyte binding and aggregation in 5dpf zebrafish larvae

1 to 2 cell zebrafish embryos with the $fga^{+/+}$ or $fga^{-/-}$ genotype, hemizygous for the *itga2b*:EGFP transgene, were microinjected for expression of fibrinogen Aα or AαE cDNA. At 5dpf laser injuries were made in the PCV and green fluorescent

thrombocytes accumulating within a 75 µm area measured over time. This is shown schematically in A, the dorsal aorta, posterior cardinal vein and laser injury position are labelled (DA, PCV, LI). In B the mean fluorescence for each experimental group is plotted as a line against time, with error bars representing the standard deviation of the mean. Despite the broad variability in this assay, similar fluorescence intensities over time were measured for *fga*$^{+/+}$, or *fga*$^{-/-}$ with expression of Aα or AαE chains. Mutant *fga*$^{-/-}$ embryos, lacking fibrinogen showed less average thrombocyte accumulation.

Figures

Figure 1



C

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          474  P  A  G  S  E  E  V  V  E  V  etc.

AB fga exon6...CCAGCGGGTCTGAGGAGGTAG...
          474  P  A  G  L  R  R stop

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AB AαE 453 DCVEIQQKHVNGGQSGMFKIKPAGLRR-stop

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          **    * *  * *** * **  **     ***** * **** * **
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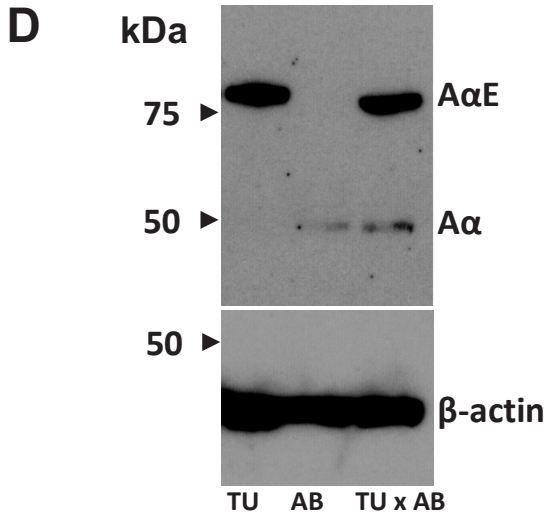
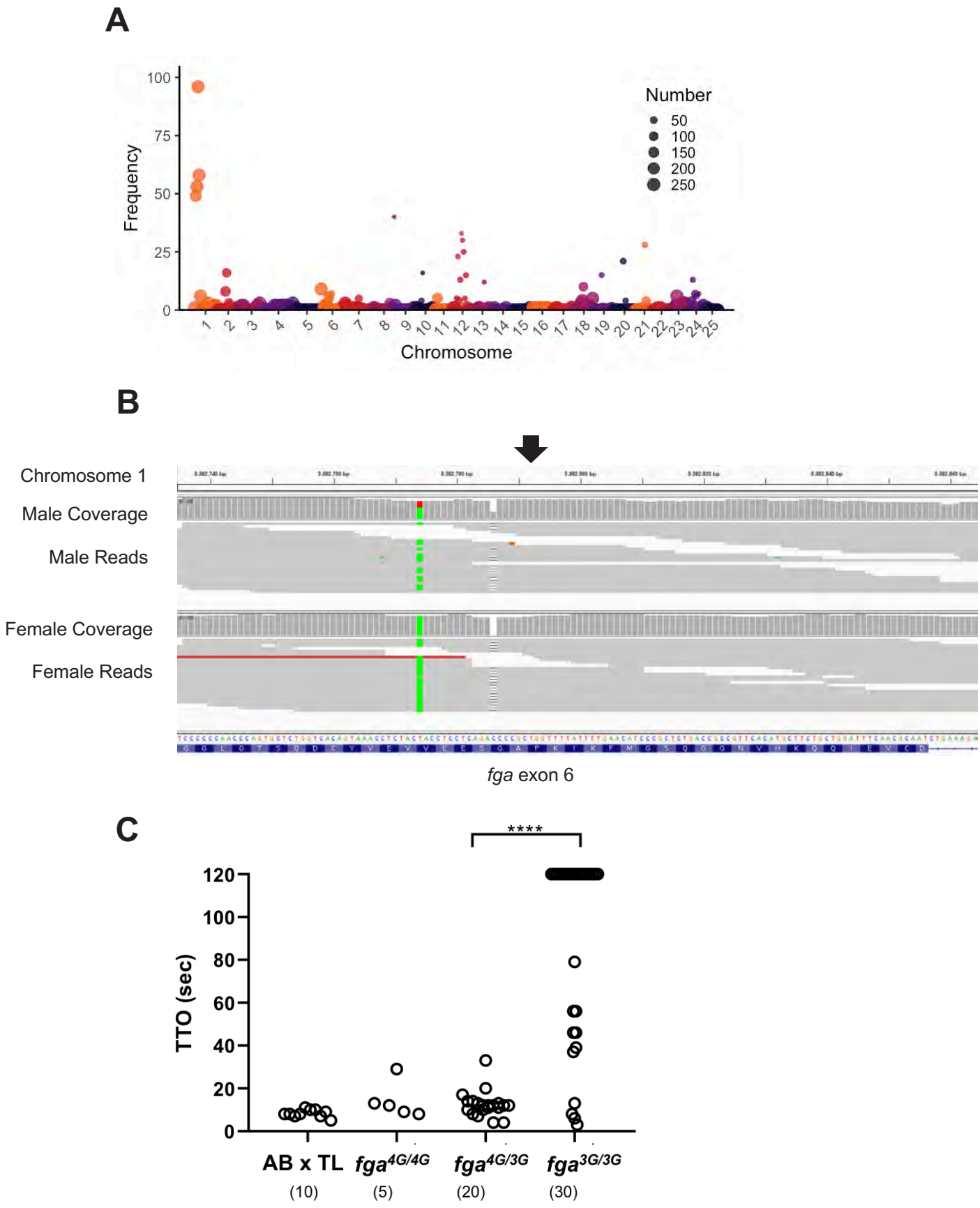


Figure 2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100
150
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250
Frequency
100
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Chromosome
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Male Coverage
Male Reads
Female Coverage
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fga exon 6
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TTO (sec)
120
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40
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AB x TL
(10)
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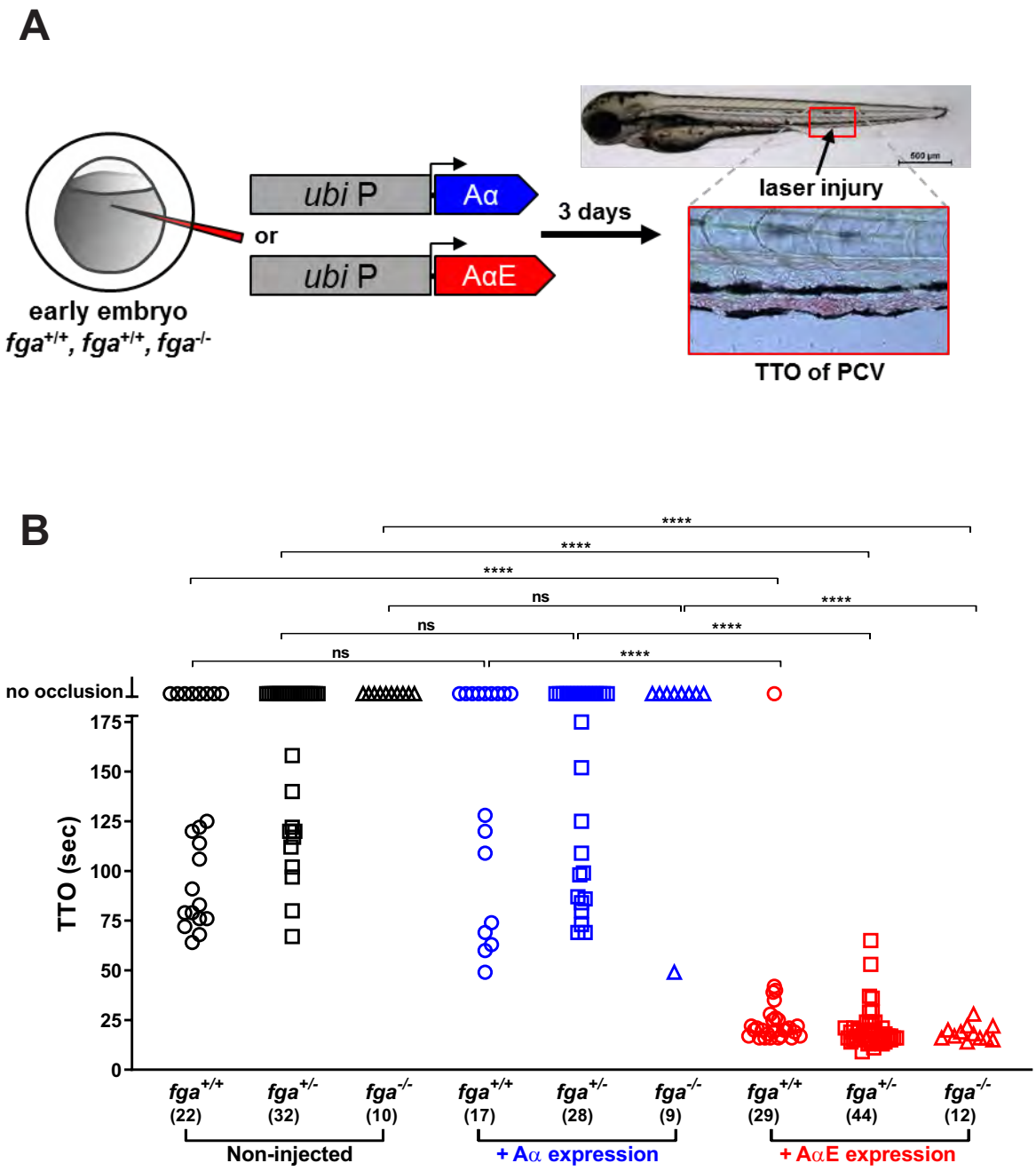
fga3G/3G
(30)

Figure 3


A
ubi P
Aα
or
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AαE
3 days
laser injury
early embryo
fga+/+, fga+/+, fga-/-
TTO of PCV
B
no occlusion
TTO (sec)
175
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125
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75
50
25
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ns
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fga+/+ (22)
fga+/- (32)
fga-/- (10)
fga+/+ (17)
fga+/- (28)
fga-/- (9)
fga+/+ (29)
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fga-/- (12)
Non-injected
+ Aα expression
+ AαE expression

