



Thèse

2020

Open Access

This version of the publication is provided by the author(s) and made available in accordance with the copyright holder(s).

Hemostasis and thrombosis in zebrafish models of human congenital fibrinogen disorders

Freire Sanz, Cristina

How to cite

FREIRE SANZ, Cristina. Hemostasis and thrombosis in zebrafish models of human congenital fibrinogen disorders. Doctoral Thesis, 2020. doi: 10.13097/archive-ouverte/unige:140844

This publication URL: <https://archive-ouverte.unige.ch/unige:140844>

Publication DOI: [10.13097/archive-ouverte/unige:140844](https://doi.org/10.13097/archive-ouverte/unige:140844)

HEMOSTASIS AND THROMBOSIS IN ZEBRAFISH MODELS OF HUMAN CONGENITAL FIBRINOGEN DISORDERS

THÈSE

présentée aux Facultés de médecine et des sciences de l'Université de Genève

pour obtenir le grade de Docteur ès sciences en sciences de la vie,

mention Life Sciences

par

Cristina FREIRE SANZ

de

Madrid (ESPAGNE)

Thèse N° 65

GENÈVE

2020

DOCTORAT ÈS SCIENCES EN SCIENCES DE LA VIE DES
FACULTÉS DE MÉDECINE ET DES SCIENCES
MENTION SCIENCES BIOMÉDICALES

Thèse de Mme Cristina FREIRE SANZ

intitulée :

**« Hemostasis and thrombosis in zebrafish models
of human congenital fibrinogen disorders »**

Les Facultés de médecine et des sciences, sur le préavis de Madame Marguerite NEERMAN-ARBEZ, professeure ordinaire et directrice de thèse (Département de Médecine Génétique et Développement), Madame Anne ANGELILLO-SCHERRER, professeure (Hôpital Universitaire de Berne, Suisse), Madame Brenda KWAK, professeure ordinaire (Département de Pathologie et Immunologie), autorisent l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 19 juin 2020

Thèse - 65 -



Le Doyen
Faculté de médecine



Le Doyen
Faculté des sciences

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	5
ABSTRACT.....	11
RESUME EN FRANÇAIS.....	17
LIST OF ABBREVIATIONS.....	23
INTRODUCTION.....	29
HEMOSTASIS:.....	31
I. Primary hemostasis.....	33
a. Vascular endothelium.....	33
b. Platelets.....	34
II. Secondary hemostasis.....	38
a. Traditional model of coagulation.....	38
Extrinsic pathway or tissue factor pathway.....	39
Intrinsic pathway or contact activation pathway.....	39
Common pathway.....	39
b. Cellular based model of coagulation.....	41
Initiation.....	41
Amplification.....	41
Propagation.....	42
c. Regulation of coagulation and termination of clotting cascade.....	43
III. Fibrinolysis.....	44

FIBRINOGEN:	45
I. Fibrinogen	45
a. Fibrinogen structure.....	45
b. Evolution of vertebrate fibrinogen.....	47
II. Fibrinogen gene regulation	48
a. Basal fibrinogen gene expression.....	48
b. Acute phase response fibrinogen gene expression.....	49
c. Enhancers.....	51
III. Fibrinogen biosynthesis	52
IV. Fibrinogen conversion to fibrin	56
a. Fibrin crosslinking.....	59
b. Fibrin clot structure.....	59
V. Fibrinogen biological functions	62
VI. Fibrinogen 420	64
a. Fibrinogen 420 structure.....	64
b. Role of Fibrinogen 420.....	65
c. Newborn and adult levels of Fibrinogen 420.....	66
FIBRINOGEN DEFICIENCIES	68
I. Hereditary fibrinogen disorders	70
a. Type I fibrinogen disorders.....	70
i. Afibrinogenemia.....	70
ii. Hypofibrinogenemia.....	71
b. Type II fibrinogen disorders.....	72
i. Dysfibrinogenemia.....	72

ii. Hypodysfibrinogenemia.....	74
II. Diagnostic testing.....	78
III. Clinical management.....	80
IV. Fibrinogen in disease.....	81
V. Animal models for fibrinogen disorders.....	83
ZEBRAFISH.....	88
I. Zebrafish as a model to study hemostasis and thrombosis.....	88
II. Functional tests to study hemostasis and thrombosis.....	90
RESULTS.....	93
I. Part I: Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders.....	95
a. Abstract.....	97
b. Aims.....	101
c. Manuscript to submit.....	105
II. Part II: A genetic modifier of venous thrombosis in the zebrafish (<i>Danio rerio</i>) reveals a functional role for the fibrinogen A α E chain in early hemostasis.....	149
a. Abstract.....	151
b. Aims.....	155
c. Submitted manuscript.....	159
DISCUSSION AND PERSPECTIVES.....	195
Part I: Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders.....	197

I.	Congenital fibrinogen disorders modelled in zebrafish.....	199
a.	Afibrinogenemic zebrafish model.....	199
b.	Dysfibrinogenemic and hypodysfibrinogenemic zebrafish models.....	202
II.	Laser injury methods to assess the phenotype of larval zebrafish models of fibrinogen disorders.....	205
III.	Role of fibrinogen versus fibrin.....	206
IV.	Future perspectives.....	207
Part II: A genetic modifier of venous thrombosis in the zebrafish (<i>Danio rerio</i>) reveals a functional role for the fibrinogen A α E chain in early hemostasis.....		
I.	Evolutionary conservation of fibrinogen from zebrafish to humans.....	211
II.	The role of Fibrinogen 420 during developmental hemostasis.....	213
III.	A genetic modifier of venous thrombosis.....	214
IV.	A α E C-terminal domain as a ligand for erythrocyte binding.....	215
V.	Mutations in the C-terminal domain the of A α -chain.....	216
VI.	Future perspectives.....	217
ADVANTAGES AND LIMITATIONS.....		221
REFERENCES.....		227
APPENDIX.....		245
I.	Appendix A: Multiple sequence alignment.....	247

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to Professor Marguerite Neerman-Arbez, my research supervisor, for giving me the opportunity to undertake a PhD in her laboratory under her supervision. Moreover, I am thankful for her invaluable advices, guidance, feedback, patience and consistent encouragement in keeping me progressing during my PhD. This research would have never been accomplished without her assistance and dedicated involvement.

I would also like to express my great appreciation to Dr. Richard Fish for his constructive and valuable suggestions during the planning and development of the research. Moreover, I am thankful for his support, advices, assistance, time and recommendations through my PhD.

Furthermore, I would like to offer my special thanks to the technician Corine di Sanza not only for her valuable technical support during my entire PhD but also for her personal support during my academic endeavors. Moreover, I would like to thank my colleague Cindy for her dedicated support and friendship throughout the years during the PhD, and my colleague Rui for his technical support.

My gratitude extends not only to the old chief of the GEDEV Department, the Professor Stylianos Antonarakis, for letting me participate in his lab meetings during the first years of my PhD, but also the new chief of the GEDEV department Professor Serge Nef. I would also like to thank Francine Chopard, secretary of MNA lab and Thérèse Tanovski, secretary of the GEDEV Department, for their help. I would like to thank my friends and colleagues in the GEDEV department, Marta, Maria, Herta, Nico amongst others for a joyfull time spent together in social events organized by the Department.

Besides my advisor, the members of my lab and the GEDEV department, I would like to thank the rest of my thesis committee: Professor Anne Angelillo-Scherrer and Professor Brenda Kwak for their positive comments about the thesis manuscript and presentation and also for their hard questions which uplifted me to expand my research knowledge from different angles. My appreciation also includes Professor Julien Bertrand for letting me participate in his lab meetings and for his valuable comments, critics and advices which were useful in shaping my experiments. Moreover, I would like to thank all the members of his lab who always helped me. Besides the members working in CMU, I extend my gratitude to Jordan Shavit, Professor at the University of Michigan, who contributed in one of the research projects.

I would also like to express my very best appreciation to the Animal Facility in the CMU and the people working to feed the animals and to maintain and clean the tanks. The CMU's Imaging Facility, specially François Prodon, for his technical help with the microscopes. Furthermore, I would like to thank other Facilities in the CMU: The Histology Facility, specially to Marie Ebrahim Malek, the Genomics Facility, specially to Natacha Civic, the Proteomics facility, and the Flow Cytometry Facility.

I would also like to express my gratitude to the PhD school of Life Sciences from the Medicine and the Science faculties, for the opportunity to undertake my studies at the GEDEV Department in the Faculty of Medicine at the University of Geneva. Many thanks to the directors of the Life Sciences PhD school, Professor Dominique Soldati and Professor Dominique Garcin, and to their collaborators Manel Essaidi and Claudine Neyen for their help guiding PhD students in every step.

I would also like to acknowledge the International Society on Thrombosis and Haemostasis (ISTH) and the Life Sciences Switzerland (LS2) for awarding me with two travel awards, which enabled me to attend the ISTH 2019 Congress in Melbourne, Australia.

I would like to express my appreciation to my colleagues from the CMU Daniel, Marta, Morgan, Lou, Ainoa, Miguel, Maria, Elsa, Vasia, Diana and Luisa amongst others for a cherished time spent together in the CMU and in social settings. My appreciation also includes my friends from Switzerland and Spain, Maria, Clara, Lara, Eva, Marc, Ade, Raquel, Maria, Emilia, Cristina, Laura, Virginia, Laura, Miriam, Eli and Gala amongst others.

I would like to deeply thank my family: my father, Angel Freire, my mother, Ursula Sanz, my sister and brother Lucia and Luis Miguel and my sister in law Elena, for their encouragement, unconditional support and for helping me in whatever way they could through my studies. It was their endless love who raised me up again when I got demotivated during this challenging period. Finally, I thank with love Stephan who has been my friend and companion, loved, encouraged, supported and helped me get through the last period of my PhD.

Pursuing this PhD has been a life-challenging experience for me, and it would not have been possible to do it without the guidance and support that I have received from the people listed here and the ones that indirectly also have contributed.

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: A α , B β 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 A α chains are replaced by two A α E isoforms, rendering a fibrinogen molecule of 420 kDa. The A α E 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 A α chain and the A α E isoform in embryonic hemostasis using different zebrafish strains (AB, TU and TL strain). It is known that the proportion of the fibrinogen-420 (A α E-containing fibrinogen) varies through life, being more abundant in a new-born human's blood than in adults. Similarly, zebrafish produce the A α E-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 A α E isoform due to a mutation occurring in the A α E-specific coding region of *fga*. We questioned if the lack of A α E in AB larvae was the reason for their prolonged TTO. We showed that the A α E isoform rescued venous occlusion in fibrinogen deficient mutants, while surprisingly the common A α chain was less efficient. Furthermore, we studied the contribution of A α chain and A α E 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 A α E 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 chaînes polypeptidiques, $\text{A}\alpha$, $\text{B}\beta$ et γ . Ces trois chaînes 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 chaînes $\text{A}\alpha$ principales sont remplacées par deux isoformes $\text{A}\alpha\text{E}$, donnant lieu à une molécule de fibrinogène de 420 kDa. Cet isoforme $\text{A}\alpha\text{E}$ 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 chaînes $A\alpha$ et l'isoforme $A\alpha E$ 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 $A\alpha E$) 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 A α E, à cause d'une mutation dans la région codant pour la chaîne A α E du gène FGA. Nous nous sommes demandés si le manque de A α E dans les larves AB était la raison pour laquelle leur TDO était prolongé. Nous avons montré que l'isoforme A α E rétablit l'occlusion veineuse chez les poissons mutants déficients en fibrinogène, tandis que la chaîne A α commune était moins efficace. De plus, nous avons étudié la contribution des isoformes A α et A α E 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 A α E dans l'hémostase embryonnaire et des différences de contribution de ces deux chaînes pour promouvoir la thrombose, dictées par la disponibilité des types cellulaires.

LIST OF ABBREVIATIONS

A α C A α C-terminal Domain

A α E A α extended isoform

A α EC A α 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].

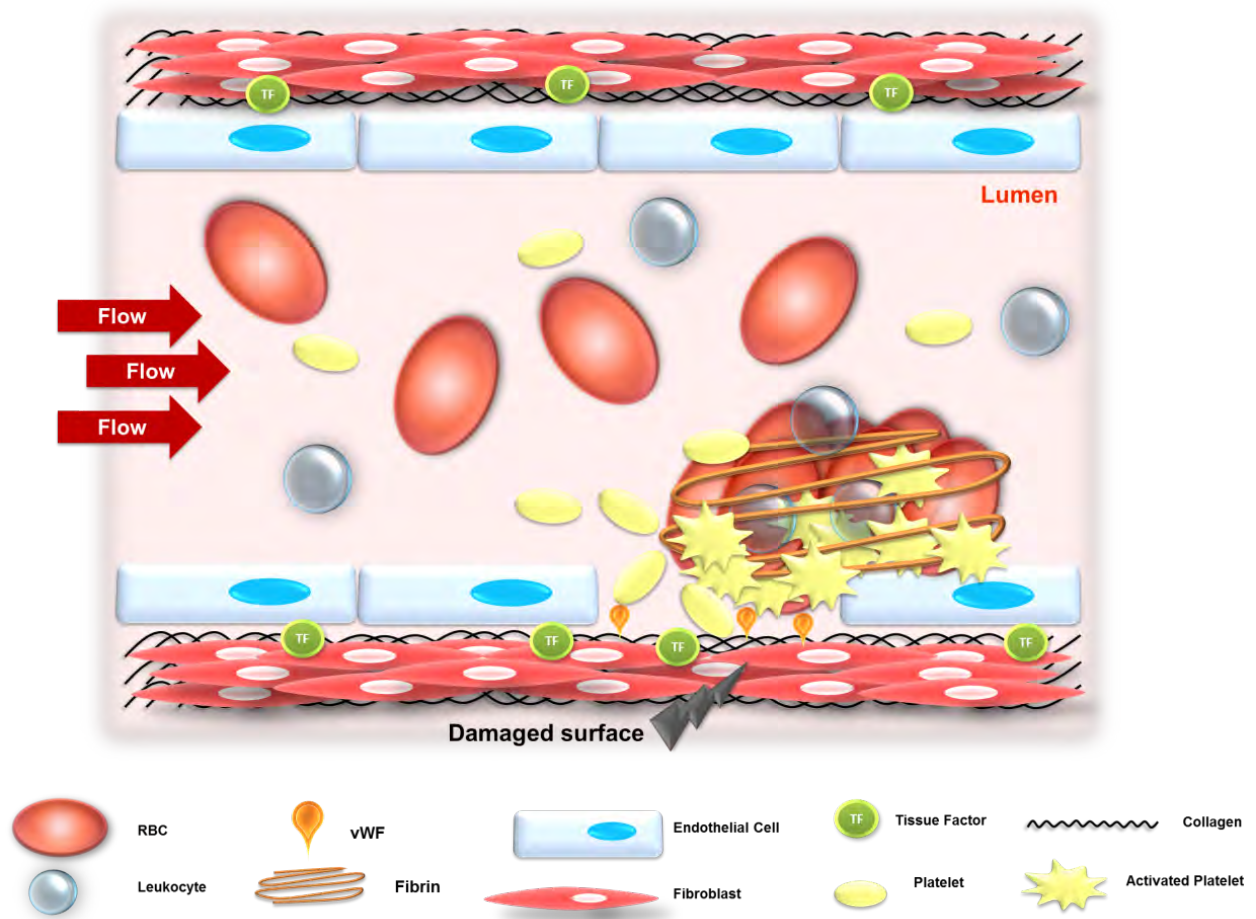


Figure 1. Hemostasis. When an injury occurs in the vasculature, it triggers the release and or exposure of clotting factors. First the formation of a platelet plug occurs and then fibrin fibers adhere to the clot to encapsulate the platelets, red blood cells and leukocytes creating an insoluble blood 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 ($< 1000 \text{ s}^{-1}$, i.e., in veins and large arteries) platelet adhesion involves binding to collagen, fibronectin (through $\alpha 5\beta 1$ integrin binding) and laminin (through $\alpha 6\beta 1$ integrin binding). Platelet-collagen interactions are mediated by two receptors: glycoprotein VI (GPVI) and the integrin $\alpha 2\beta 1$. 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 ($> 1000 \text{ s}^{-1}$, i.e., in the microvasculature and small

arteries), the interaction between the platelet surface receptor glycoprotein Ib α (GPIb α) 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 GPIb α 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 ($< 1000 \text{ s}^{-1}$), 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 GPIb α and $\alpha_{IIb}\beta_3$. At high shear rates ($> 10,000 \text{ s}^{-1}$), platelet aggregation is

mediated by VWF–GPIIb α 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].

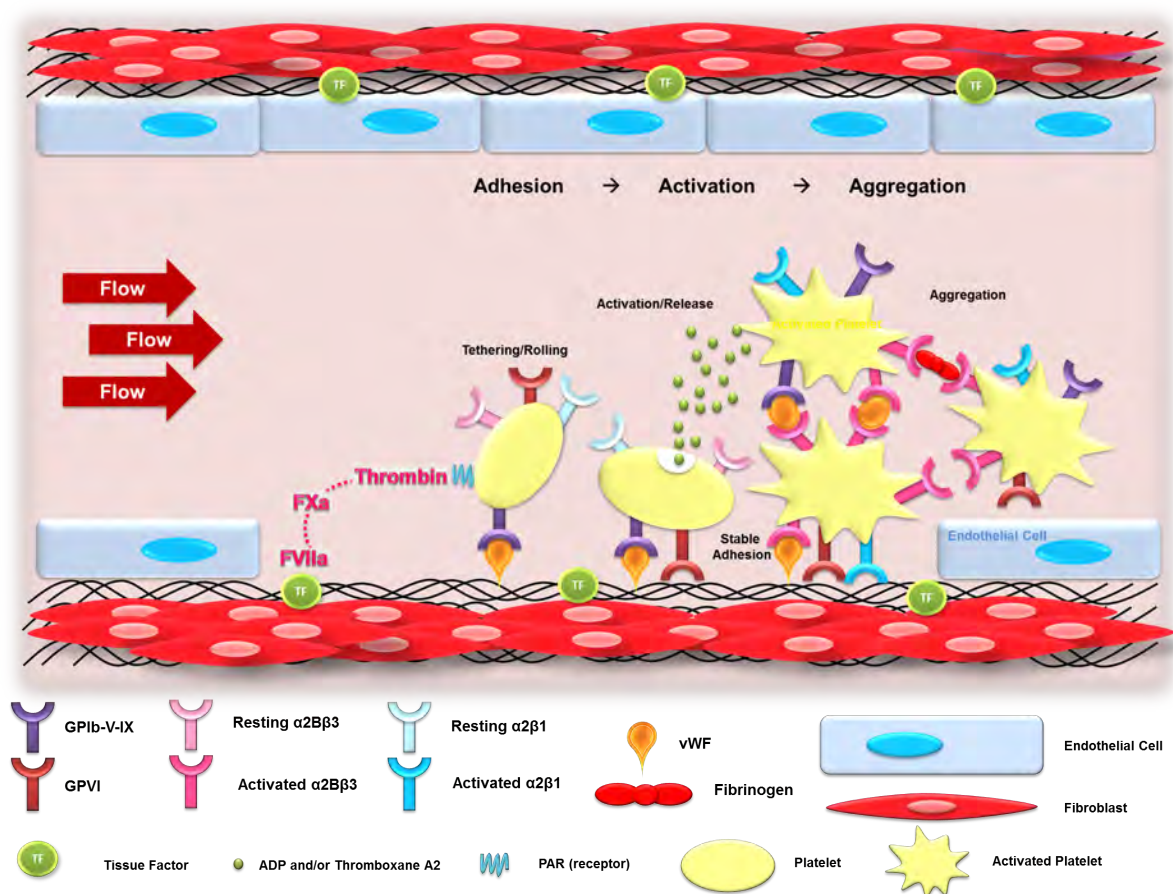


Figure 2. Platelet adhesion and aggregation. The first interaction enables platelet tethering and it is mediated by the binding of vWF with the receptor complex GPIb-V-IX. This interaction enables GPVI binding with collagen. This leads to a shift in the integrin receptors to a high affinity state (activation), 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.

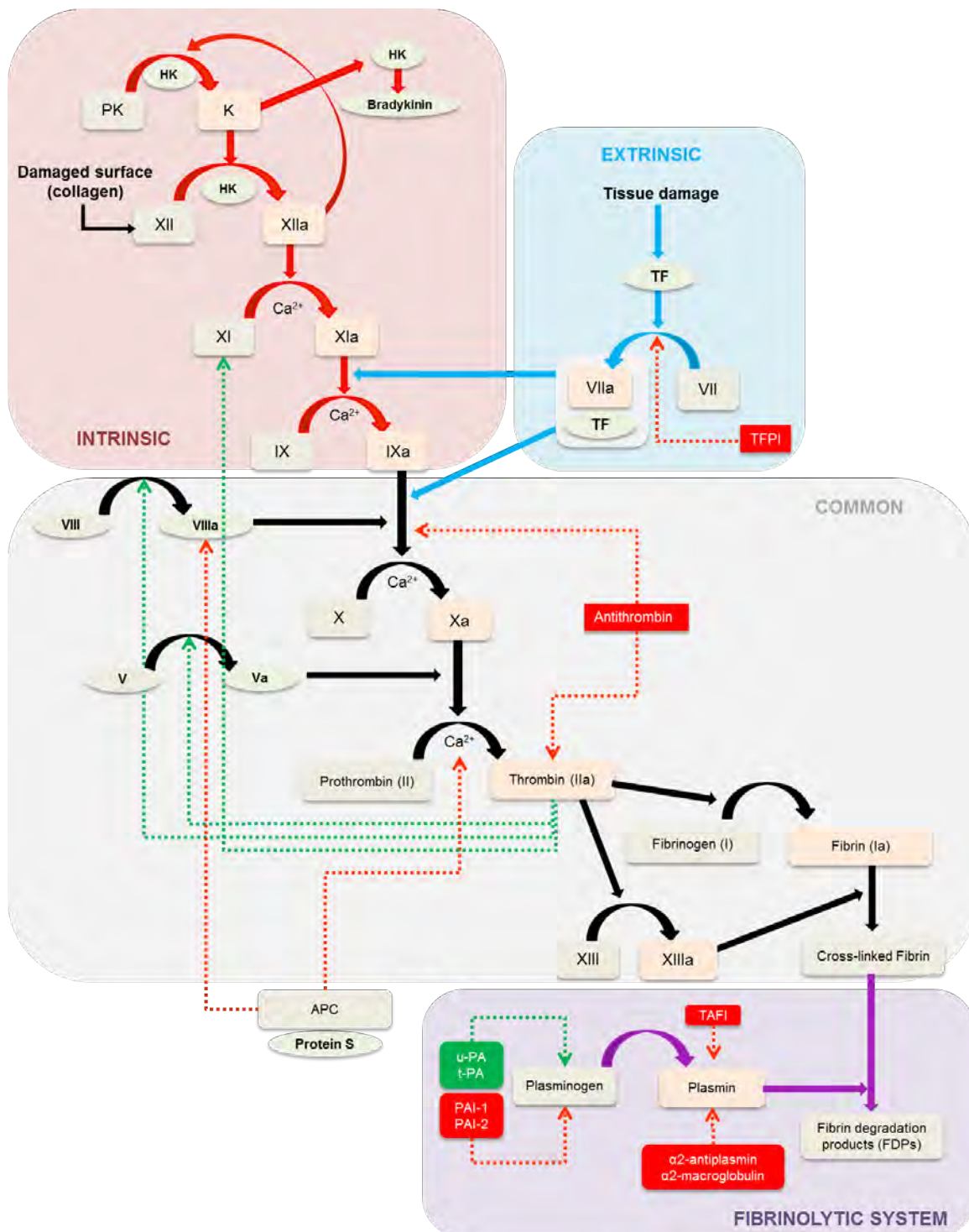


Figure 3. The Coagulation Cascade. In the diagram appears the traditional model of coagulation cascade (intrinsic, extrinsic and common pathways) and the fibrinolytic system. Arrows in green represent the positive feedback. Arrows in red represent the negative feedback.

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].

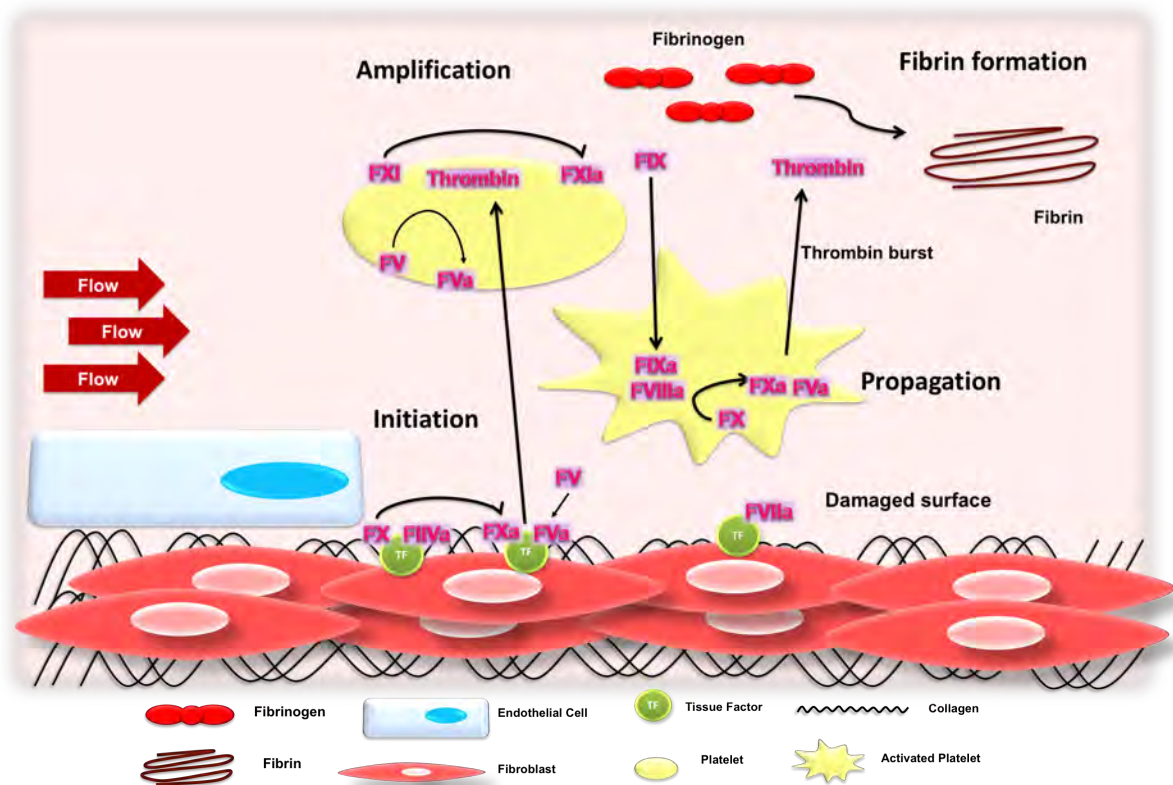


Figure 4. The cell-based model of coagulation is divided in three overlapping phases. (I) The initiation phase starts when an injury exposes TF (from TF-bearing cell) to blood flow, leading to generation of small amounts of thrombin and FIXa. (II) In the amplification phase, the small amounts of thrombin are sufficient to activate platelets and to generate FVa, FVIIIa, and FIXa. (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 ($A\alpha$ -(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 α_2 -macroglobulin, or serpins such as PAI-1, PAI-2, and α_2 -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: $A\alpha$, $B\beta$ 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 $A\alpha$ -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 $A\alpha$, $B\beta$ and γ chains are homologous, but the random coil C-terminal domain of the common $A\alpha$ -chain is different from the highly conserved globular C-terminal domains present in $B\beta$ 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 (γ A-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, VRPEHPAET EYDSL YPEDDL (Figure 5) [49].