Figure 4

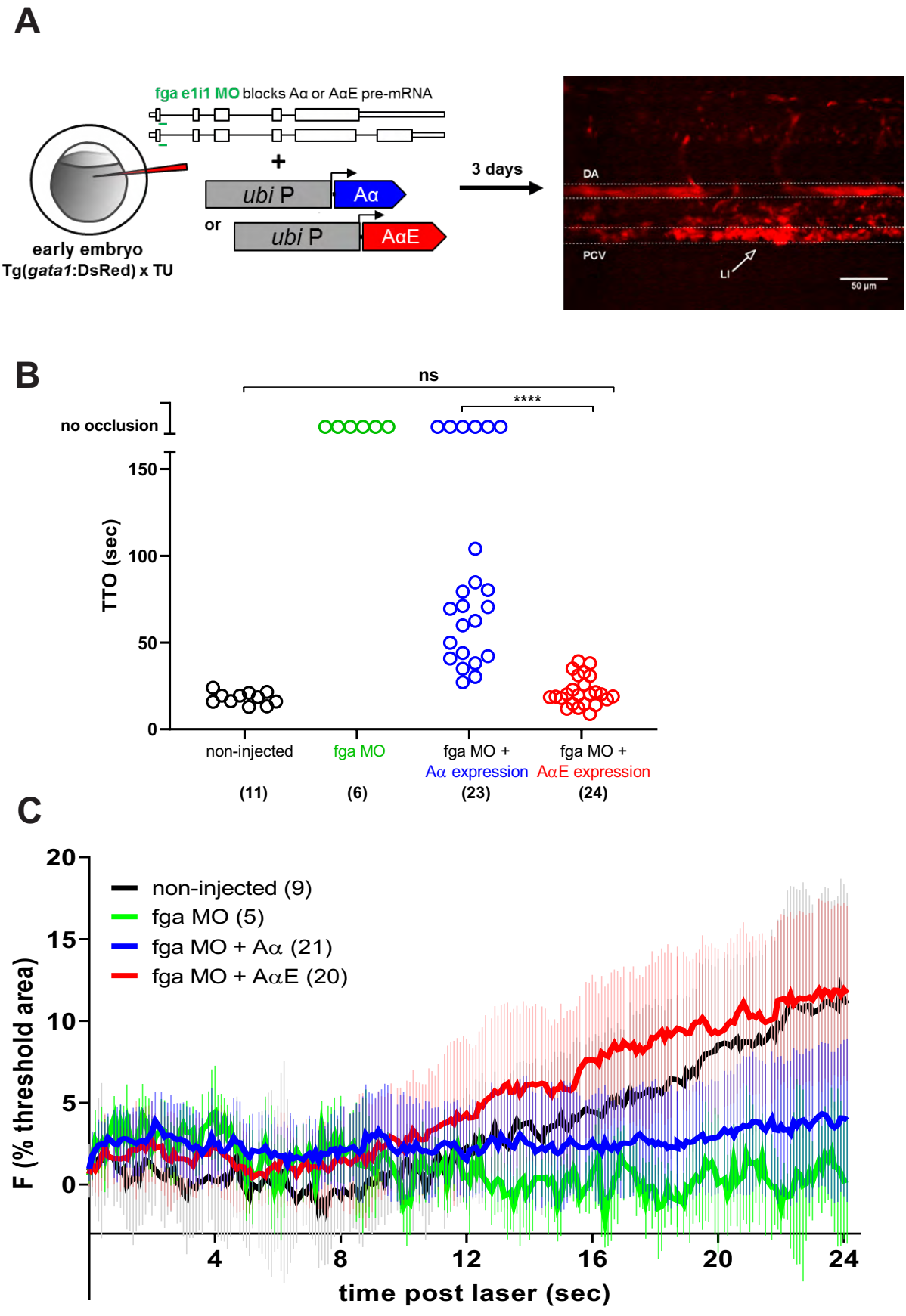
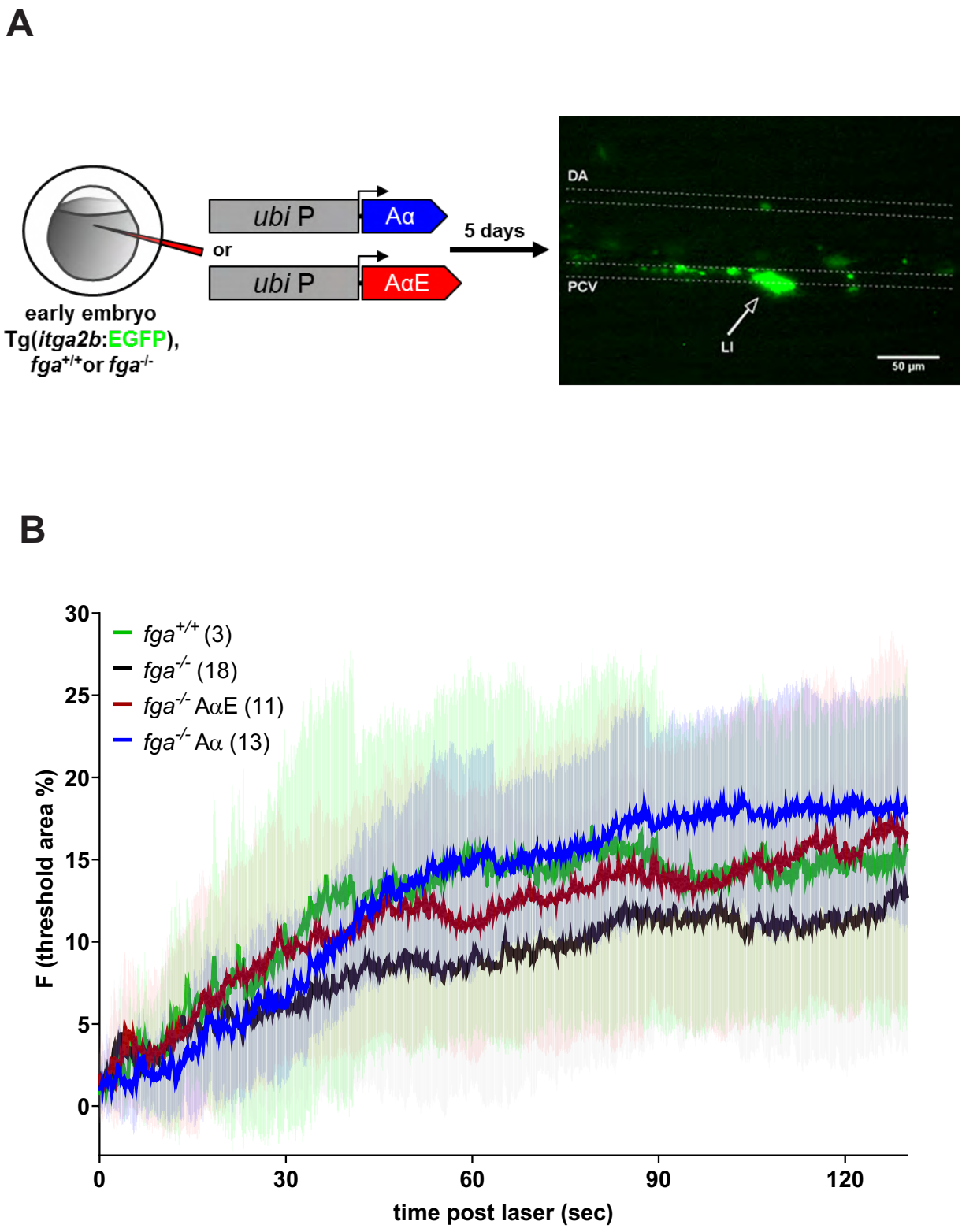

A
fga e1i1 MO blocks Aα or AαE pre-mRNA
ubi P
Aα
or
ubi P
AαE
3 days
early embryo
Tg(gata1:DsRed) x TU
DA
PCV
LI
50 µm
B
ns
****
no occlusion
150
100
50
0
TTO (sec)
non-injected
fga MO
fga MO + Aα expression
fga MO + AαE expression
(11)
(6)
(23)
(24)
C
20
15
10
5
0
F (% threshold area)
non-injected (9)
fga MO (5)
fga MO + Aα (21)
fga MO + AαE (20)
4
8
12
16
20
24
time post laser (sec)

Figure 5


A
ubi P
Aα
or
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AαE
5 days
early embryo
Tg(itga2b:EGFP),
fga+/+ or fga-/-
DA
PCV
LI
50 µm
B
fga+/+ (3)
fga-/- (18)
fga-/- AαE (11)
fga-/- Aα (13)
F (threshold area %)
time post laser (sec)
0
5
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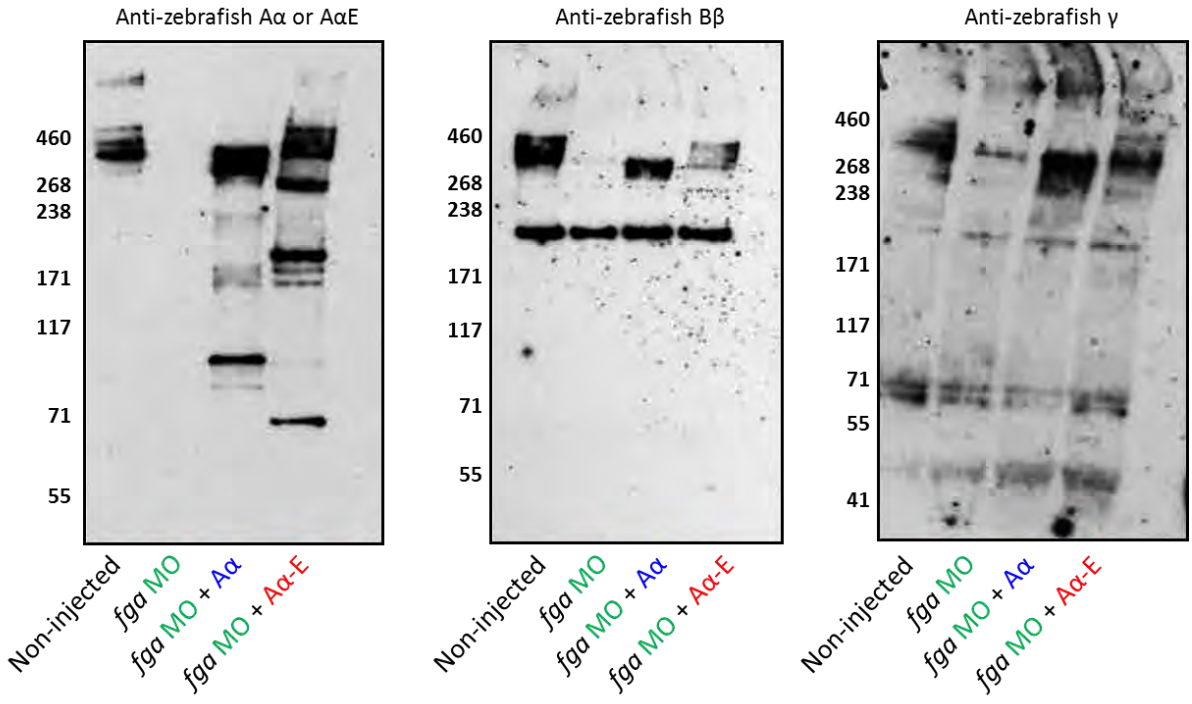
60
90
120

Supplementary Data

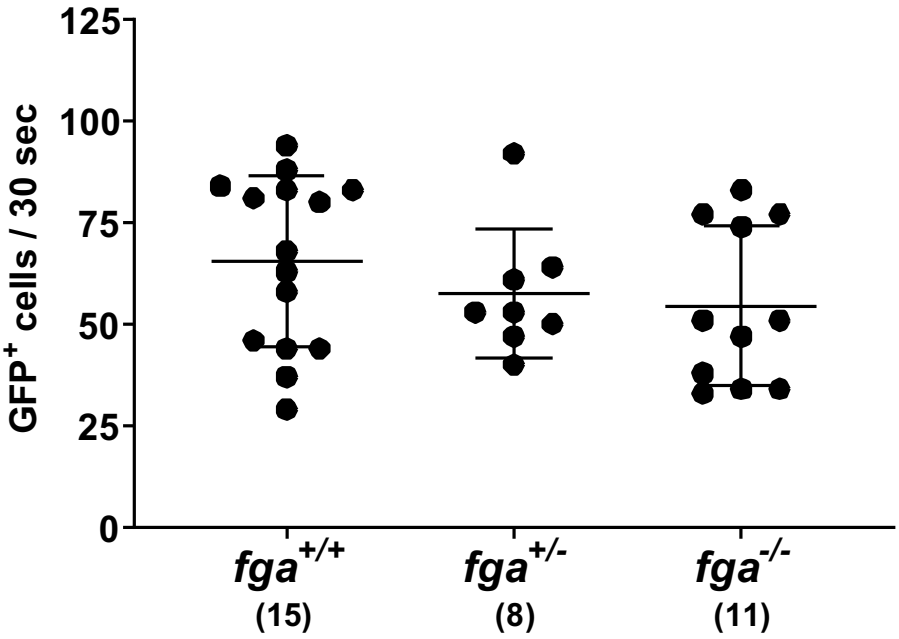
Supplementary Figure 1 - Immunoblots of fibrinogen production in zebrafish larvae after morpholino targeting of fibrinogen alpha chain mRNA and transgenic expression of fibrinogen Aα or AαE

1 to 2 cell embryos were injected with an fga mRNA-targeting morpholino with and without expression of fibrinogen Aα or AαE. Whole embryo lysates from pooled embryos were subjected to immunoblotting after SDS-PAGE separation in non-reducing conditions. Blot membranes were probed with antibodies to zebrafish Aα (or AαE), Bβ or γ chains. Without considering post-translational modifications, the



expected molecular weight for zebrafish fibrinogen (AαBβγ)2 is approximately 295 kDa and 346 kDa with the AαE chains (AαEBβγ)2. Reactive bands at fibrinogen hexamer size can be seen in the blot for each chain in non-injected embryos lysates, which are almost absent after fga morpholino injection. Predicted hexamer sized bands, at similar intensities, can be seen with expression of Aα or AαE cDNA with the

morpholino. Smaller mass bands are proposed to be a combination of fibrinogen assembly intermediates, fibrin fragments from the lysate preparations or non-specific bands such as that seen at ˜200 kDa in the Bβ chain blot.