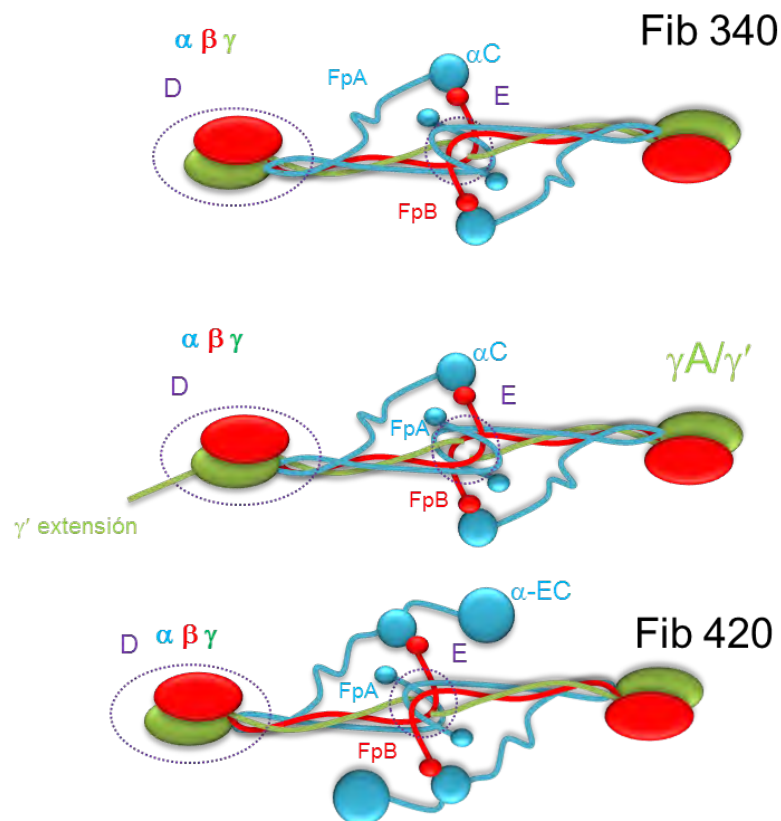


Figure 5. Fibrinogen structure. α chains are shown in blue. β chains are shown in red and γ chains are shown in green. FpA and FpB are fibrinopeptides A and B, respectively. E is the E central region and D is the D domain. The common fibrinogen

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 $A\alpha$, $B\beta$ 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 million 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 $B\beta$ chains is more recent because the homology between these chains is higher when compared to the $A\alpha$ -chain. This fact implies that $A\alpha$, pre- $B\beta$ and pre- γ genes are originated from the duplication of an ancestral gene around 1 billion years ago. The opposite gene orientation of $B\beta$ gene can be explained by the inversion of $A\alpha$ and γ genes or inversion of $B\beta$ 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 B β 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-1 β 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-1 β -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-1 β 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)- κ B which are activated by IL-1 and IL-6 or IL-1 and NF- κ B, respectively. The transcription factors C/EBP and NF- κ B 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].

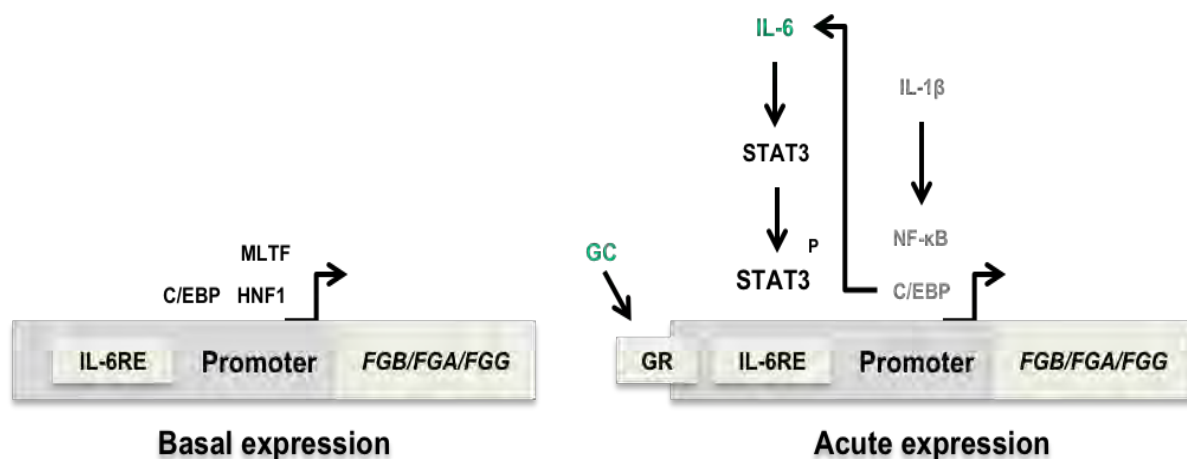


Figure 6. Fibrinogen gene expression regulation. Left: fibrinogen gene regulation under basal conditions. Represented are the three fibrinogen genes (*FGA*, *FGB*, *FGG*) under the control of a promoter region, and the transcription factors i.e., HNF1, C/EBP and MLTF which bind to the promoter near the TSS (arrow). Right: fibrinogen gene expression under the acute phase inflammatory response. Glucocorticoids (GC), IL-1 β 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-1 β activates the

transcription factor (NF)- κ B, 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 $(\text{A}\alpha\text{B}\beta\gamma)_2$. $\text{A}\alpha$, $\text{B}\beta$ 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- $\text{A}\alpha$ chain (644 aa residues), pre-pro- $\text{B}\beta$ 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 ($\text{A}\alpha$ - γ and $\text{B}\beta$ - γ), secondly a third chain is added to the two chain complexes, either by combining a $\text{B}\beta$ chain with an $\text{A}\alpha$ - γ dimer or an $\text{A}\alpha$ chain with a $\text{B}\beta$ - γ dimer, forming trimeric half molecules ($\text{A}\alpha\text{B}\beta\gamma$). 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.

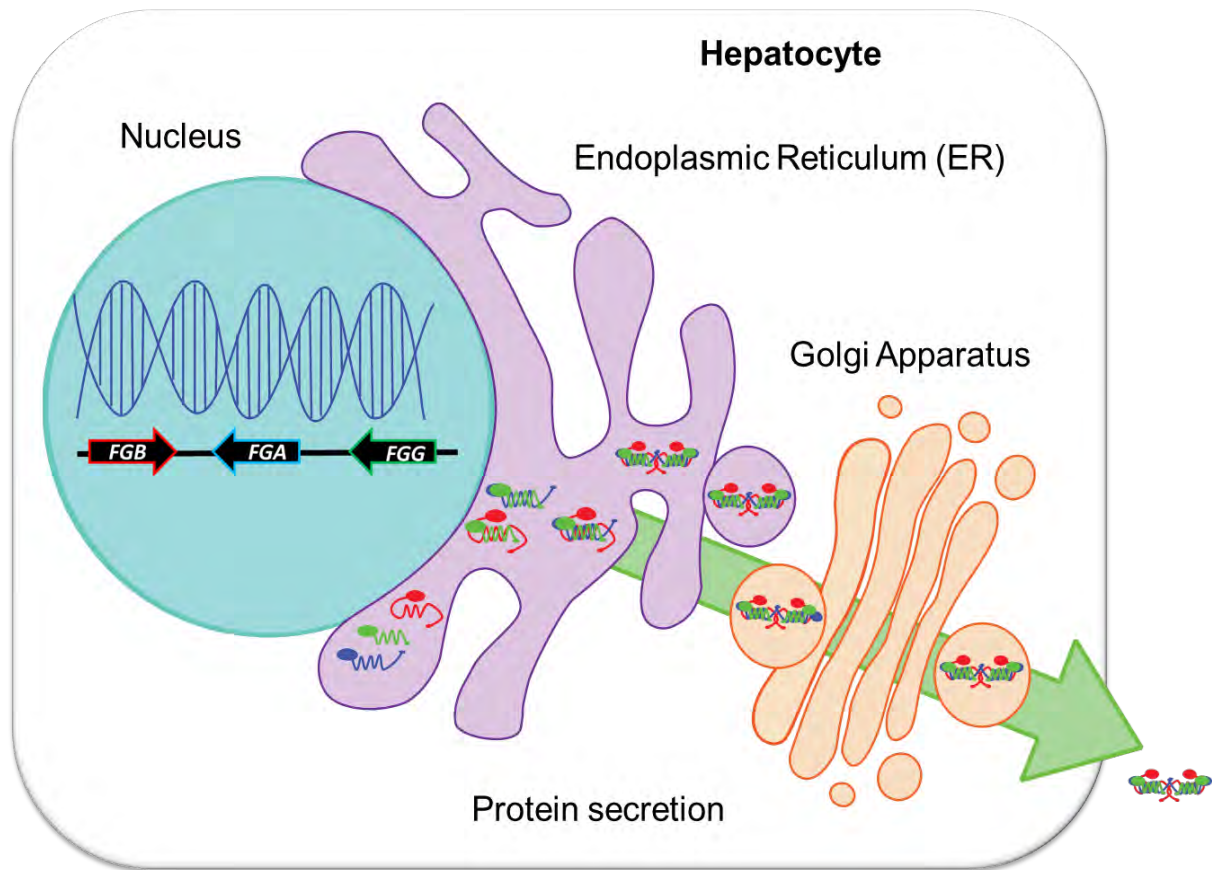


Figure 7. Fibrinogen synthesis and expression. Fibrinogen is composed by two copies of three polypeptide chains ($A\alpha$, $B\beta$ and γ) which are encoded by *FGA*, *FGB* and *FGG* genes, respectively, which are clustered on human chromosome 4. After translation, fibrinogen chains are assembled in a stepwise manner in the endoplasmic reticulum (ER). Single chains assemble into $A\alpha$ - γ or $B\beta$ - 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. $A\alpha$ chains are showed in blue. $B\beta$ chains are showed in red and γ chains are showed in green.

The fibrinogen molecule contains one central E region, two terminal D regions, two α C regions and two B β 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 A α , B β and γ chains; (II) one funnel-shape domain formed by the N-terminal portions of two A α and two B β 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 A α , B β 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 B β and γ chains, respectively. Furthermore, each fibrinogen half molecule has two other regions: an α C region and a B β N region. Although the structure of these regions is not present in the crystal structure, it is known from NMR studies. In turn, the α C 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 α C region to the rest of the molecule, and (II) an α C folded compact domain which contains the C-terminal part (residues Aa392–610). The B β N region (functional B β N-domain) is composed by the N-terminal portion of the B β chain. Disruption of the α C region affects the hydrodynamic behavior of fibrinogen (Figure 8) [85] [86] [87] [88].

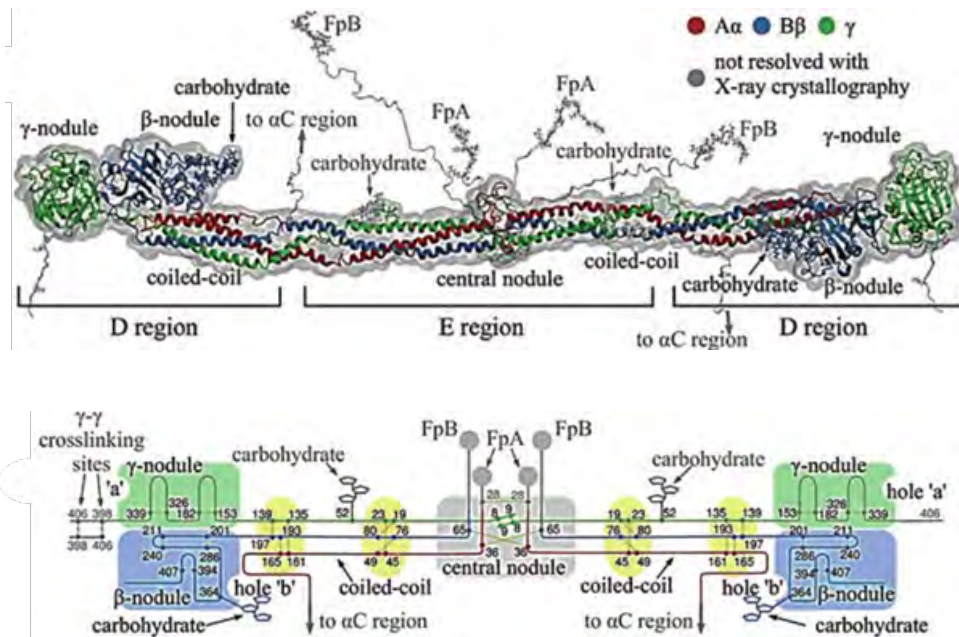


Figure 8. Upper figure. X-ray crystallography structure of the fibrinogen molecule (PDB Entry: 3GHG) is shown and the portions missing from the crystal structure were reconstructed computationally (the N-terminal parts of αA and βB chains with FpA and FpB in the central nodule and the beginning of the αC regions). Bottom figure. Diagram of the three fibrinogen polypeptide chains αA , βB 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 $A\alpha$ and $B\beta$ 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 $A\alpha\text{Arg16-Gly17}$ (without signal peptide), and Fibrinopeptide B (FpB) is cleaved at the peptide bond $B\beta\text{Arg14-Gly15}$ (without signal peptide) [86]. Thus, the polypeptide composition of fibrinogen molecule after the fibrinopeptide thrombin cleavage is designated as $(\alpha\beta\gamma)_2$. No peptides are cleaved from γ chains; thus, the polypeptide composition of fibrin is $(\alpha\beta\gamma)_2$ and the conversion of fibrinogen to fibrin can be described as: $(A\alpha B\beta\gamma)_2 \rightarrow (\alpha\beta\gamma)_2 + 2\text{FpA} + 2\text{FpB}$ [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 $(\alpha B\beta\gamma)_2$ and then fibrinopeptide B is removed to produce desAB fibrin $(\alpha\beta\gamma)_2$ [87]. More rarely, fibrinopeptide B is removed first to produce desB fibrin $(A\alpha\beta\gamma)_2$. This distinction is due to spatial restrictions of the thrombin binding to fibrinogen where the N-terminal of the $A\alpha$ chain is more accessible to thrombin active site than the one of $B\beta$ 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 γ Trp315-Trp330, γ Trp335-Asn365 and γ Phe295-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.

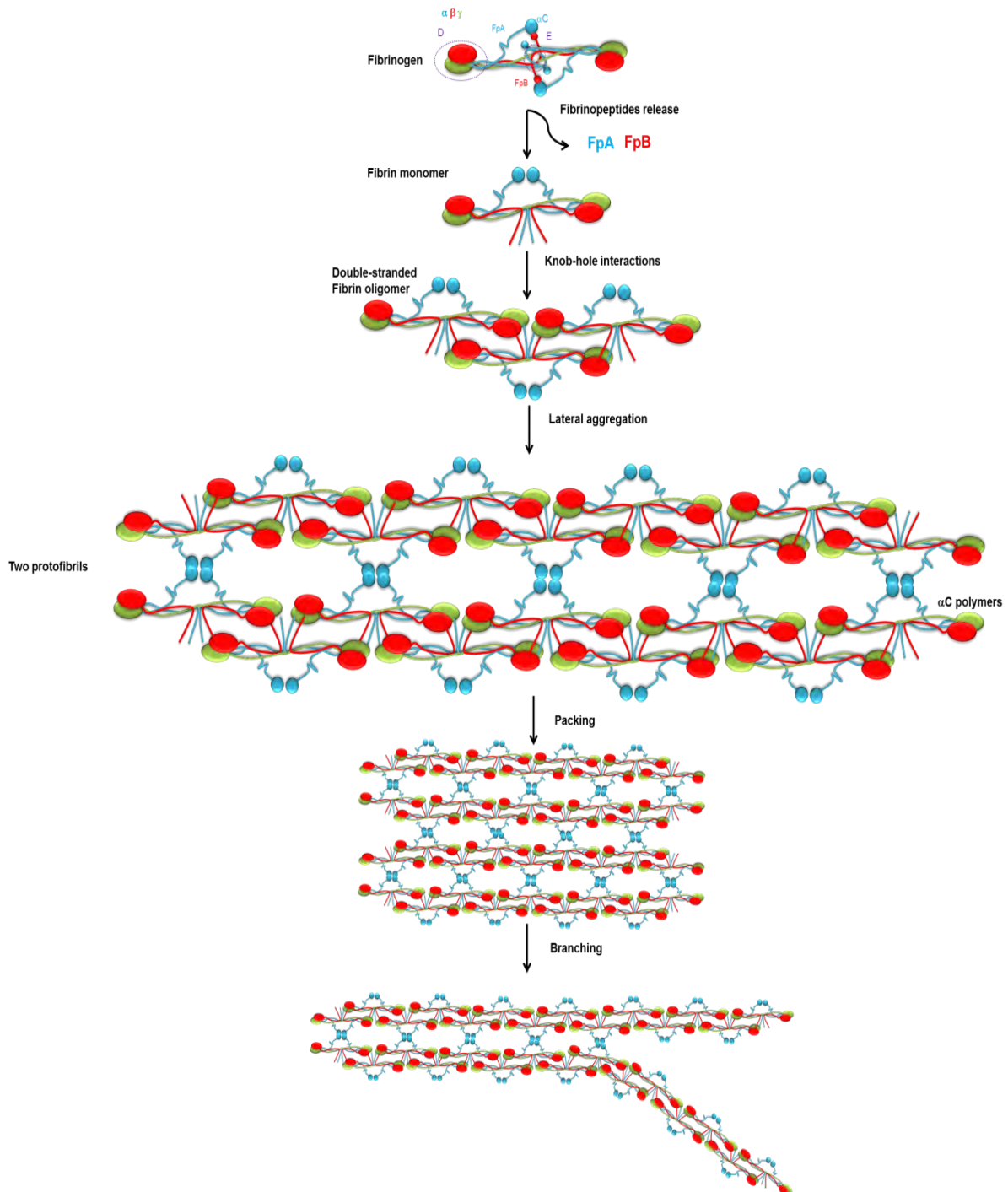


Figure 9. Fibrin polymerization. Thrombin removes fibrinopeptides A and B from A α and B β chains respectively, then knob-hole interaction occurs forming oligomers

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 Ca^{2+} . 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 A α -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 β_3 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 A α -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-1 β). 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 A α -chain leading to the A α E isoform (Figure 10) [143]. The A α E chain is more than 50% longer than the common A α 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 B β and γ chains [143] [144]. Fibrinogen molecules, in which each of the two A α -chains is replaced by an A α E isoform, are symmetrical molecules (homodimers) of the structure (A α EB β γ)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 A α E C-terminal globular domain is the single largest conserved portion of the A α -chain. Therefore, the preservation of this fibrinogen subclass throughout vertebrate evolution suggests a vital physiological role that remains to be identified.

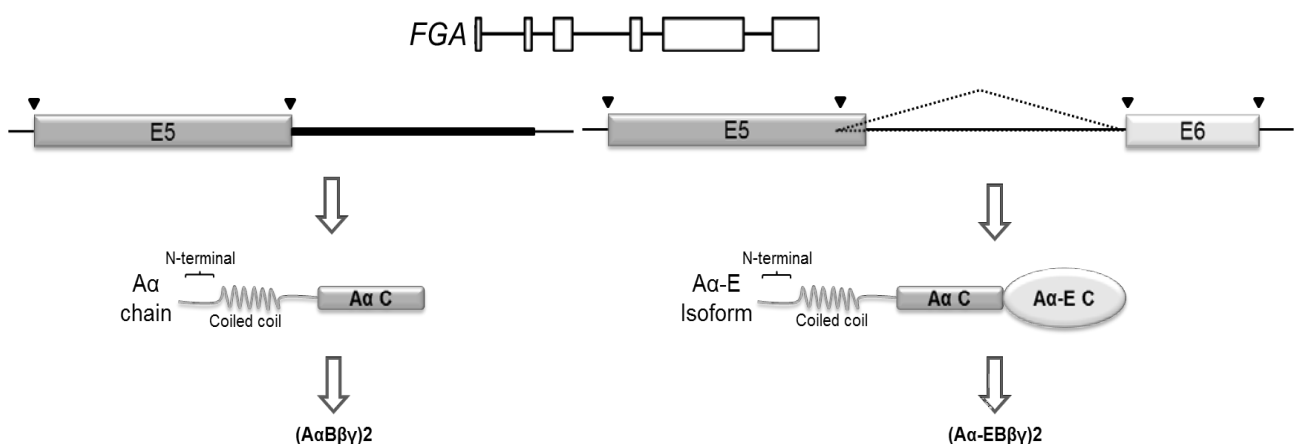


Figure 10. Fibrinogen 340 and 420 produced from FGA gene. The FGA gene consists of six exons and three different mRNA transcripts are produced. In this figure appear two of them: the transcript encoding for the common A α chain (on the left) and the transcript encoding for the A α E isoform (on the right)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

Role of Fibrinogen 420

The A α E isoform has some distinguishable features which can be attributed to its possible functions. Because the A α E 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 A α -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 A α E 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 A α E 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 A α E isoform is less susceptible to proteolytic degradation due to the inaccessibility of A α E 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 A α E 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 A α -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,

ante partum or post partum 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 B β -chain Gly472Val missense change identified in a woman who had experienced thrombosis episodes and miscarriages [180], and the fibrinogen B β -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 γ Gly284Arg (*FGG* c928G>C/A) [183] which was the first discovered, and fibrinogen Aguadilla γ Arg375Trp (*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 A α Arg38Ser 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 A α -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 A α Arg16Cys. 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 example is the heterozygous dysfibrinogenemia mutation fibrinogen Sumida (A α Cys472Ser), 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 (γ Arg554Cys), 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 A α 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 hypodysfibrinogenemia occur within the C-terminal end

of the fibrinogen A α 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].

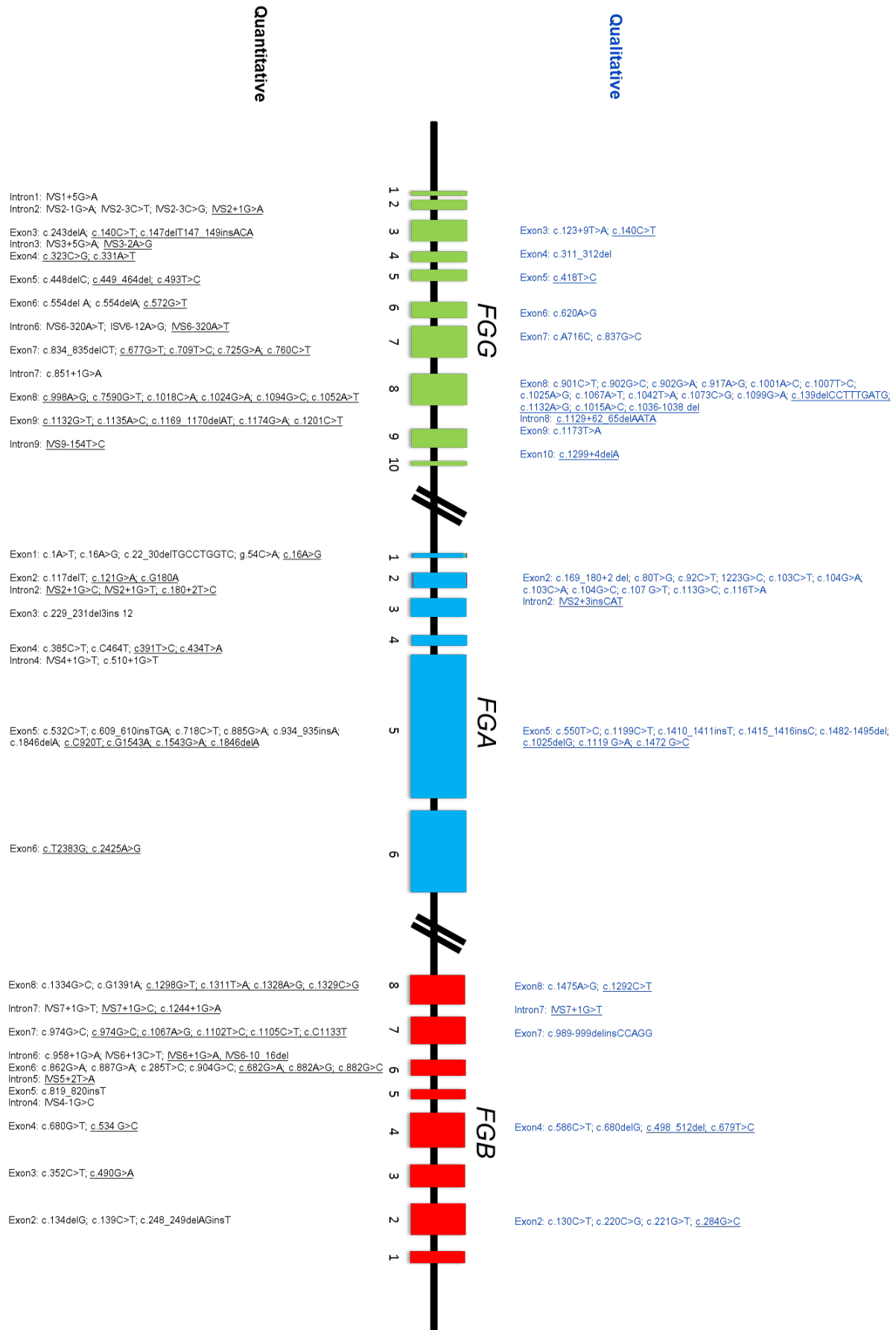


Figure 11. *Fibrinogen gene mutations accounting for afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia and hypodysfibrinogenemia. In black quantitative: afibrinogenemia (normal font), hypofibrinogenemia, in blue: qualitative: dysfibrinogenemia (normal font), hypodysfibrinogenemia (underlined). References 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 $\gamma A/\gamma'$ has been shown to be an etiologic risk factor for CVDs. Several studies have associated elevated circulating levels of the $\gamma A/\gamma'$ fibrinogen isoform with coronary artery disease [210] [206], myocardial infarction [211], and