Supplementary Figure 2 - Assessment of circulating thrombocytes in *Tg(itga2b:EGFP), fga*$^{+/+}$*, fga*$^{+/-}$ and *fga*$^{-/-}$ zebrafish

To control for possible effects of fibrinogen mutation on thrombocyte development, the number of GFP-positive cells passing the PCV in 30 seconds was measured in 5dpf *Tg(itga2b:EGFP), fga*$^{+/+}$, *fga*$^{+/-}$ and *fga*$^{-/-}$ larvae. The number of larvae assessed is given in brackets under the *fga* genotype, individual larvae are shown by circles with the mean and standard deviation shown. In unpaired t-tests, differences between the groups did not reach statistical significance ($p>0.05$).

DISCUSSION AND PERSPECTIVES

PART I: Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders

I. Congenital fibrinogen disorders modelled in zebrafish

Mutations in fibrinogen genes lead to several types of CFDs with various biological and clinical impacts such as bleeding and/or thrombosis. It is important to determine the clinical impact of molecular defects in fibrinogen in order to better confirm and elaborate a diagnosis, evaluate the risk of thrombosis and bleeding before they occur, perform prenatal diagnosis, and tailor the therapeutic protocol for each patient [163].

In the first part of this thesis, we generated zebrafish models of quantitative (afibrinogenemia) and qualitative (dysfibrinogenemia) human fibrinogen disorders to characterize venous thrombosis in zebrafish embryos via a venous endothelial mediated laser-induced injury method.

Afibrinogenemic zebrafish model

The clinical manifestations of afibrinogenemic patients are spontaneous bleeding and bleeding after trauma or surgical intervention; however, paradoxically, patients also show spontaneous thrombotic complications. Afibrinogenemic patients cannot form a normal fibrin clot after a vascular injury, so they lack the antithrombin activity of fibrin [174] [173]. As a result, these patients experience increased prothrombin activation and a consequent increase in thrombin generation [175]. The increased thrombin generation in these patients may stimulate platelet aggregation and thereby contribute to the development of thrombotic events [176] [177].

Several murine and non-murine models have been generated to study the pathophysiology of afibrinogenemia. A fibrinogen-deficient mice model generated by disruption of the fibrinogen *Fga* gene (*Fga*$^{-/-}$) has been associated with severe bleeding [223], and also with a thrombotic phenotype. When the authors performed an arterial injury on these mice, they observed the formation of an unstable thrombus that detached repeatedly from the sub-endothelium causing downstream occlusion events [280]. The first zebrafish model of a human bleeding disorder was the afibrinogenemic zebrafish (*fga*$^{-/-}$), generated in our laboratory with ZFN technology [260] by introducing targeted mutations into exon 2 of the zebrafish *fga* gene. Adult *fga*$^{-/-}$ fish showed spontaneous hemorrhages and a reduced survival rate (~40%) compared to wild-type zebrafish. However, in the early stages of development, afibrinogenemic (*fga*$^{-/-}$) embryos showed no evidence of apparent blood extravasation.

In this part of the thesis, we used *fga*$^{-/-}$ afibrinogenemic zebrafish to study the impact of complete fibrinogen deficiency on venous thrombosis, by means of the TTO assay and the thrombocyte recruitment assay (thrombocyte adhesion and accumulation). For the first assay, we used zebrafish embryos at 3dpf where the clotting was mainly dependent on fibrinogen-to-fibrin conversion and RBCs (erythrocytes) aggregation. For the second assay, we used 5dpf embryos. At this developmental stage, most of the mature thrombocytes (the cellular equivalent to mammalian platelets) are now in circulation and therefore in this setting, clotting was dependent on fibrinogen, thrombocytes, and RBCs.

After performing a vascular laser-mediated injury, we observed that 3dpf *fga*$^{-/-}$ afibrinogenemic zebrafish larvae presented no sign of thrombus formation; similar results have been observed by another group in another *fga*$^{-/-}$ zebrafish model of

afibrinogenemia [281]. At 5dpf we observed that thrombocyte binding and accumulation were affected in *fga*$^{-/-}$ afibrinogenemic zebrafish after performing a vascular injury. Here, unstable thrombocyte aggregates were formed, but were continually detaching from the injury site to flow along the vessel, causing downstream occlusion. This thromboembolic phenotype was comparable to the phenotype observed in the previously mentioned *Fga*$^{-/-}$ afibrinogenemic mice model [223] [280]. In contrast, heterozygous fish (*fga*$^{+/-}$) showed thrombus formation after vascular injury at 3dpf and thrombocyte adhesion and accumulation at the injury site at 5dpf.

The phenotypes seen in our *fga*$^{-/-}$ afibrinogenemic zebrafish larvae recapitulate the effect of this mutation in homozygosity and resemble the thromboembolic complications seen in afibrinogenemic human patients [282] [283] [284] [285]. When considering these findings collectively, we can confirm that our *fga*$^{-/-}$ afibrinogenemic zebrafish model is adequate in studying the pathophysiology of the human afibrinogenemia disorder.

Fibrinogen plays a role in platelet adhesion and aggregation during platelet plug formation. In humans, the first contact between the platelets and the damaged endothelium is mediated by the binding of collagen-immobilized vWF and the platelet integrin receptor (GPIb-V-IX). Platelet adhesion is then stabilized by fibrinogen binding to the $\alpha_{IIb}\beta_3$ platelet receptor. Following platelet adhesion, platelet aggregation occurs [286] when soluble fibrinogen crosslinks with two $\alpha_{IIb}\beta_3$ receptors from two different platelets [287] [288] [289]. In our afibrinogenemic zebrafish model, the absence of fibrinogen seems to be detrimental in the adhesion of thrombocytes to the damaged endothelium and its aggregation, even in the presence of other proteins involved in these processes. We suggest that in our afibrinogenemic zebrafish model,

the unstable thrombocyte adhesion to the endothelium may be mediated by collagen, vWF, and/or fibronectin, while the thrombocyte–thrombocyte aggregation may be due to $\alpha_{IIb}\beta_3$ vWF and/or fibronectin binding [290].

Dysfibrinogenemic and hypodysfibrinogenemic zebrafish models

One of the most common dysfibrinogenemia mutations is the hotspot mutation *FGA* c.103C→T, leading to Aα R35C amino acid changes (Fibrinogen Metz I) [291] [188] [292] [187]. Patients with this mutation manifest a variable phenotype, with 30% experiencing episodes of bleeding and 15% suffering from thrombotic events [189]. It is known that fibrinopeptide A release plays an important role in the process of fibrin polymerization (protofibril elongation and lateral aggregation) and defects in this process directly impact fibrin clot structure (architecture) and function [293]. This mutation occurs in the thrombin cleavage site (RG) at the N-terminal end of the fibrinogen Aα chain. Consequently, mutations in R35 lead to prolonged fibrin polymerization, as a result of delayed or impaired fibrinopeptide A release [291]. The structure of fibrin clots in patients with this mutation is disorganized with a high density of thicker [291] and thinner fibrin fibers and small pores [294]. When analyzing the function of these aberrant clots, it was found that the clots presented fibrinolytic and proteolytic resistance [189], diminished platelet aggregation and enhanced inhibition of fibrin assembly [187]. The disorganized structure of these clots affect their biophysical properties and biological functions, which is consistent with the bleeding and thrombotic phenotypes seen in patients [189].

Another mutation affecting the fibrin polymerization process was identified by our group in patients heterozygous for the *FGA* IVS2+3insCAT mutation (Fibrinogen Montpellier II). These patients were hypodysfibrinogenemic and produced fibrinogen that lacked the FpA, the knob A, and the thrombin cleavage site in the N-terminal end of the fibrinogen Aα chain. The clots formed from patient samples were non-uniform with clusters of thinner fibers and larger pores [200]. Although patients with *FGA* R35C and *FGA* IVS2+3insCAT mutations showed aberrant fibrin clot architecture, the clots were still formed, suggesting that 'B:b' knob-hole interactions still occur when 'A:a' knob-hole interactions are disrupted [295] [294].

In this part of the thesis, we also aimed to generate a zebrafish model of dysfibrinogenemia by introducing the equivalent human mutation (*FGA* R35C) in zebrafish (*fga* R28C), using CRISPR/Cas9 genome editing technology. Unexpectedly, we found that the genome editing led to an *fga* exon 2 skipping event in our *fga* R28C mutant zebrafish. The predicted Aα-chain translated from this exon-skipped transcript lacked amino acids 19 to 56 in the N-terminal of the fibrinogen Aα chain (corresponding to the amino acids encoded by exon 2), but this did not result in a translational frameshift. As a result, our *fga* R28C mutant zebrafish, now named *fga* Δ19–56, have an Aα-chain with no FpA, knob A, or thrombin cleavage site. Interestingly, the hypodysfibrinogenemia *FGA* IVS2+3insCAT mutation (mentioned above) [200] also leads to an *FGA* exon 2 skipping event, so we used this mutant zebrafish in heterozygosity ($fga^{+/\Delta 19–56}$) to model the human *FGA* IVS2+3insCAT hypodysfibrinogenemia mutation.

When studying venous thrombosis in hypodysfibrinogenemic ($fga^{+/\Delta 19–56}$) zebrafish larvae at 3dpf, they showed a prolonged venous TTO. The presence of a single mutated allele in these mutants prolonged the venous TTO, demonstrating the

dominant negative effect of the mutation. At 5dpf, hypodysfibrinogenemic zebrafish larvae showed a decrease in thrombocyte adhesion and accumulation at the injury site, demonstrating again the dominant negative effect of the mutated allele in heterozygosity (*fga*$^{+/\Delta 19–56}$) on thrombocyte adhesion and accumulation.

Finally, after our previous unsuccessful attempt with CRISPR/Cas9, we aimed to model the human dysfibrinogenemia mutation *FGA* R35C [291] in zebrafish, via a different approach. To achieve this, we used transgenic expression of *fga* R28C cDNA in *fga* knock-down or *fga*$^{-/-}$ mutants. Similar to the phenotype observed in hypodysfibrinogenemic (*fga*$^{+/\Delta 19–56}$) zebrafish larvae, dysfibrinogenemic (R28C) zebrafish larvae showed a prolonged venous TTO after vascular injury at 3dpf, demonstrating the dominant negative effect of AαE R28C expression on venous occlusion. Moreover, at 5dpf, dysfibrinogenemic (R28C) larvae showed reduced thrombocyte adhesion and accumulation at the injury site, demonstrating once more the dominant negative effect of AαE R28C expression on thrombocyte adhesion and accumulation. Similarly, the phenotypes observed in our dysfibrinogenemic zebrafish larvae correlated to those observed in the *Fib*^{AEK} mice [225]. These mice displayed the formation of an aberrant fibrin-like clot after liver injury and were unable to form a stable occlusive thrombus after a $FeCl_3$ injury of the carotid arteries.

Since hypodysfibrinogenemia and dysfibrinogenemia mutations lacking the thrombin cleavage site in the N-terminal end of the fibrinogen Aα chain affect FpA release and fibrin polymerization [291], the prolonged venous thrombosis seen in larval zebrafish models of both hypodysfibrinogenemia and dysfibrinogenemia is consistent with impairments in the fibrin polymerization process. Furthermore, since human hypodysfibrinogenemia and dysfibrinogenemia mutations lead to fibrin-like clots with an altered structure [291] that affects platelet interaction [187], the decreased

thrombocyte adhesion and accumulation at the injury site seen in hypodysfibrinogenemia and dysfibrinogenemia zebrafish models is consistent with those observations. Moreover, the phenotypes observed in our hypodysfibrinogenemia and dysfibrinogenemia zebrafish models recapitulated the dominant negative effect of these mutations in heterozygosity. Considering all these findings collectively, we concluded that venous thrombosis assays performed in zebrafish larvae are a valuable method in examining the pathophysiological phenotypes of zebrafish models of qualitative congenital fibrinogen disorders.

II. Laser injury methods to assess the phenotype of larval zebrafish models of fibrinogen disorders

Taking into consideration all the observations, we propose that our two laser injury assays (TTO and thrombocyte recruitment assay) are good methods to screen for the different zebrafish phenotype profiles between the models of quantitative versus qualitative fibrinogen disorders.