ischemic stroke [212]. Furthermore, the abnormal fibrin clot structure formed from the $\gamma A/\gamma'$ 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 A α E 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 A α 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* ^{γ Δ5}, was generated 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* ^{γ Δ319,320} 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 *Fib $\gamma^{390-396A}$* 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
<u>Coagulation</u>			
<i>Fib</i> ^{-/-}	Afibrinogenemia	Loss of clotting and platelet aggregation, spontaneous hemorrhagic episodes, absorptions, wound healing defects	[223] [224]
<i>Fib</i> ^{AEK}	Mice with fibrinogen form that cannot support fibrin formation	Bleeding complications	[225]
<i>Fbg</i> ^{Δ5}	Mice with fibrinogen without α _{IIb} β ₃ binding motif in γ-chain	Impaired platelet aggregation and increased bleeding time	[226]
<i>Fbg</i> ^{Δ319,320}	Dysfibrinogenemia mutation Fibrinogen Vlissingen/Frankfurt IV	Fibrinogen molecule defective in calcium binding, fibrin polymerization and platelet aggregation	[227] [228]
<i>Fibrinogen transgene (FT) mice</i>	Hyperfibrinogenemia	Normal phenotype compared to wild type mice	[229]
<u>Inflammation</u>			
<i>Fib</i> ^{-/-}	Pulmonary fibrosis	Increase in neutrophil infiltration at early days during inflammation	[234].
	Host defense	Compromised pathogen clearance after microbial infection	[235]
<i>Fib</i> ^{AEK}	Mice with fibrinogen form that cannot support fibrin formation	Compromised pathogen clearance after microbial infection	[225]
<i>Fib</i> ^{390-396A}	Mice with fibrinogen without α _M β 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.

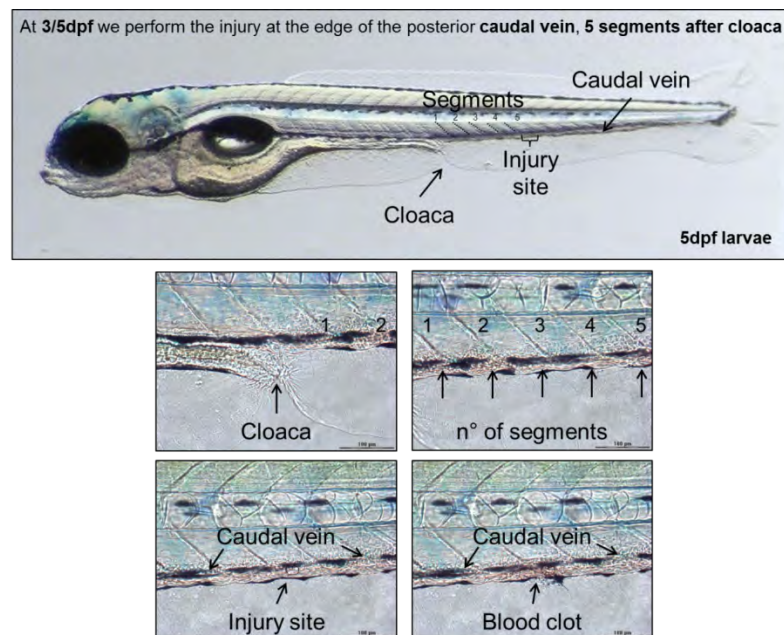


Figure 12. Laser induced vessel injury in zebrafish larvae. 5 days post fertilization (dpf) larvae bright field images (20X) taken from the microscope Leica Laser Microdissection (LMD). Larvae cloaca, n° of segments, caudal vein, site of injury and blood clot formation images are shown.

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 A α 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*^{+/ Δ 19-56}) 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*^{+/ Δ 19-56}) 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 A α thrombin cleavage site which prevents normal fibrin formation, as seen in dysfibrinogenemic patients with the A α 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. Freire¹, R.J. Fish¹, Corinne Di Sanza¹, Marguerite Neerman-Arbez¹

¹Department 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 Freire¹, Richard J. Fish¹, Corinne Di Sanza¹, Marguerite Neerman-Arbez¹

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 A α 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 A α R28C, equivalent to the human R35C dysfibrinogenemia mutation, we detected in-frame skipping of exon 2 in the *fga* mRNA, thereby encoding A α ^{Δ 19-56}. This mutation is similar to Fibrinogen Montpellier II which causes hypodysfibrinogenemia. A α ^{+/ Δ 19-56} 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. A α R28C expression had similar dominant effects on TTO and thrombocyte activity as A α ^{+/ Δ 19-56}. We conclude that thrombosis assays in larval

zebrafish can distinguish between quantitative and qualitative 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, $(\alpha\beta\gamma)_2$, 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 (A α R28C in zebrafish), we detected *fga* transcripts with exon 2 skipping and an mRNA encoding an A α chain lacking amino acids 19 to 56 (A α ^{Δ 19-56}). This resembles fibrinogen Montpellier II, found in hypodysfibrinogenemia. Venous thrombosis occurred almost normally in A α ^{+/ Δ 19-56} larvae but thrombocyte accumulation at laser injury sites was reduced, a dominant effect of the mutation. With no possibility for A α chain fibrinopeptide A cleavage, A α ^{Δ 19-56/ Δ 19-56} 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 A α R28C cDNA. Fibrinogen with the R28C mutation gave a similar thrombotic phenotype to A α ^{Δ 19-56}, 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 μ S 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 α ^{Δ 19-56}, 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 A α R35C mutation prevents FpA cleavage by thrombin. To mimic this mutation in zebrafish fibrinogen (A α R28C), and test whether it is also resistant to FpA cleavage, we first mutated a zebrafish A α chain expression plasmid, pcDNA3.1-ZF-A α , 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 A α plasmids, were co-transfected into HEK-293T cells in 10 cm cell culture dishes with plasmids for the expression of zebrafish fibrinogen B β 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 A α or B β antibodies, as described previously [25].

Generation of zebrafish producing fibrinogen A α ^{Δ 19-56}

We aimed to produce a zebrafish line expressing fibrinogen A α 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 MgSO₄, 0.6 mM Ca(NO₃)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 A α 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
 CTTGTGCCTTGAAGCCGTGCTCAATAGGACGAGCGCCGCAAGGGTTAACCACG
 GTATCCTCCTCGGCCTAAAAAGTAAGTACTTTATAAC3'

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 Aα^{Δ19-56}.

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 A α E or A α E R28C, injection mixes included 25 ng of pTol2ubi-A α E, pTol2ubi-A α E R28C and approximately 35 ng of 5'capped, in vitro-polyadenylated Tol2 transposase mRNA. Where noted injection included 12.5 ng pTol2ubi-A α E or a mixture of 12.5 ng pTol2ubi-A α E + 12.5 ng pTol2ubi-A α E R28C.

Genotyping

Mutations were detected by PCR-genotyping of adult tail fin clips or embryo lysates using *fga* oligonucleotides for PCR (*fgaset1*F, 5'AATGGCCTATGTTGGCAGAC3' and *fgaset1*R, 5'CAGTGGTTATCAGCTGACAG3') and restriction digests with BceAI for the previously-described *fga* mutants [25] or HpaI to detect A $\alpha^{\Delta 19-56}$.

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 $\alpha^{\Delta 19-56}$ (*fga*^{+/+}, *fga*^{+/ $\Delta 19-56$} , *fga* ^{$\Delta 19-56$ / $\Delta 19-56$}), as described previously [25], a protocol adapted from [39]. Reduced sample preparations of dilute plasma were subjected to western blotting with anti-zebrafish fibrinogen A α 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*^{+/-} Δ^{19-56} , *fga* ^{$\Delta^{19-56}/\Delta^{19-56}$} 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 A α chain R35C amino acid change. A α R35 (R16 in the mature protein after signal peptide cleavage) is part of

the A α chain thrombin cleavage site. When present with the non-mutated A α chain, A α 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 A α R35C. This corresponds to R28C in the zebrafish A α (Figure 3A). Thrombin catalyzed fibrinopeptide A (FpA) release does not occur in the human A α R35C chain. To verify that zebrafish A α R28C also prevents thrombin cleavage, we transfected HEK-293T cells with plasmids for expression of wild-type or A α 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 A α and B β chain antibodies, respectively. Size shifts were seen in the zebrafish A α and B β chain with thrombin treatment but not in the A α 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 A α 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 A α and A α E 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 A α R28C, the predicted A α chain translated from this exon 2-skipped transcript lacks amino acids 19 to 56 but retains the natural open reading frame, encoding A α Δ 19-56.

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 ESEs in the mutated sequence (Supplementary Figure 3) providing a possible source for changes in *fga* mRNA splicing.

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

⁵⁶ plasma but, when compared with ceruloplasmin, may be at lower levels compared to *fga*^{+/+} and *fga*^{+/Δ19-56} (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*^{Δ19-56/Δ19-56} 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*^{+/Δ19-56} and *fga*^{Δ19-56/Δ19-56} 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 (A α E) in larvae (CF, RJF, MNA, submitted). All *fga*^{+/ Δ 19-56} 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*^{+/ Δ 19-56} (n=42) gave a *P*-value of 0.361. No occlusion was measured in 11 of 16 *fga* ^{Δ 19-56/ Δ 19-56} larvae. The mean TTO in the remaining 5 *fga* ^{Δ 19-56/ Δ 19-56} larvae that supported occlusion was 77 sec. t-tests comparing the TTO from this sub-group of 5 *fga* ^{Δ 19-56/ Δ 19-56} larvae and the *fga*^{+/+} or *fga*^{+/ Δ 19-56} larvae gave *P*-values of <0.0001 for both analyses. We conclude that the presence of a single *fga*^{+/ Δ 19-56} allele prolongs TTO, compared to wild-type, but homozygous *fga* ^{Δ 19-56/ Δ 19-56} 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* ^{Δ 19-56/ Δ 19-56} 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* ^{Δ 19-56/ Δ 19-56} larvae, compared to *fga*^{+/+} controls. When occlusion occurred in *fga* ^{Δ 19-56/ Δ 19-56} 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* ^{Δ 19-56/ Δ 19-56}). Even in *fga* ^{Δ 19-56/ Δ 19-56} 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*^{+/ Δ 19-56} and *fga* ^{Δ 19-56/ Δ 19-56} 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 $fga^{\Delta 19-56/\Delta 19-56}$ 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 $fga^{+/+}$, $fga^{+/\Delta 19-56}$ heterozygotes gave an intermediate thrombocyte binding and accumulation phenotype, with the fluorescence curve measured over time clearly intermediate between $fga^{+/+}$ and $fga^{+/\Delta 19-56}$ (Figure 6A). This demonstrates a dominant effect on thrombocyte activity for the mutated allele in $fga^{+/\Delta 19-56}$. Examples for fluorescent thrombocyte activity in $fga^{+/+}$ and $fga^{\Delta 19-56/\Delta 19-56}$ 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 A α E chain cDNA or A α E R28C. The MO cannot target the cDNA sequence. The A α E 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 A α E 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 A α E R28C permitted occlusion in 16/22 larvae but the TTO was prolonged compared to non-injected or A α E-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 A α E and A α E R28C plasmids. Venous occlusion was measured in 20/21 larvae, with shorter TTO than A α E R28C alone, but markedly prolonged TTO compared to A α E alone (mean TTO 62 sec, n=20). To control for the half quantity of A α E plasmid injected in the A α E + A α E R28C condition we also injected $\frac{1}{2}$ quantity of A α E plasmid with the MO and measured TTO at 3dpf. All larvae supported occlusion with a mean TTO considerably shorter than the A α E + A α E R28C mix and marginally longer than the initial MO + A α E condition. This demonstrates the dominant effect of A α E R28C expression, seen when comparing the TTO in A α E + A α E R28C versus A α E alone, is not due to lower quantities of injected A α E plasmid in the mixed condition.

To monitor the effect of the fibrinogen A α E R28C mutation on laser-induced thrombocyte adhesion and aggregation we used transgenic expression of A α E and A α E 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 A α E 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 A α E R28C, or a mix of A α E + A α E R28C to mimic a heterozygous state, were largely similar to non-injected *fga*^{-/-} controls, demonstrating a failure of

A α E R28C to complement fibrinogen deficiency and a dominant effect of A α E R28C in the mixed condition. Injecting half the quantity of A α E to control for the amount of A α E in the A α E + A α E R28C condition gave a similar result to A α E, reiterating the dominant effect of A α E 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*^{+/ Δ 19-56}), 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*^{+/ Δ 19-56}) lack the A α 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*^{+/ Δ 19-56}, compared to *fga*^{+/+} controls, and the longer TTO measured in *fga*-targeted morpholino knock-down larvae with A α E + A α E R28C expression, compared to A α E 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* ^{Δ 19-56/ Δ 19-56} or expression of A α E 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.

References

1. Neerman-Arbez, M., P. de Moerloose, and A. Casini, *Laboratory and Genetic Investigation of Mutations Accounting for Congenital Fibrinogen Disorders*. Semin Thromb Hemost, 2016. **42**(4): p. 356-65.
2. Casini, A. and P. de Moerloose, *Can the phenotype of inherited fibrinogen disorders be predicted?* Haemophilia, 2016. **22**(5): p. 667-75.
3. Casini, A., et al., *Diagnosis and classification of congenital fibrinogen disorders: communication from the SSC of the ISTH*. J Thromb Haemost, 2018. **16**(9): p. 1887-1890.
4. Neerman-Arbez, M., *Molecular basis of fibrinogen deficiency*. Pathophysiol Haemost Thromb, 2006. **35**(1-2): p. 187-98.
5. Neerman-Arbez, M. and P. de Moerloose, *Mutations in the fibrinogen gene cluster accounting for congenital afibrinogenemia: an update and report of 10 novel mutations*. Hum Mutat, 2007. **28**(6): p. 540-53.
6. Casini, A., et al., *Dysfibrinogenemia: from molecular anomalies to clinical manifestations and management*. J Thromb Haemost, 2015. **13**(6): p. 909-19.
7. Peyvandi, F., *Epidemiology and treatment of congenital fibrinogen deficiency*. Thromb Res, 2012. **130 Suppl 2**: p. S7-11.
8. Korte, W., et al., *Thrombosis in Inherited Fibrinogen Disorders*. Transfus Med Hemother, 2017. **44**(2): p. 70-76.
9. Vorjohann, S., et al., *Hypodysfibrinogenaemia due to production of mutant fibrinogen alpha-chains lacking fibrinopeptide A and polymerisation knob 'A'*. Thromb Haemost, 2010. **104**(5): p. 990-7.
10. Ariens, R.A., *Fibrin(ogen) and thrombotic disease*. J Thromb Haemost, 2013. **11 Suppl 1**: p. 294-305.
11. de Moerloose, P., A. Casini, and M. Neerman-Arbez, *Congenital fibrinogen disorders: an update*. Semin Thromb Hemost, 2013. **39**(6): p. 585-95.
12. Mosesson, M.W., *Update on antithrombin I (fibrin)*. Thromb Haemost, 2007. **98**(1): p. 105-8.
13. de Bosch, N.B., et al., *Inhibition of thrombin generation in plasma by fibrin formation (Antithrombin I)*. Thromb Haemost, 2002. **88**(2): p. 253-8.
14. Dupuy, E., et al., *Embolized ischemic lesions of toes in an afibrinogenemic patient: possible relevance to in vivo circulating thrombin*. Thromb Res, 2001. **102**(3): p. 211-9.
15. Liu, C.Y., J.A. Koehn, and F.J. Morgan, *Characterization of fibrinogen New York 1. A dysfunctional fibrinogen with a deletion of B beta(9-72) corresponding exactly to exon 2 of the gene*. J Biol Chem, 1985. **260**(7): p. 4390-6.
16. De Marco, L., et al., *von Willebrand factor interaction with the glycoprotein IIb/IIIa complex. Its role in platelet function as demonstrated in patients with congenital afibrinogenemia*. J Clin Invest, 1986. **77**(4): p. 1272-7.

17. Endenburg, S.C., et al., *Plasma fibrinogen inhibits platelets adhesion in flowing blood to immobilized fibrinogen*. *Arterioscler Thromb Vasc Biol*, 1996. **16**(5): p. 633-8.
18. Koopman, J., et al., *Molecular basis for fibrinogen Dusart (A alpha 554 Arg-->Cys) and its association with abnormal fibrin polymerization and thrombophilia*. *J Clin Invest*, 1993. **91**(4): p. 1637-43.
19. Marchi, R., et al., *Fibrinogen Caracas V, an abnormal fibrinogen with an Aalpha 532 Ser-->Cys substitution associated with thrombosis*. *Thromb Haemost*, 2000. **84**(2): p. 263-70.
20. Lozier, J.N. and T.C. Nichols, *Animal models of hemophilia and related bleeding disorders*. *Semin Hematol*, 2013. **50**(2): p. 175-84.
21. Jagadeeswaran, P., et al., *Animal Models of Thrombosis From Zebrafish to Nonhuman Primates: Use in the Elucidation of New Pathologic Pathways and the Development of Antithrombotic Drugs*. *Circ Res*, 2016. **118**(9): p. 1363-79.
22. Jagadeeswaran, P. and J.P. Sheehan, *Analysis of blood coagulation in the zebrafish*. *Blood Cells Mol Dis*, 1999. **25**(3-4): p. 239-49.
23. Jagadeeswaran, P. and Y.C. Liu, *A hemophilia model in zebrafish: analysis of hemostasis*. *Blood Cells Mol Dis*, 1997. **23**(1): p. 52-7.
24. Weyand, A.C. and J.A. Shavit, *Zebrafish as a model system for the study of hemostasis and thrombosis*. *Curr Opin Hematol*, 2014. **21**(5): p. 418-22.
25. Fish, R.J., C. Di Sanza, and M. Neerman-Arbez, *Targeted mutation of zebrafish fga models human congenital afibrinogenemia*. *Blood*, 2014. **123**(14): p. 2278-81.
26. Grzegorski, S.J., et al., *Disruption of the kringle 1 domain of prothrombin leads to late onset mortality in zebrafish*. *Sci Rep*, 2020. **10**(1): p. 4049.
27. Hu, Z., et al., *Loss of fibrinogen in zebrafish results in an asymptomatic embryonic hemostatic defect and synthetic lethality with thrombocytopenia*. *J Thromb Haemost*, 2019. **17**(4): p. 607-617.
28. Hu, Z., et al., *Genome editing of factor X in zebrafish reveals unexpected tolerance of severe defects in the common pathway*. *Blood*, 2017. **130**(5): p. 666-676.
29. Iyer, N., et al., *Knockout of von Willebrand factor in Zebrafish by CRISPR/Cas9 mutagenesis*. *Br J Haematol*, 2019. **186**(4): p. e76-e80.
30. Liu, Y., et al., *Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function*. *Blood*, 2014. **124**(1): p. 142-50.
31. Weyand, A.C., et al., *Analysis of factor V in zebrafish demonstrates minimal levels needed for early hemostasis*. *Blood Adv*, 2019. **3**(11): p. 1670-1680.
32. Jagadeeswaran, P., et al., *Identification and characterization of zebrafish thrombocytes*. *Br J Haematol*, 1999. **107**(4): p. 731-8.
33. Khandekar, G., S. Kim, and P. Jagadeeswaran, *Zebrafish thrombocytes: functions and origins*. *Adv Hematol*, 2012. **2012**: p. 857058.

34. Gregory, M., R. Hanumanthaiah, and P. Jagadeeswaran, *Genetic analysis of hemostasis and thrombosis using vascular occlusion*. Blood Cells Mol Dis, 2002. **29**(3): p. 286-95.
35. Hwang, W.Y., et al., *Efficient genome editing in zebrafish using a CRISPR-Cas system*. Nat Biotechnol, 2013. **31**(3): p. 227-9.
36. Mosimann, C., et al., *Ubiquitous transgene expression and Cre-based recombination driven by the ubiquitin promoter in zebrafish*. Development, 2011. **138**(1): p. 169-77.
37. Ablain, J., et al., *A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish*. Dev Cell, 2015. **32**(6): p. 756-64.
38. Kwan, K.M., et al., *The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs*. Dev Dyn, 2007. **236**(11): p. 3088-99.
39. Babaei, F., et al., *Novel blood collection method allows plasma proteome analysis from single zebrafish*. J Proteome Res, 2013. **12**(4): p. 1580-90.
40. Jagadeeswaran, P., et al., *Laser-induced thrombosis in zebrafish*. Methods Cell Biol, 2011. **101**: p. 197-203.
41. Hanss, M. and F. Biot, *A database for human fibrinogen variants*. Ann N Y Acad Sci, 2001. **936**: p. 89-90.
42. Flood, V.H., et al., *Incorporation of fibrin molecules containing fibrinopeptide A alters clot ultrastructure and decreases permeability*. Br J Haematol, 2007. **138**(1): p. 117-24.
43. Okumura, N., et al., *B:b interactions are essential for polymerization of variant fibrinogens with impaired holes 'a'*. J Thromb Haemost, 2007. **5**(12): p. 2352-9.
44. Flood, V.H., H.A. Al-Mondhiry, and D.H. Farrell, *The fibrinogen Aalpha R16C mutation results in fibrinolytic resistance*. Br J Haematol, 2006. **134**(2): p. 220-6.

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 A α chains. A α R35 and A α 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 nucleotides 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^{+/Δ19-56}* and *fga^{Δ19-56/Δ19-56}* 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^{Δ19-56}*, 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^{+/Δ19-56}*, and *fga^{Δ19-56/Δ19-56}* 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^{Δ19-56/Δ19-56}* 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^{+/Δ19-56}* and *fga^{Δ19-56/Δ19-56}* 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^{Δ19-56/Δ19-56}* 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 A α E, A α E R28C, A α E + A α E R28C or a half quantity of A α E (1/2 A α E). 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 A α E and A α E 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 A α E or A α E 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 A α E or A α E R28C, A α E + A α E R28C or a half quantity of A α E (A α E 1/2). 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