The prolonged venous TTO shown in *fga*$^{+/-}$ and *fga*$^{+/\Delta 19\text{-}56}$ compared to wild type zebrafish, and the prolonged venous TTO shown in *fga* knock-down larvae with AαE + AαE R28C expression compared to *fga* knock-down larvae with AαE expression, suggests that the TTO values are affected by the fibrinogen quantity or quality. Therefore, the TTO values will be prolonged in quantitative and qualitative congenital fibrinogen disorders in the heterozygous state. The thrombocyte recruitment assay may be useful discriminating between the quantitative and qualitative congenital fibrinogen disorders. The phenotypic differences are clearly observed in the reduced

accumulation of thrombocytes at the injury site, which is shown in dysfibrinogenemia and hypodysfibrinogenemia zebrafish larvae in the heterozygous state, compared to the unaffected thrombocyte accumulation at the injury site, which is shown in *fga*$^{+/-}$ and *fga*$^{+/+}$ larvae. However, this thrombocyte assay was unable to discriminate between the dysfibrinogenemia and hypodysfibrinogenemia disorders.

Despite the limitation of the zebrafish larvae model measuring the concentration of plasma fibrinogen (zebrafish larvae presents low blood volume), the TTO and thrombocyte assays are valuable methods of detecting detrimental functional defects of a fibrinogen mutation. However, they are not useful for precisely correlating between the fibrinogen quantity, quality and the pathophysiological phenotype shown in zebrafish larvae.

III. Role of fibrinogen versus fibrin

Fibrinogen and fibrin play overlapping roles in coagulation, inflammation and wound healing. After fibrinogen has been converted to fibrin, the protein conformation changes, leading to the exposure of new binding sites for other molecules and cells. These new interactions will determine the fibrin structure (thickness, porosity, and permeability) [296] [297] [298] [299] and function. Variations in fibrin clot formation, structure, and stability can be a result of a variety of conditions such as hereditary and acquired variations in fibrinogen structure, environmental conditions of polymerization, cellular effects and hydrodynamic flow [300]. Moreover, they are critical determinants in the development of several pathologies related to hemostasis, thrombosis, inflammation, and other processes [301] [302].

The final aim of the first part of this thesis was to unravel the different roles of fibrinogen versus fibrin. Regarding the role of fibrinogen and fibrin in venous thrombosis, we observed that fibrinogen mutations in the thrombin cleavage site of the Aα-chain affecting the FpA release process, needed for fibrin polymerization, leads to unstable clot formation, subsequently affecting vascular hemostasis. Concerning the role of fibrinogen and fibrin in thrombocyte adhesion and accumulation, in contrast to wild-type zebrafish larvae, afibrinogenemic larvae showed a dramatic decrease in thrombocyte adhesion and accumulation at the injury site. Comparable phenotypes were also observed in embryos expressing AαE R28C. These findings suggest that thrombocytes interact strongly with fibrin during adhesion and accumulation processes. Interestingly, our findings correlate with results observed in a recent study analyzing the binding strength between the integrin receptor $\alpha_{IIb}\beta_3$ with fibrinogen and monomeric fibrin [303]. This study showed that monomeric fibrin displayed a greater probability of interaction with $\alpha_{IIb}\beta_3$ and a higher binding strength than fibrinogen.

IV. Future perspectives

In the future, we hope to use our afibrinogenemic zebrafish model to test a range of *FGA* fibrinogen variants found in patients with congenital fibrinogen disorders, and study their pathophysiologic phenotype in intravascular hemostasis and venous thrombosis. Moreover, we would like to analyze the ultrastructure of fibrin clots formed from fibrinogen human variants modeled in zebrafish, and then correlate their aberrant structure with the pathologic phenotype.

We would also like to study the contribution of other cell types to hemostasis, in the presence of normal or aberrant fibrinogen and in the absence of fibrinogen. For instance, we could use the transgenic zebrafish line *Tg(gata1:dsRed)* [266], which expresses dsRed cDNA under the control of the *gata1* promoter, to study the contribution of erythrocytes on thrombus formation in this setting. Furthermore, we would like to study the different roles of fibrinogen versus fibrin during inflammation and wound healing [304] [305]. For example, we could examine the role of macrophages and neutrophils in inflammatory responses after pathogen infection and their role in wound healing after performing an epithelial injury by tracking their migration into the wound. For this purpose we could use the transgenic zebrafish lines *Tg(mpeg1:mCherry)* and *Tg(mpx:EGFP)* to track the migration of macrophages and neutrophils, respectively.

Finally, beyond studies into pathophysiologic phenotypes linked to genetic disorders, zebrafish models can be used for chemical screenings and to discover candidate drugs to combat diseases. We could test thrombolytic and antithrombotic drug therapies in our models and evaluate their effect on intravascular hemostasis and venous thrombosis.

Using venous thrombosis assays in zebrafish models of quantitative and qualitative CFDs to study the pathophysiology of these disorders will help us to better understand bleeding and thrombosis risk in patients, and also to correlate the pathological phenotype of these zebrafish models with clinical manifestations in patients.

PART II: A genetic modifier of venous thrombosis in the zebrafish (*Danio rerio*) reveals a functional role for the fibrinogen AαE chain in early hemostasis

I. Evolutionary conservation of fibrinogen from zebrafish to humans

The origin and evolution of fibrinogen have been studied throughout the vertebrate kingdom, from lampreys (the most primitive form of vertebrates) to humans. The amino acid sequences of all three fibrinogen chains (Aα, Bβ and γ) have been compared across vertebrate species (Appendix, Figure A-1 and Figure A-2), and it was concluded that all vertebrates present fibrinogen molecules composed of three non-identical polypeptide chains: Aα, Bβ and γ [306].

While the three fibrinogen genes were different from each other, they presented similarities in their amino acid sequences, revealing that they must have evolved from the duplication of a common ancestor gene (paralogs), deriving ultimately by speciation (orthologs) [307] [308]. Orthologs share similar domain and crystal structures, and are believed to have been under strict evolutionary constraints, retaining equivalent function in different organisms [309] [310] [311]. In contrast, paralogs tend to evolve, acquiring new functions due to adaptation. The evolutionary conservation of fibrinogen orthologs in vertebrate organisms has been comprehensively studied [148].

Not only is Fib340 conserved between vertebrates, but so is Fib420 [312]. When studying the conservation of the common Aα-chain and AαE isoform between zebrafish and humans, we noted that the percentage of identity for AαE isoform was greater (32.53%) than that of the common Aα-chain (18.46%) (Appendix, Figure A-3), suggesting that the common Aα-chain has evolved more than the AαE isoform due to adaptation. Upon comparing the percentage of identity between the AαE C-terminal

domain of zebrafish and its human counterpart we observed an identity of 61.98% (Appendix, Figure A-4). This indicates that the AαE C-terminal domain is conserved to a great extent during vertebrate evolution. Since the AαE isoform is under high evolutionary constraint, it is likely that Fib420 fulfils an important physiological function.

The 30 kDa C-terminal globular domains of the Bβ and γ chains also called fibrinogen-like globe (FBG) domains, are known to play vital roles in the initial stages of wound healing as they interact with a large variety of molecules, thus mediating different cell responses (e.g., angiogenesis, fibroblast and keratinocytes cell migration and proliferation, wound closure, and matrix remodeling) [313] [314]. In vertebrates, the C-terminal globular domain of the AαE isoform shows high amino acid sequence similarity with the C-terminal globular domain of the Bβ and γ chains [312] (Appendix Figure A-5 and Figure A-6). When comparing the C-terminal globular domains of the three AαE, Bβ and γ chains from humans and zebrafish, we observed a similarity between the globular domains of approximately 40%, for both humans and zebrafish (Appendix Figure A-7). The high similarity of the C-terminal globular domain between the three chains (AαE, Bβ and γ) in humans and zebrafish highlights the possibility that the cellular binding sites (and binding sites for different molecules) found in the C-terminal domain of the Bβ and γ chains may also be present in the AαE C-terminal domain. For instance, it is known that some amino acid residues found in the C-terminal globular domain of human fibrinogen γ chains that are involved in fibrinogen binding to leukocytes through the $\alpha_M\beta_2$ integrin receptor [136], are also present in the C-terminal globular domain of the AαE isoform [134] (Appendix Figure A-8). As expected, we have observed that these residues seem to be conserved as well in zebrafish (Appendix Figure A-9).

Therefore, the conservation of both Fib340 and Fib420 in humans and zebrafish make zebrafish a useful model organism in which to study the impacts of Fib420 on hemostasis and other physiological processes.

II. The role of Fibrinogen 420 during developmental hemostasis

Hemostasis is an age-dependent dynamic process that begins in utero and continues throughout life [315] [316]. This process was termed “developmental hemostasis” in the 1980s by Dr Maureen Andrew [317]. The neonatal hemostatic system undergoes several changes during the first six months of life in order to reach average adult values [318] [319]. Apart from factors I, V, VIII and vWF, all other coagulation factors are reduced at birth [320] [321] [322] [323]. However, the reduced levels of pro-coagulant factors are balanced by reduced levels of anti-coagulant factors, apart from α_2-macroglobulin (the concentration of which is increased in newborns) [153] [323] [324], which may contribute to the thromboprotective state seen in newborns [325] [326].

One distinctive feature of the neonatal hemostatic system is the presence of factors with unique functions, such as Fib420 and fetal hemoglobin. Fetal hemoglobin has a different composition from the adult hemoglobin form, which allows it to attach to oxygen more strongly. This way, fetal hemoglobin is involved in transporting oxygen from the bloodstream of the mother to the fetus [327]. The levels remain high after birth, decreasing gradually to reach adult levels [315] [328]. In contrast to fetal hemoglobin, Fib420 is not only a fetal form of fibrinogen – it persists throughout adult

life [153], albeit in a lower concentration. Fib420 levels drop by six months after birth at a time when other components of the coagulation system mature [92] [154]. Although there is no known explanation for this decrease, it may be that Fib420 compensates for the absence in neonatal hemostasis of other coagulation factors.

III. A genetic modifier of venous thrombosis

In the second part of this thesis we wanted to unravel the functional role of the AαE isoform in embryonic hemostasis. To achieve this we used different laboratory zebrafish strains: the AB strain, which has a naturally-occurring single nucleotide deletion in *fga*, preventing the formation of AαE isoform; and the TU and TL strains without the deletion. Thanks to the genetic variability of these strains, we were able to see differences in venous thrombosis, and we could demonstrate that the mutation (which resides in exon 6 of *fga*) preventing the production of the AαE isoform is a genetic modifier of venous thrombosis in zebrafish.

We suggested that AαE isoform plays a role in embryonic hemostasis. During early blood coagulation (developmental hemostasis), Fib420 may act as a mechanism to compensate for the absence of other coagulation factors, perhaps by binding different cells or factors available for clotting. For example, Fib420 could compensate for reduced platelet function, seen in newborns [329] [330] and zebrafish larvae [267], by binding erythrocytes through its extra AαE C-terminal globular domain, generating an erythrocyte-rich thrombus after a vascular injury has occurred.

IV. AαE C-terminal domain as a ligand for erythrocyte binding

Several research groups have studied the role played by AαE C-terminal globular domain in protein folding and stability [143] [331], the binding of macromolecules and cells [146] [149] [150] (e.g., as a ligand for $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrin receptors from leukocytes [134]), and in fibrin polymerization, crosslinking, and degradation [143] [146] [147] (e.g., fibrin clot structure and/or function [149]). Nevertheless, there has to date been no evidence that the Fib420 molecule has either beneficial or detrimental effects on hemostasis.

As stated above, we suggested that AαE isoform may play a role in early blood coagulation (developmental hemostasis) through its binding to erythrocytes, possibly acting as a mechanism to compensate for the absence of other coagulation factors in the early stages of development. Since the AαE isoform has an extra C-terminal globular domain, which is thought to be involved in binding to other macromolecules and cells, we would suggest that this domain could also be implicated in erythrocyte-fibrinogen binding (erythrocyte aggregation) in zebrafish embryos [131] [332] [333] [334].