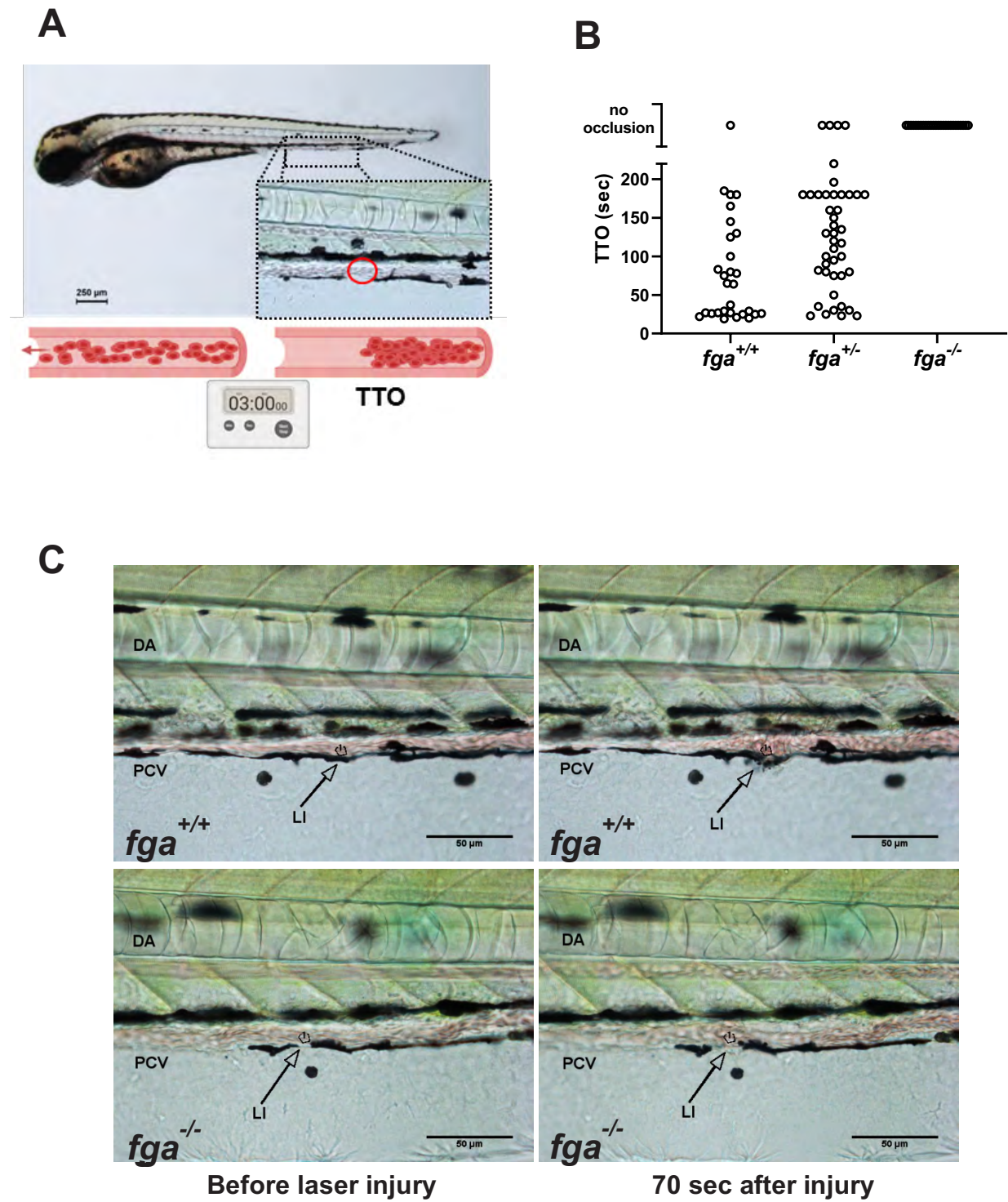


Figure 2

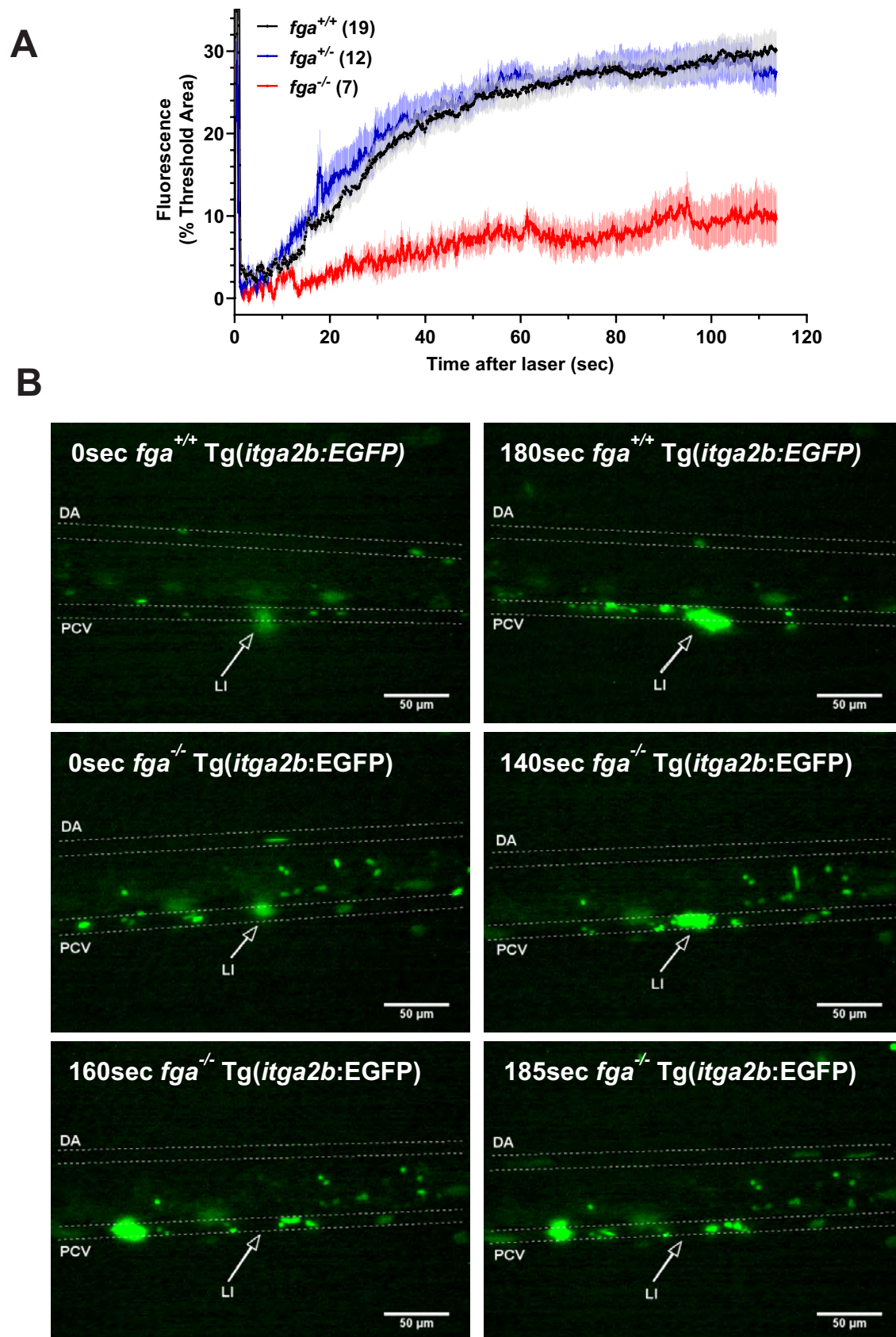


Figure 3

A

Fibrinogen A α

Human	MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGV	35	RGPRVVERHQSACKDSDWPFCSDWDWN...
Zebrafish	MKFTHSLLCLFVAVSSALAEEDTVVNP	28	RGARPIEHGFKAQDTCQTKWPGCTDDDWDG...

B

sgRNA **GACACAGUGGUGAACCCUAG...**

fga
exon 2 ...GCCGAGGAGGACACAGTGGTGAACCCTAG**AGG**CGCTCGTCCT

ssODN ...GGATACCGTGGTTAACCCCT**TG**CGG...

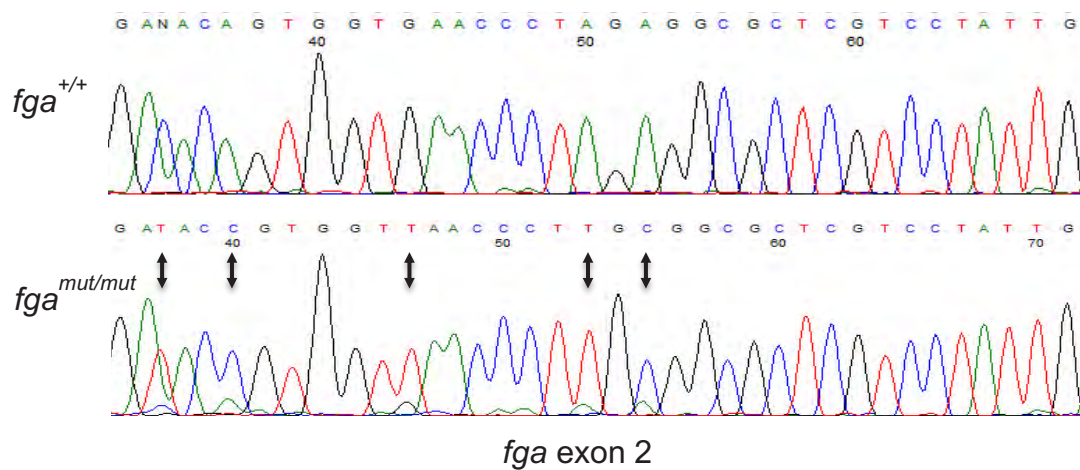
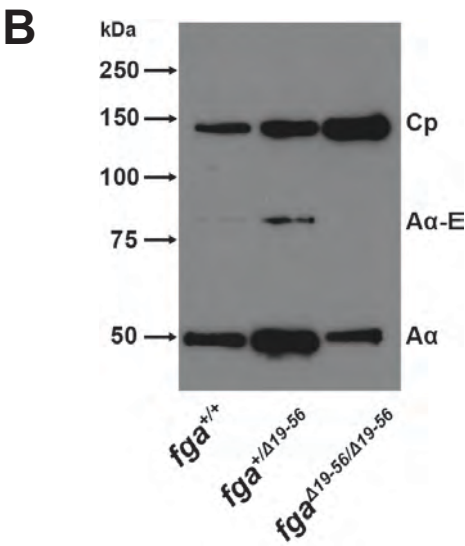
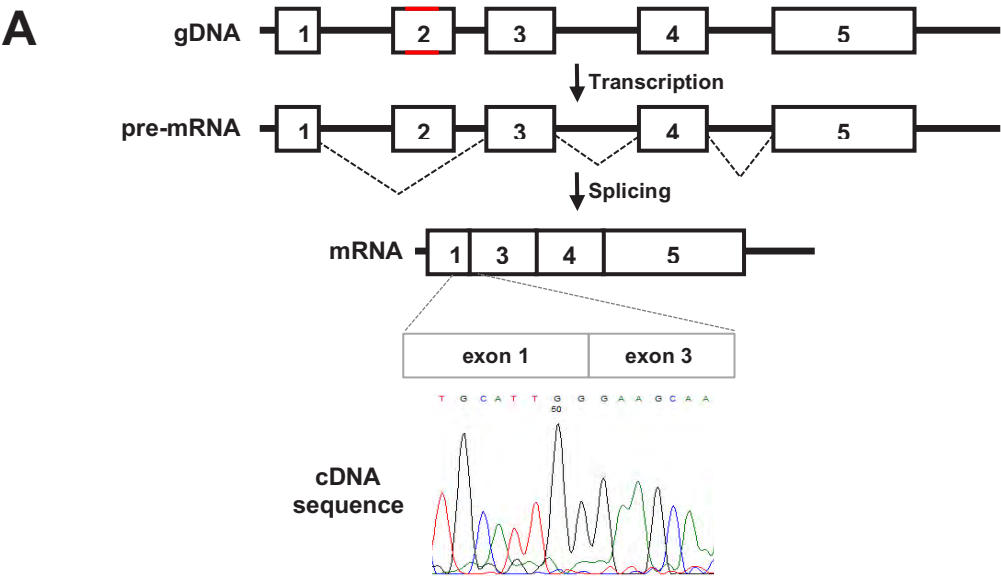
C

Figure 4



C

Human Aα^{Δ19-60} MFSMRIVCLVLSVVGTAWNYKCPSGCRMKGLIDEVNQDFTNRINKLKNLSLFYQKNNKDSHS...

Zebrafish Aα^{Δ19-56} MKFTHSLCLFVAVSSALGSKCPSGCRVQGLMDKADHDLLKKIEKIRRLLEGGQKLYRSAD...