Erythrocyte-fibrinogen binding takes place between the $\alpha_V\beta_3$ integrin receptor from erythrocytes [130] [132] [133] [335] and the RGD motif from the fibrinogen Aα-chain [336] (the same motif that binds the fibrinogen Aα-chain with the platelet $\alpha_{IIb}\beta_3$ integrin receptor). It has been reported that the platelet $\alpha_{IIb}\beta_3$ integrin receptor has a relaxed specificity for both RGD and AGD ligands [125] [337] [338]. Moreover, it has been shown that the RGD or the AGD ligand interaction with the integrin β_3 subunit

from $\alpha_{IIb}\beta_3$ is enough for stable ligand binding [339] [340]. Therefore, as for the platelet $\alpha_{IIb}\beta_3$ integrin, not only could RGD motifs be involved in the fibrinogen-erythrocyte binding through the $\alpha_V\beta_3$ integrin, but AGD motifs as well. Since the C-terminal globular domain from the AαE isoform presents an AGD motif (in both humans and zebrafish), we suggest that the presence of this motif could be implicated in the increased affinity of the AαE isoform for erythrocytes in zebrafish larvae [335].

V. Mutations in the C-terminal domain of the Aα-chain

Until this point, we have discussed the evolutionary conservation of Fib420, its implication in developmental hemostasis and the role of the AαE isoform in venous thrombosis perhaps as a ligand for erythrocyte binding during embryonic hemostasis. Since Fib420 is retained throughout evolution in the vertebrate kingdom, its role in hemostasis and other physiological processes must be important – but is it essential?

Upon looking for mutations affecting the C-terminal domain of the AαE isoform, none were found. However, there are patients who present frameshift, nonsense, or point mutations leading to a truncation of the Aα C-terminal domain or resulting in the absence of part of the Aα C-terminal domain. For instance, the Fibrinogen Marburg mutation, which is a nonsense mutation that results in an Aα C-terminal domain that lacks the 461-610 amino acid segment, is not lethal but leads to thrombotic and bleeding episodes in homozygous patients [341] [342]. There is also, the Fibrinogen Milano III, which results in an Aα chain that lacks Aα 454-610 and has two new C-terminal amino acids (W452-S453), which is not lethal but leads to thromboembolism

in homozygous patients [343]. And there is the Fibrinogen Nieuwegein, that results in an Aα chain that lacks Aα 454-610 with deletion of the transglutaminase (TG) crosslinking site in the Aα C-terminal domain, which is asymptomatic in homozygous patients [344]. Given this, as for Fibrinogen Marburg, Milano III, and Nieuwegein, a mutation occurring in the AαE C-terminal domain will be problematic, but not lethal. Thus, we concluded that Fib420 plays an important but non-essential role.

VI. Future perspectives

There are several questions we would like to address so that we may better comprehend the role of Fib420 in hemostasis and other physiological processes such as inflammation and wound healing.

We have suggested that the Fib420 molecule might have another binding site to erythrocytes in the C-terminal globular domain of the AαE chains, like the AGD motif. Our goal for the future is to find out which residue(s) are involved in the binding of the AαE C-terminal globular domain with erythrocyte surface receptors, using zebrafish as a model organism. To achieve this, we will first outline predictions of possible candidate residues in the C-terminal globular domain of AαE that might be involved in binding with integrin (e.g., the AGD motif) and non-integrin erythrocyte receptors. These will then be mutated and generated by site-directed mutagenesis, incorporated into vectors for ubiquitous expression, and then injected into one cell stage *fga* ko zebrafish embryos. Injected embryos will be subjected to laser-induced vascular injury at 3dpf, with measurement of erythrocyte binding to the damaged endothelia being taken subsequently. Then, we will screen for larvae that present

abnormal phenotypes, which binding may be impaired a cause of the mutation in the candidate residue.

Alongside from the role of Fib420 in hemostasis, we would like to study its role in inflammation and wound healing, as it has been shown that fibrinogen plays a role in the inflammatory response [215] [216] wherein fibrinogen's ability to bind leukocytes, which is mediated by the C-terminal globular domain of the γ-chain, is dependent upon. As could have been expected, it was recently reported that the fibrinogen AαE C-terminal domain can also support the strong adhesion and migration of leukocytes (i.e., monocytes, neutrophils, and activated lymphocytes), expressing $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins [134] [136]. We hypothesize that the AαE C-terminal domain of Fib420 may also play a role during inflammation. To test this hypothesis, we will evaluate the inflammatory response of both our AB (which due to a mutation in *fga* exon 6 produces only the Aα containing fibrinogen form throughout the embryonic development onwards until adult stages) and the TU (which larvae produce predominantly AαE containing fibrinogen) zebrafish strains after subjecting them to a pathogenic infection (virus or bacteria) [345] [314] [346]. In zebrafish, the adaptive immune response takes four to six weeks to fully develop [347], so their embryos possess only the innate immune response during the first weeks of development [348]. Therefore, we will use zebrafish embryos to study the innate immune response after pathogen infection. After infection of the embryos (by static immersion), we will measure their responses by studying signs of viral/bacterial infection, pathology and clinical disease (e.g., mortality, anorexia, hemorrhage and erythema, and histopathological analysis). Moreover, thanks to the optical clarity of zebrafish embryos, we will be able to measure the infection and the cellular activity during the immune response by *in vivo* imaging microscopy [349]. For instance, we can

measure infection [349] by injecting a virus or bacteria expressing fluorescent protein reporters, such as dsRed or EGFP [350] [351], and subsequently measuring the cellular response (i.e., migration) by using transgenic zebrafish lines with macrophages [352] [353] or neutrophils [354] [355] expressing a fluorescent protein reporter, e.g., *Tg(mpeg1:mCherry)* and *Tg(mpx:EGFP).*

Finally, we would like to study the role of Fib420 in wound healing. The FBG domains from Bβ and γ chains are known to participate in wound healing [313] [314]. Since the AαE C-terminal globular domains from Fib420 molecules show high homology with the C-terminal domain from Bβ and γ chains, we suggest that they also play a role in wound healing processes. To study the function of Fib420 in wound healing we could study wound closure or track leukocyte cell migration (macrophages and neutrophils) into the wound after inducing a laser injury in *fga* ko zebrafish embryos injected with vectors for exogenous expression of either Aα or AαE chains. For this purpose, transgenic reporters can be used to monitor macrophage (*Tg(mpeg1:mCherry)* zebrafish line) and neutrophil (*Tg(mpx:EGFP)* zebrafish line) migration.

ADVANTAGES AND LIMITATIONS

We have previously mentioned in the introduction that zebrafish have been widely used as models to study genetics, developmental biology and several human disorders [356] [357] [358], partly due to their high fecundity, the optical transparency of their embryos, their tissue accessibility, their quick maturation and the simplicity involved in genetically manipulating their genomes, since they are suitable for reverse, forward and chemical genetics [255]. However, zebrafish being used as model organisms presents some limitations.

Anatomically, zebrafish blood cells present different morphology (e.g., they contrast with human blood cells – zebrafish thrombocytes and erythrocytes are nucleated). Despite the morphological differences between zebrafish and mammalian blood cells, zebrafish thrombocytes and erythrocytes are the hemostatic homologues of mammalian platelets [359] and RBCs [265] [360] [361], respectively, and are functionally equivalent to them [255] [268]. Nowadays, there are several studies that use transgenic zebrafish with GFP fluorescent thrombocytes to study their role in hemostasis [247] and hematopoiesis [265] [362] [363]. Moreover, several zebrafish mutants have been generated to model human erythropoiesis diseases [361] [363]. For instance, the zebrafish mutant *dracula* drc^{m248}, which has a G→T transition at a splice donor site in the ferrochelatase gene, leading to a premature stop codon, was used to study erythropoietic protoporphyria [364]; the zebrafish mutant *zinfandel* zin^{te207} was used to study hypochromic anemia [365]; and the zebrafish mutant *merlot* mot^{tu275}, which has a C→T transition in the mot gene, leading to a premature stop codon and causing a truncated protein, was used to study hemolytic anemia [366].

In terms of genetic techniques, the use of morpholinos (MOs) for gene knock-down show some drawbacks. For example, the efficacity of MOs is limited to three to five

days, can cause toxic effects in the injected embryos, and give rise to false phenotypes, misleading the interpretation of the results [367]. Despite these limitations, MOs have several advantages e.g., they are a good approach for studies on loss of function, are precise for spatial targeting (tissue-specific), and are a good technique to study dose-dependent effects (titration) [368] [369].

Moreover, the use of genome editing techniques e.g., CRISPR/Cas9, also presents some drawbacks because when a mutant or knock-out is generated, the phenotypes observed can be due to off-target effects rather than the mutation introduced [370] [371]. Despite this limitation, CRISPR/Cas9 technique has several advantages e.g., its simplicity and specificity, and its applicability in biomedical research [369] [372] [373] [374].

In terms of similarities between the zebrafish and human genomes [252], although there are many orthologs found between these two species, not all genes from the human genome are found in the zebrafish genome and not all human genes have a single-copy gene in the zebrafish genome; some of them have more than one copy in the zebrafish genome (duplicated genes) [367] [375]. Therefore, not all human genes can be modeled in zebrafish [256]. However, human fibrinogen genes (*FGA*, *FGB* and *FGG*) are found in the zebrafish genome as single-copy genes (*fga*, *fgb* and *fgg*) [376], and it is one of the reasons why we have used the zebrafish as a model organism.

Another important limitation to consider when using zebrafish as model organisms in thrombosis and hemostasis is that the contact system pathway is absent. While the majority of coagulation factors are conserved in zebrafish, factors XI, XII and prekallikrein, from the contact system pathway, have not been found [253] [377].

However, other genes encoding proteins involved in hemostasis and coagulation, such as vWF, fibronectin, vitronectin, laminin [378], collagen, and some integrins such as $\alpha_{IIb}\beta_3$, GPIb, GPV, GPIX, $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_6\beta_1$ (with the exception of the collagen receptor GPVI [379]), have also been identified in zebrafish [248] [252].

Despite all these limitations, the majority of the hemostatic pathways involved in coagulation are conserved in zebrafish, suggesting this organism is a good model to study mammalian hemostasis and thrombosis [247] [248] [275] [380]; and to model blood coagulation disorders [252].

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APPENDIX

Appendix A: Multiple sequence alignment

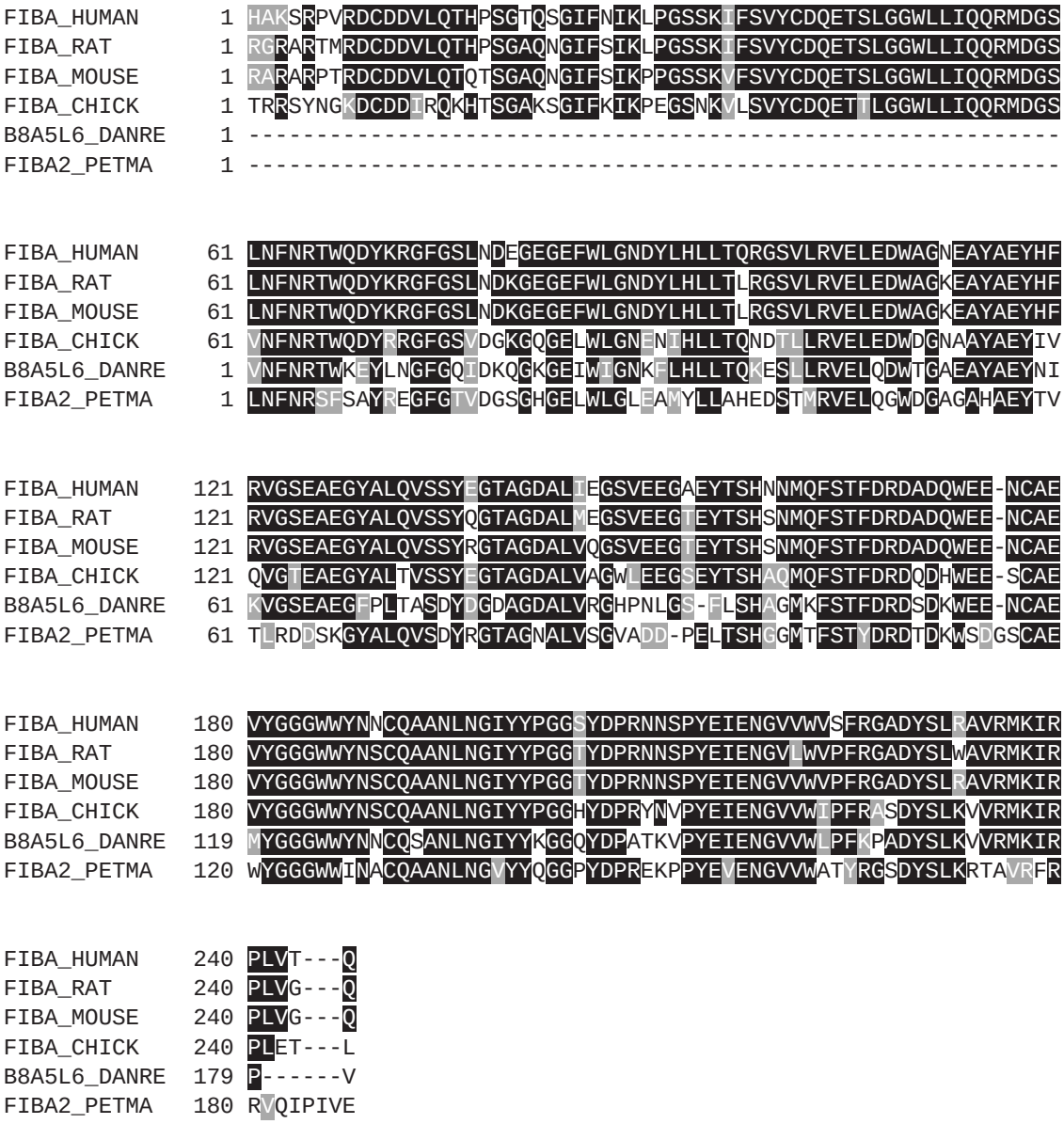

Figure A-1. *Multiple protein sequence alignment of AαE C-terminal domains from several vertebrate species (human, rat, mouse, chicken, zebrafish (Danio rerio) and Petromyzon marinus) was generated using T-Coffee server and then the figure was formatted using the BoxShade server.*

(a)

```
FIBB_HUMAN      1 NIPVVSGKECEEIIRKGGETSEMYLIQPDSSVKPYRVYCDMNTENGGWTVIQNRQDGSVD
Q6NYE1_DANRE    1 PIPVVSGKECEDIIRKGGEDSQMYIIRPDPLGTPYKVFCDQTSKNGGWVLIQNRMDGSVD

FIBB_HUMAN     61 FGRKWDPYKQGFGNVATNTDGKNYCGLPGEYWLGNDKISQLTRMGPTELLIEMEDWKGDK
Q6NYE1_DANRE   61 FGRRWDDYRRGFGNIAFDV-GKGHCQTPGEYWLGNDRISQLSKMGATELLVEMEDWSGSK

FIBB_HUMAN    121 VKAHYGGFTVQNEANKYQISVNKYRGTAGNALMDGASQLMGENRTMTIHNGMFFSTYDRD
Q6NYE1_DANRE  120 VYAQYEQFSMQGEASNYILGVGRYSGTAGNTFLEGATELFGENRTMTIHNGMMFSTYDRD

FIBB_HUMAN    181 NDGWLTSDPRKQCSKEDGGGWWYNRCHAANPNGRYYWGGQYTWDMAKHGTDDGVVWMNWK
Q6NYE1_DANRE  180 NDKWIPGDPSKQCSKEDGGGWWYNRCHSCNPNGRYYWGGAYTKYMAKHGTDDGIVWMNWK

FIBB_HUMAN    241 GSWYSMRKMSMKIRPFFPQQ
Q6NYE1_DANRE  240 GSWYSLKTISMKIRPYFKQK
```

(b)

```
FIBG_HUMAN      1 QIHDITGKDCQDIANKGAKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDGSVD
Q7ZVG7_DANRE    1 EIQTVTGKDCQDIANKGGKVSGLYYVKPARAPEAFLVYCEIDSFGRGWTVLQRRRDGSVD

FIBG_HUMAN     61 FKKNWIQYKEGFGHLSPTGTTEFWLGNEKIHLISTQSAIPYALRVELEDWNGRTSTADYA
Q7ZVG7_DANRE   61 FSKNWIQYKEGFGYLSPDDRTEFWLGNEKIHLLSVQSSVPYVLRIEMVDWEGNKKYADYA

FIBG_HUMAN    121 MFKVGPEADKYRLTYAYFAGGDAGDAFDGFDFGDDPSDKFFTSHNGMQFSTWDNDNDKFE
Q7ZVG7_DANRE  121 TFKLGPEVDAYRLTYAYYFGGDAGDAFDGYDFGDDPSDKFYTSHNGMQFSTVDRDNDKFQ

FIBG_HUMAN    181 GNCAEQDGSGWWMNKCHAGHLNGVYYQGGTYSKASTPNGYDNGIIWATWKTRWYSMKKTT
Q7ZVG7_DANRE  181 GHCAQQDGSGWWMNRCHAAHLNGKYYQGGKYTEKDAESGYDNGIIWATWHSRWYSLKETT

FIBG_HUMAN    241 MKIIPFNRLTIGEGQQHHLGGAKQA---GDV
Q7ZVG7_DANRE  241 MKIIPINRIGATGGQES---GSKQFGGIGDI
```

Figure A-2. *Multiple protein sequence alignment of the C-terminal domains from Bβ (a) and γ (b) chains of humans and zebrafish were generated using T-Coffee server and then the figures were formatted using the BoxShade server. CLUSTAL O (1.2.4) multiple sequence alignment software was used to calculate the percentage of identity between the aligned sequences. (a) The percentage of identity of the C-terminal domain from Bβ chains between humans and zebrafish is 69.50%. (b) The percentage of identity of the C-terminal domain from γ chains between humans and zebrafish is: 69.40%.*

(a)

```
FIBA_HUMAN      1 M-FSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDED
E9QCD1_DANRE    1 MKFTHSLLCLFVAVSSA-----LAEEDTVVNPRGARPIEHGFKAQDTCQTKEWPGCTDDD

FIBA_HUMAN     60 WNYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSS
E9QCD1_DANRE   56 WGSKCPSGCRVQGLMDKADHDLLKKIEKIRRLLVEGQKLYRSADEVSKTTYTYLREKLTS

FIBA_HUMAN    120 ANNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRS
E9QCD1_DANRE  116 NSGYDNRYMSLAEQLRQRITDIKIKIDRQLRLLDALKSQVKDQVVVMQRLEVDIDIKLRT

FIBA_HUMAN    180 CRGSCSRALAREVDLKDYEDQQKQLEQVIAKDLLPSRDRQHLPLIKMKPVPD-LVPGNFK
E9QCD1_DANRE  176 CKGSCASYSEFSVDRESYVTLDKQMDNLNAMRVQSVETVSSLGIMKSRPLKDVLVPTIYK

FIBA_HUMAN    239 SQLQKVPPEWKALTDMPQMRMELERPGGNEITRGGSTSYGTGSETESPRNPSSAGSWNSG
E9QCD1_DANRE  236 SGTGKAEQK-LLFGDVGQMQLSLEAEGSTAESAATVS----------------------

FIBA_HUMAN    299 SSGPGSTGNRNPGSSGTGGTATWKPGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTG
E9QCD1_DANRE  272 ----------------------KFP-------------TSGTGST--------------

FIBA_HUMAN    359 TWNPGSSERGSAGHWTSESSVSGSTGQWHSESGSFRPDSPGSGNARPNNPDWGTFEEVSG
E9QCD1_DANRE  282 ------------------------------------------------------------

FIBA_HUMAN    419 NVSPGTRREYHTEKLVTSKGDKELRTGKEKVTSGSTTTTRRSCSKTVTKTVIG-PDGHKE
E9QCD1_DANRE  282 ----------------------------------SQTSSKQCTKSVRKVITHTKDGPV-

FIBA_HUMAN    478 VTKEVVTSEDGSDCPEAMDLGTLSGIGTLDGFRHRHPDEAAFFDTASTGKTFPGFFSPML
E9QCD1_DANRE  306 --EKVEVSSSGPGCDD---LGKL-GI-----------SDSEFLTAAKDGKDLSR------

FIBA_HUMAN    538 GEFVSETESRGSESGIFTNTKESSSHHPGIAE--FPSR--------------------GK
E9QCD1_DANRE  343 -DGLKITVTGGDEKS-LTSLSHSTSDLGGFGGDFFKNLGTDNRKYASSSSSTSTKTVSDG

FIBA_HUMAN    576 SSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEADHEGTHSTKRGHAKSRPVRG-IHT
E9QCD1_DANRE  401 SKSYSKTTLTSD-------------PIFGDDLGAFMRGDVEEDMPDIHARSVKSQDDRKA

FIBA_HUMAN    635 SPLGKPSLSP
E9QCD1_DANRE  448 GFVGGGTLE-
```

(b)

```
FIBA_HUMAN      1 M-FSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDED
B8A5L6_DANRE    1 MKFTHSLLCLFVAVSSAL-----AEEDTVVNPRGARPIEHGFKAQDTCQTKEWPGCTDDD

FIBA_HUMAN     60 WNYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSS
B8A5L6_DANRE   56 WGSKCPSGCRVQGLMDKADHDLLKKIEKIRRLLVEGQKLYRSADEVSKTTYTYLREKLTS

FIBA_HUMAN    120 ANNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKIRS
B8A5L6_DANRE  116 NSGYDNRYMSLAEQLRQRITDIKIKIDRQLRLLDALKSQVKDQVVVMQRLEVDIDIKLRT

FIBA_HUMAN    180 CRGSCSRALAREVDLKDYEDQQKQLEQVIAKDLLPSRDRQHLPLIKMKPVPD-LVPGNFK
B8A5L6_DANRE  176 CKGSCASYSEFSVDRESYVTLDKQMDNLNAMRVQSVETVSSLGIMKSRPLKDVLVPTIYK

FIBA_HUMAN    239 SQLQKVPPEWKALTDMPQMRMELERPGGNEITRGGSTSYGTGSETESPRNPSSAGSWNSG
B8A5L6_DANRE  236 SGTGKAEQK-LLFGDVGQMQLSLEAEGSTAESAATVS----------------------

FIBA_HUMAN    299 SSGPGSTGNRNPGSSGTGGTATWKPGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTG
B8A5L6_DANRE  272 ----------------------KFPT------------SGTGST--------------
```

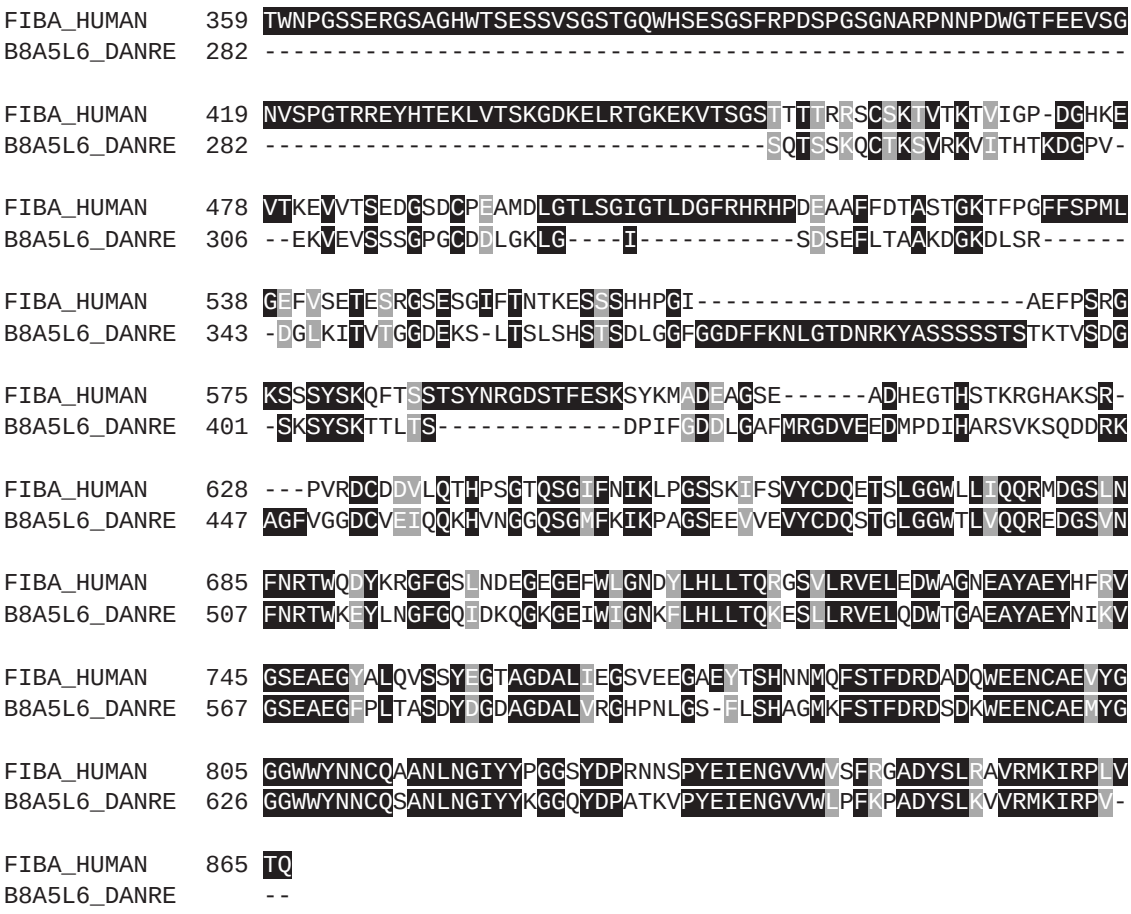

Figure A-3. *Multiple protein sequence alignment of Aα (a) and AαE (b) chains from human and zebrafish was generated using T-Coffee program and then the figures were formatted using the program Boxshade. CLUSTAL O (1.2.4) multiple sequence alignment software was used to calculate the identity between the aligned sequences. (a) The percentage of identity of the Aα fibrinogen chain between humans and zebrafish is 18.457%. (b) The percentage of identity of the AαE fibrinogen chain between humans and zebrafish is: 32.534%.*

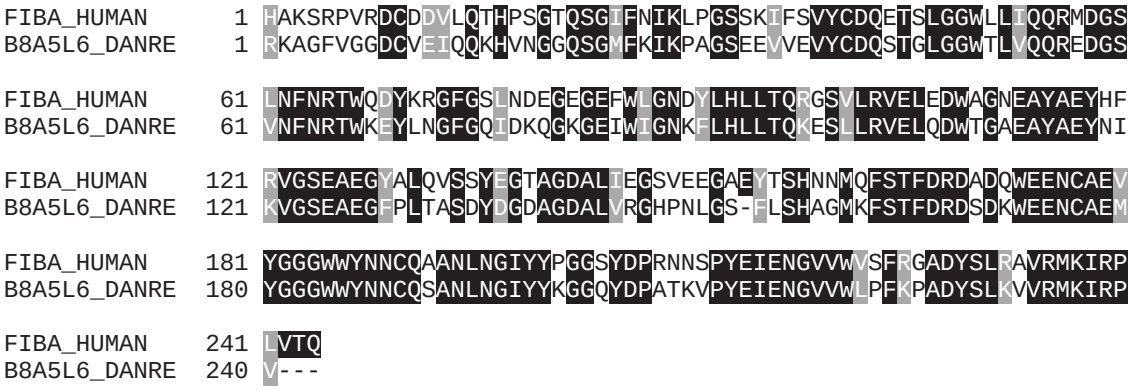

Figure A-4. *Multiple protein sequence alignment of the AαE C-terminal domains from human and zebrafish was generated using T-Coffee server and then the figure was formatted using the BoxShade server. CLUSTAL O (1.2.4) multiple sequence alignment software was used to calculate the identity between the aligned sequences; which is: 61.983%.*

(a)

```
FIBA_HUMAN    1 HAKSRPVRDCDDVLQTHPSGTQSGIFNIKLPGSSKIFSVYCDQETSLGGWLLIQQRMDGS
FIBB_HUMAN    1 NIPVVSGKECEEIIRK--GGETSEMYLIQPDSSVKPYRVYCDMNTENGGWTVIQNRQDGS

FIBA_HUMAN   61 LNFNRTWQDYKRGFGSLNDE--EGEGEFWLGNDYLHLLTQRGS-VLRVELEDWAG
FIBB_HUMAN   59 VDFGRKWDPYKQGFGNVATNTDGKNYCGLPGEYWLGNDKISQLTRMGPTELLIEMEDWKG

FIBA_HUMAN  112 NEAYAEYH-FRVGSEAEGYALQVSSYEGTAGDALIEGSVE---EGAEYTSHNNMQFSTFD
FIBB_HUMAN  119 DKVKAHYGGFTVQNEANKYQISVNKYRGTAGNALMDGASQLMGENRTMTIHNGMFFSTYD

FIBA_HUMAN  168 RDADQWE-----ENCAEVYGGGWWYNNCQAANLNGIYYPGGSYDPRNNSPYEIENGVVWV
FIBB_HUMAN  179 RDNDGWLTSDPRKQCSKEDGGGWWYNRCHAANPNGRYYWGGQYT-WDMAKHGTDDGVVWM

FIBA_HUMAN  223 SFRGADYSLRAVRMKIRPLVT-Q
FIBB_HUMAN  238 NWKGSWYSMRKMSMKIRPFFPQQ
```

(b)

```
FIBA_HUMAN    1 HAKSRPVRDCDDVLQTHPSGTQSGIFNIKLPGSSKIFSVYCDQETSLGGWLLIQQRMDGS
FIBG_HUMAN    1 QIHDITGKDCQDIAN--KGAKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDGS

FIBA_HUMAN   61 LNFNRTWQDYKRGFGSLNDEGEGEFWLGNDYLHLLTQRG---SVLRVELEDWAGNEAYAE
FIBG_HUMAN   59 VDFKKNWIQYKEGFGHLSPTGTTEFWLGNEKIHLISTQSAIPYALRVELEDWNGRTSTAD

FIBA_HUMAN  118 Y-HFRVGSEAEGYALQVSSYEG-TAGDALI---EGSVEEGAEYTSHNNMQFSTFDRDADQ
FIBG_HUMAN  119 YAMFKVGPEADKYRLTYAYFAGGDAGDAFDGFDFGDDPSDKFFTSHNGMQFSTWDNDNDK

FIBA_HUMAN  173 WEENCAEVYGGGWWYNNCQAANLNGIYYPGGSYDPRNNSPYEIENGVVWVSFRGADYSLR
FIBG_HUMAN  179 FEGNCAEQDGSGWWMNKCHAGHLNGVYYQGGTYS-KASTPNGYDNGIIWATWKTRWYSMK

FIBA_HUMAN  233 AVRMKIRPLVTQ------------------
FIBG_HUMAN  238 KTTMKIIPFNRLTIGEGQQHHLGGAKQAGDV
```

(c)

```
FIBB_HUMAN    1 NIPVVSGKECEEIIRKGGETSEMYLIQPDSSVKPYRVYCDMNTENGGWTVIQNRQDGSVD
FIBG_HUMAN    1 QIHDITGKDCQDIANKGAKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDGSVD

FIBB_HUMAN   61 FGRKWDPYKQGFGNVATNTDGKNYCGLPGEYWLGNDKISQLTRM--GPTELLIEMEDWKG
FIBG_HUMAN   61 FKKNWIQYKEGFGHLSPT-------G-TTEFWLGNEKIHLISTQSAIPYALRVELEDWNG

FIBB_HUMAN  119 DKVKAHYGGFTVQNEANKYQISVNKYRG-TAGNALMDGASQ-LMGENRTMTIHNGMFFST
FIBG_HUMAN  113 RTSTADYAMFKVGPEADKYRLTYAYFAGGDAGDA-FDGFDFGDDPSDKFFTSHNGMQFST

FIBB_HUMAN  177 YDRDNDGWLTSDPRKQCSKEDGGGWWYNRCHAANPNGRYYWGGQYTWDMAKHGTDDGVVW
FIBG_HUMAN  172 WDNDNDKF-----EGNCAEQDGSGWWMNKCHAGHLNGVYYQGGTYSKASTPNGYDNGIIW

FIBB_HUMAN  237 MNWKGSWYSMRKMSMKIRPFFPQQ------------------
FIBG_HUMAN  227 ATWKTRWYSMKKTTMKIIPFNRLTIGEGQQHHLGGAKQAGDV
```

Figure A-5. *Multiple protein sequence alignment of the C-terminal globular domain from: (a) AαE and Bβ human fibrinogen chains, (b) AαE and γ human fibrinogen chains; and (c) Bβ and γ human fibrinogen chains. Multiple protein sequence alignment was generated using T-Coffee server and then the figures were formatted using the BoxShade server.*

(a)

```
B8A5L6_DANRE     1 RKAGFVGGDCVEIQQKHVNGGQSGMFKIKPAGSEEVVEVYCDQSTGLGGWTLVQQREDGS
Q6NYE1_DANRE     1 PIPVVSGKECEDIIRKG--GEDSQMYIIRPDPLGTPYKVFCDQTSKNGGWVLIQNRMDGS

B8A5L6_DANRE    61 VNFNRTWKEYLNGFGQID-------KQGKGEIWIGNKFLHLLTQ-KESLLRVELQDWTGA
Q6NYE1_DANRE    59 VDFGRRWDDYRRGFGNIAFDVGKGHCQTPGEYWLGNDRISQLSKMGATELLVEMEDWSGS

B8A5L6_DANRE   113 EAYAEYN-IKVGSEAEGFPLTASDYDGDAGDALVRGHPNLG----SFLSHAGMKFSTFDR
Q6NYE1_DANRE   119 KVYAQYEQFSMQGEASNYILGVGRYSGTAGNTFLEGATELFGENRTMTIHNGMMFSTYDR

B8A5L6_DANRE   168 DSDKWE-----ENCAEMYGGGWWYNNCQSANLNGIYYKGGQYDPATKVPYEIENGVVWLP
Q6NYE1_DANRE   179 DNDKWIPGDPSKQCSKEDGGGWWYNRCHSCNPNGRYYWGGAYTKY-MAKHGTDDGIVWMN

B8A5L6_DANRE   223 FKPADYSLKVVRMKIRPV----
Q6NYE1_DANRE   238 WKGSWYSLKTISMKIRPYFKQK
```

(b)

```
B8A5L6_DANRE     1 RKAGFVGGDCVEIQQKHVNGGQSGMFKIKPAGSEEVVEVYCDQSTGLGGWTLVQQREDGS
Q7ZVG7_DANRE     1 EIQTVTGKDCQDIANK--GGKVSGLYYVKPARAPEAFLVYCEIDSFGRGWTVLQRRRDGS

B8A5L6_DANRE    61 VNFNRTWKEYLNGFGQIDKQGKGEIWIGNKFLHLLTQKES---LLRVELQDWTGAEAYAE
Q7ZVG7_DANRE    59 VDFSKNWIQYKEGFGYLSPDDRTEFWLGNEKIHLLSVQSSVPYVLRIEMVDWEGNKKYAD

B8A5L6_DANRE   118 YN-IKVGSEAEGFPLTASDY-DGDAGDALV----RGHPNLGSFLSHAGMKFSTFDRDSDK
Q7ZVG7_DANRE   119 YATFKLGPEVDAYRLTYAYYFGGDAGDAFDGYDFGDDPSDKFYTSHNGMQFSTVDRDNDK

B8A5L6_DANRE   172 WEENCAEMYGGGWWYNNCQSANLNGIYYKGGQYDPATKVPYEIENGVVWLPFKPADYSLK
Q7ZVG7_DANRE   179 FQGHCAQQDGSGWWMNRCHAAHLNGKYYQGGKYTEK-DAESGYDNGIIWATWHSRWYSLK

B8A5L6_DANRE   232 VVRMKIRPV----------------------
Q7ZVG7_DANRE   238 ETTMKIIPINRIGATGGQESGSKQFGGIGDI
```

(c)

```
Q6NYE1_DANRE     1 PIPVVSGKECEDIIRKGGEDSQMYIIRPDPLGTPYKVFCDQTSKNGGWVLIQNRMDGSVD
Q7ZVG7_DANRE     1 EIQTVTGKDCQDIANKGGKVSGLYYVKPARAPEAFLVYCEIDSFGRGWTVLQRRRDGSVD

Q6NYE1_DANRE    61 FGRRWDDYRRGFGNIAFDVGKGHCQTPGEYWLGNDRISQLSKMGATE--LLVEMEDWSGS
Q7ZVG7_DANRE    61 FSKNWIQYKEGFGYLSPDDR-------TEFWLGNEKIHLLSVQSSVPYVLRIEMVDWEGN

Q6NYE1_DANRE   119 KVYAQYEQFSMQGEASNYILGVGRYS-GTAGNTFLEGATELFGE---NRTMTIHNGMMFS
Q7ZVG7_DANRE   114 KKYADYATFKLGPEVDAYRLTYAYYFGGDAGDAFD-GY--DFGDDPSDKFYTSHNGMQFS

Q6NYE1_DANRE   175 TYDRDNDKWIPGDPSKQCSKEDGGGWWYNRCHSCNPNGRYYWGGAYTKYMAKHGTDDGIV
Q7ZVG7_DANRE   171 TVDRDNDKF-----QGHCAQQDGSGWWMNRCHAAHLNGKYYQGGKYTEKDAESGYDNGII

Q6NYE1_DANRE   235 WMNWKGSWYSLKTISMKIRPYFKQK------------------
Q7ZVG7_DANRE   226 WATWHSRWYSLKETTMKIIPINRIGATGGQESGSKQFGGIGDI
```

Figure A-6. *Multiple protein sequence alignment of the C-terminal globular domain from: (a) AαE and Bβ zebrafish fibrinogen chains, (b) AαE and γ zebrafish fibrinogen chains; and (c) Bβ and γ zebrafish fibrinogen chains. Multiple protein sequence*

alignment was generated using T-Coffee server and then the figures were formatted using the BoxShade server.

(a)

```
FIBA_HUMAN    1 HAKSRPVRDCDDVLQTHPSGTQSGIFNIKLPGSSKIFSVYCDQETSLGGWLLIQQRMDGS
FIBB_HUMAN    1 NIPVVSGKECEEIIR--KGGETSEMYLIQPDSSVKPYRVYCDMNTENGGWTVIQNRQDGS
FIBG_HUMAN    1 QIHDITGKDCQDIAN--KGAKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDGS

FIBA_HUMAN   61 LNFNRTWQDYKRGFGSLNDE--G------EGEFWLGNDYLHLLTQRG---SVLRVELEDW
FIBB_HUMAN   59 VDFGRKWDPYKQGFGNVATNTDGKNYCGLPGEYWLGNDKISQLTRMGP--TELLIEMEDW
FIBG_HUMAN   59 VDFKKNWIQYKEGFGHLSPT--G------TTEFWLGNEKIHLISTQSAIPYALRVELEDW

FIBA_HUMAN  110 AGNEAYAEY-HFRVGSEAEGYALQVSSYEG-TAGDALIE---GSV---EEGAEYTSHNNM
FIBB_HUMAN  117 KGDKVKAHYGGFTVQNEANKYQISVNKYRG-TAGNALMD---GASQLMGENRTMTIHNGM
FIBG_HUMAN  111 NGRTSTADYAMFKVGPEADKYRLTYAYFAGGDAGDAFDGFDFGDD---PSDKFFTSHNGM

FIBA_HUMAN  162 QFSTFDRDADQW-----EENCAEVYGGGWWYNNCQAANLNGIYYPGGSYDPRNNSPYEIE
FIBB_HUMAN  173 FFSTYDRDNDGWLTSDPRKQCSKEDGGGWWYNRCHAANPNGRYYWGGQYT-WDMAKHGTD
FIBG_HUMAN  168 QFSTWDNDNDKF-----EGNCAEQDGSGWWMNKCHAGHLNGVYYQGGTYS-KASTPNGYD

FIBA_HUMAN  217 NGVVWVSFRGADYSLRAVRMKIRPLVTQ------------------
FIBB_HUMAN  232 DGVVWMNWKGSWYSMRKMSMKIRPFFPQQ-----------------
FIBG_HUMAN  222 NGIIWATWKTRWYSMKKTTMKIIPFNRLTIGEGQQHHLGGAKQAGDV


FIBG_HUMAN    100.00   41.08   41.30
FIBA_HUMAN     41.08  100.00   43.98
FIBB_HUMAN     41.30   43.98  100.00
```

(b)

```
B8A5L6_DANRE    1 RKAGFVGGDCVEIQQKHVNGGQSGMFKIKPAGSEEVVEVYCDQSTGLGGWTLVQQREDGS
Q6NYE1_DANRE    1 PIPVVSGKECEDIIRKG--GEDSQMYIIRPDPLGTPYKVFCDQTSKNGGWVLIQNRMDGS
Q7ZVG7_DANRE    1 EIQTVTGKDCQDIANKG--GKVSGLYYVKPARAPEAFLVYCEIDSFGRGWTVLQRRRDGS

B8A5L6_DANRE   61 VNFNRTWKEYLNGFGQIDK-------QGKGEIWIGNKFLHLLTQKES---LLRVELQDWT
Q6NYE1_DANRE   59 VDFGRRWDDYRRGFGNIAFDVGKGHCQTPGEYWLGNDRISQLSKMGAT--ELLVEMEDWS
Q7ZVG7_DANRE   59 VDFSKNWIQYKEGFGYLSP-------DDRTEFWLGNEKIHLLSVQSSVPYVLRIEMVDWE

B8A5L6_DANRE  111 GAEAYAEYN-IKVGSEAEGFPLTASDY-DGDAGDALVRGH----PNLGSFLSHAGMKFST
Q6NYE1_DANRE  117 GSKVYAQYEQFSMQGEASNYILGVGRY-SGTAGNTFLEGATELFGENRTMTIHNGMMFST
Q7ZVG7_DANRE  112 GNKKYADYATFKLGPEVDAYRLTYAYYFGGDAGDAFDGYDFGDDPSDKFYTSHNGMQFST

B8A5L6_DANRE  165 FDRDSDKWE-----ENCAEMYGGGWWYNNCQSANLNGIYYKGGQYDPATKVPYEIENGVV
Q6NYE1_DANRE  176 YDRDNDKWIPGDPSKQCSKEDGGGWWYNRCHSCNPNGRYYWGGAYTKY-MAKHGTDDGIV
Q7ZVG7_DANRE  172 VDRDNDKF-----QGHCAQQDGSGWWMNRCHAAHLNGKYYQGGKYTEK-DAESGYDNGII

B8A5L6_DANRE  220 WLPFKPADYSLKVVRMKIRPV----------------------
Q6NYE1_DANRE  235 WMNWKGSWYSLKTISMKIRPYFKQK-----------------
Q7ZVG7_DANRE  226 WATWHSRWYSLKETTMKIIPINRIGATGGQESGSKQFGGIGDI


B8A5L6_DANRE  100.00   40.93   40.51
Q6NYE1_DANRE   40.93  100.00   42.51
Q7ZVG7_DANRE   40.51   42.51  100.00
```

Figure A-7. *Multiple protein sequence alignment of the C-terminal globular domain from: (a) AαE, Bβ and γ human fibrinogen chains; and (b) AαE, Bβ and γ zebrafish fibrinogen chains. Multiple protein sequence alignment was generated using T-*

Coffee server and then the figures were formatted using the BoxShade server. CLUSTAL O (1.2.4) multiple sequence alignment software was used to calculate the percentage of identity between the aligned sequences.

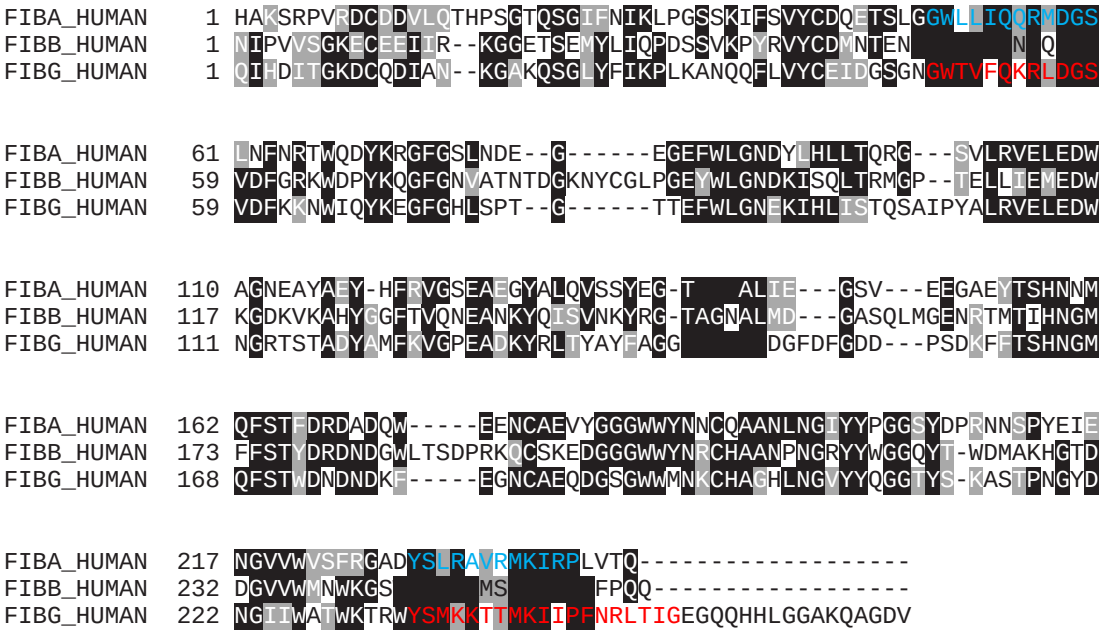

Figure A-8. *Multiple protein sequence alignment of the C-terminal globular domain from AαE, Bβ and γ human fibrinogen chains. Highlighted in red are the residues from the fibrinogen γ chain that are known to bind leukocytes through the integrin $\alpha_M\beta_2$. Highlighted in red are the equivalent amino acid residues from AαE chain that are known to bind leukocytes through the integrin $\alpha_M\beta_2$.*

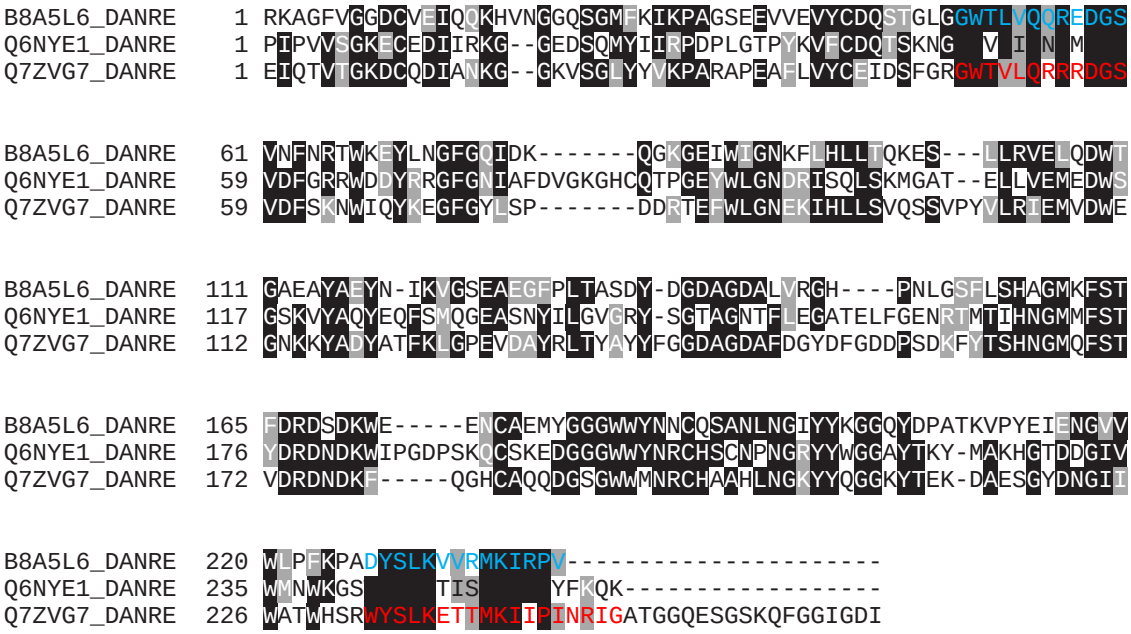

Figure A-9. *Multiple protein sequence alignment of the C-terminal globular domain from AαE, Bβ and γ zebrafish fibrinogen chains. Highlighted in red (γ-chain) and blue (AαE chain) are the equivalent residues that may be involved in fibrinogen leukocyte binding through the integrin* $\alpha_M\beta_2$ *in zebrafish.*