Figure 5

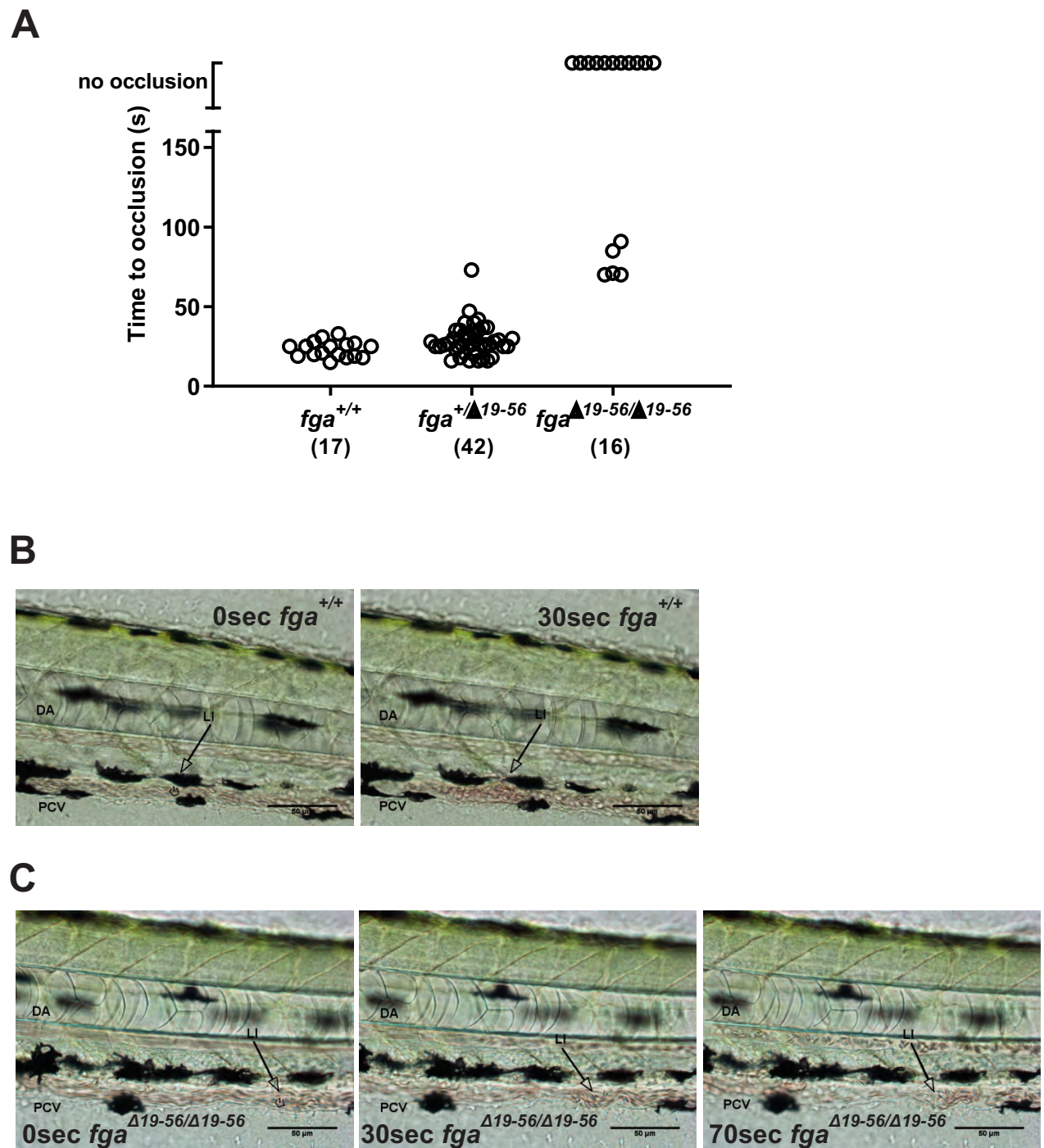


Figure 6

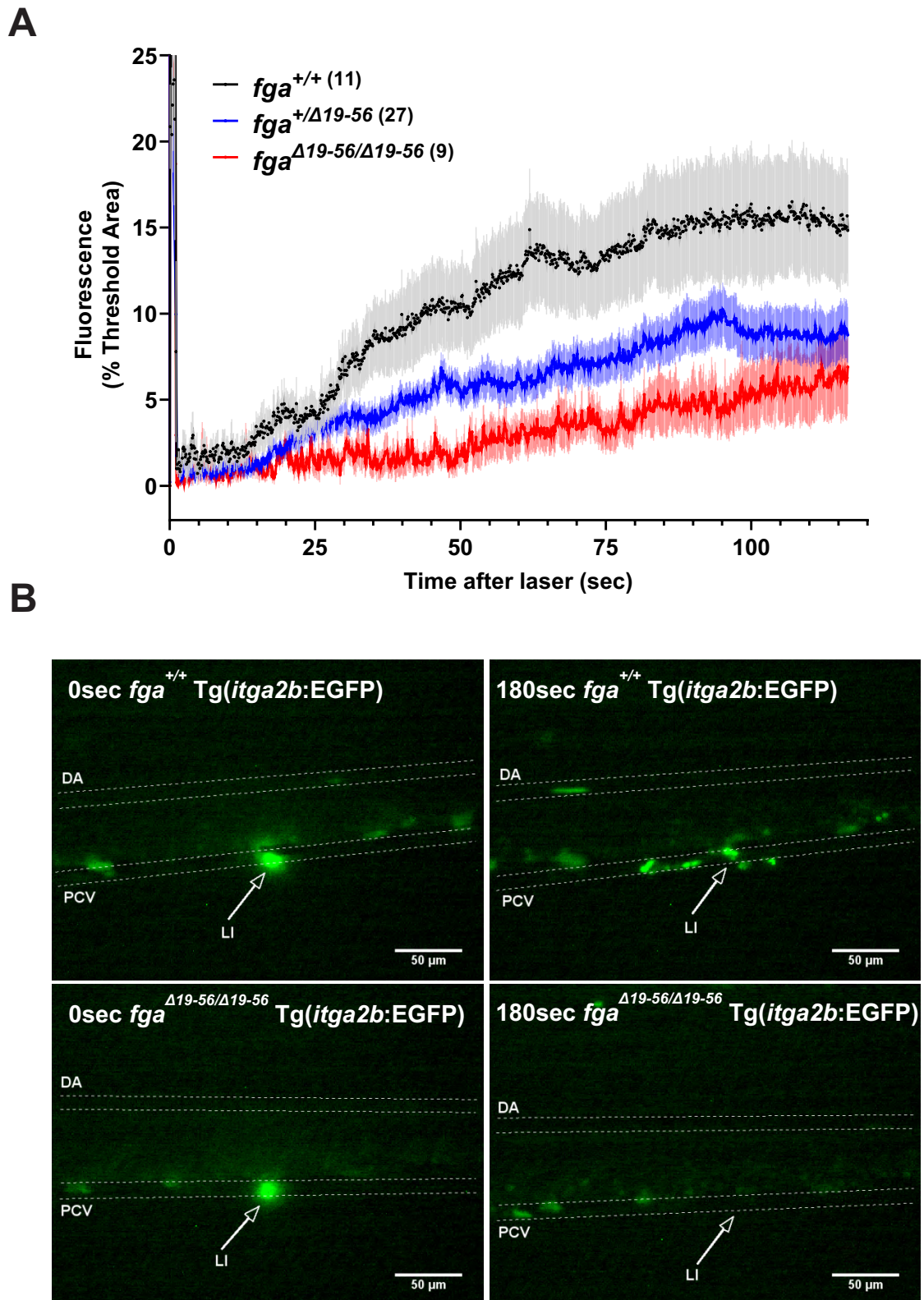
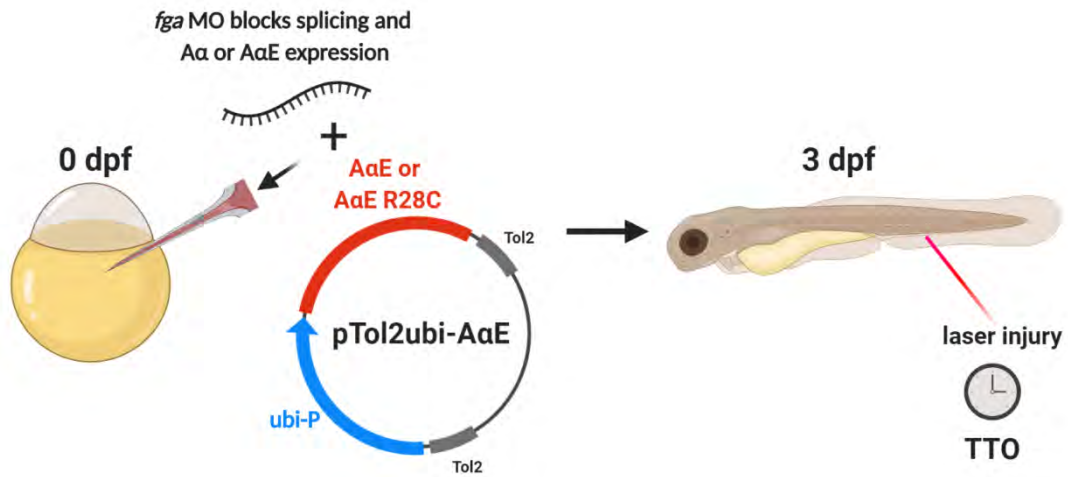


Figure 7

A



B

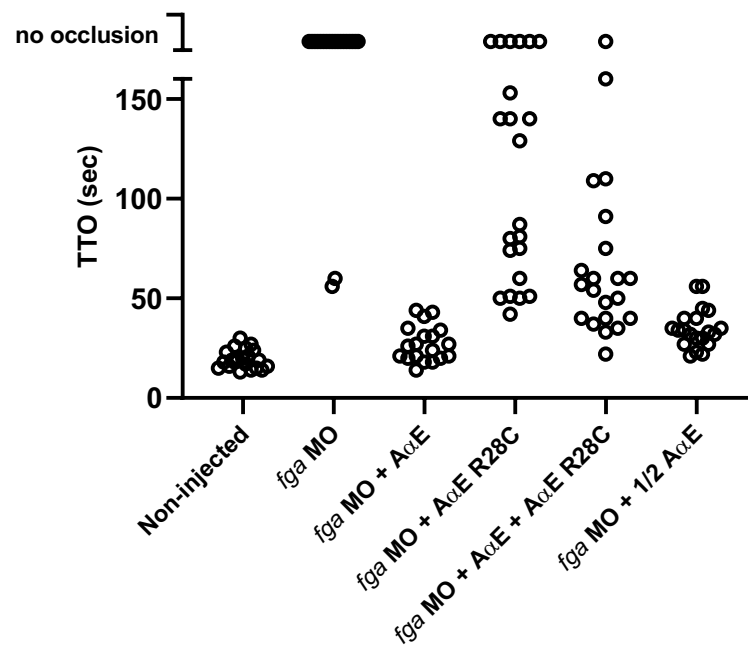
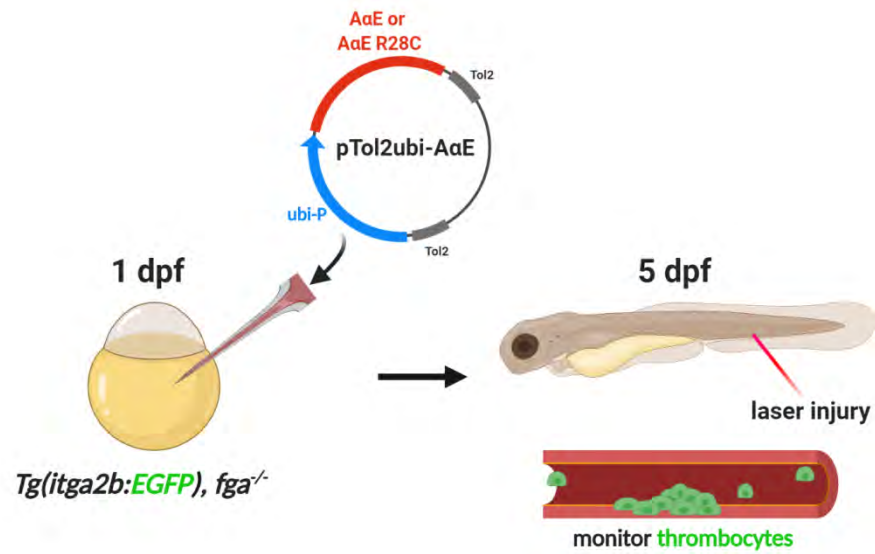
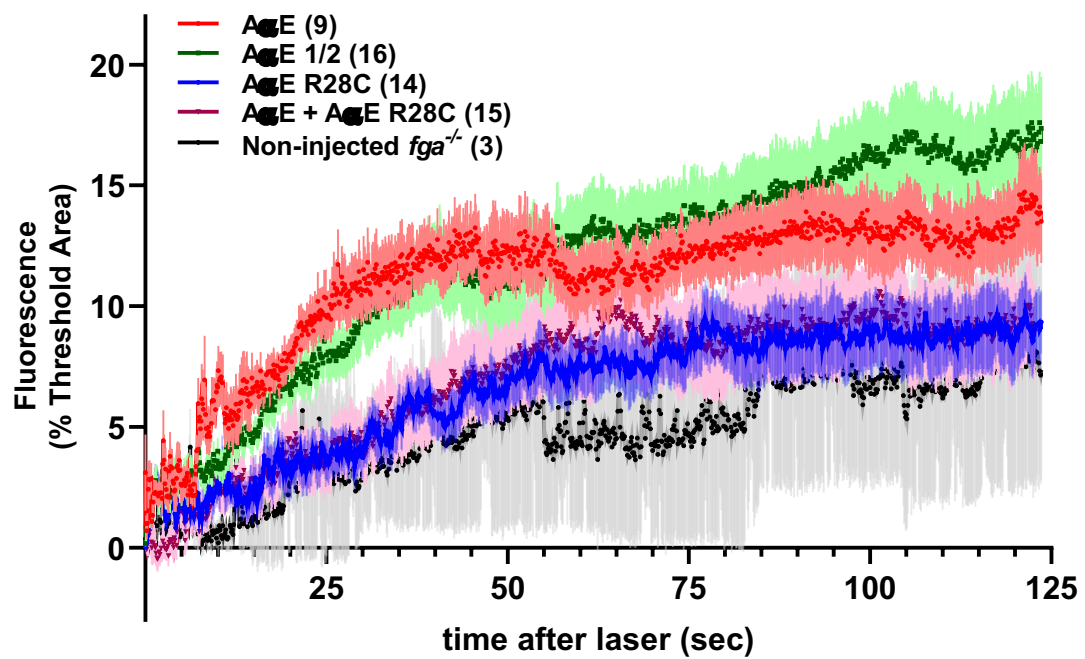


Figure 8

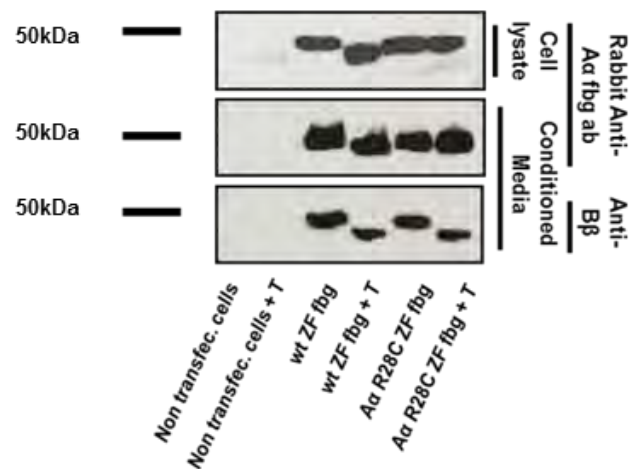
A



B

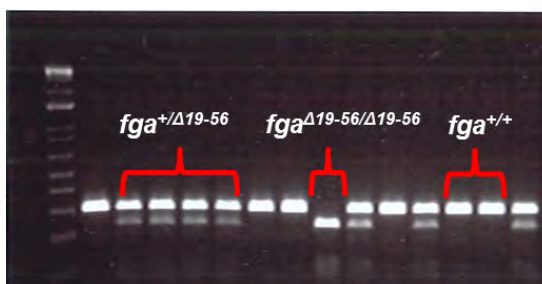


Supplementary Data

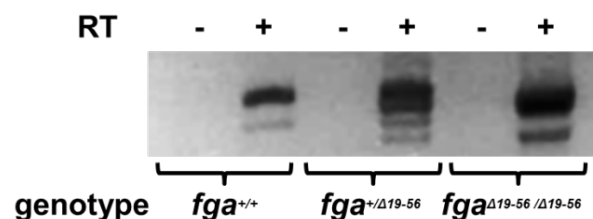


Supplementary Figure 1 –Thrombin cleavage of zebrafish fibrinogen and fibrinogen Aα R28C Immunoblot of zebrafish fibrinogen in cell lysates and conditioned media from transfected HEK-293T cells with and without thrombin treatment. Anti-Aα and anti-Bβ fibrinogen antibodies are used. Controls were non-transfected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

A



B

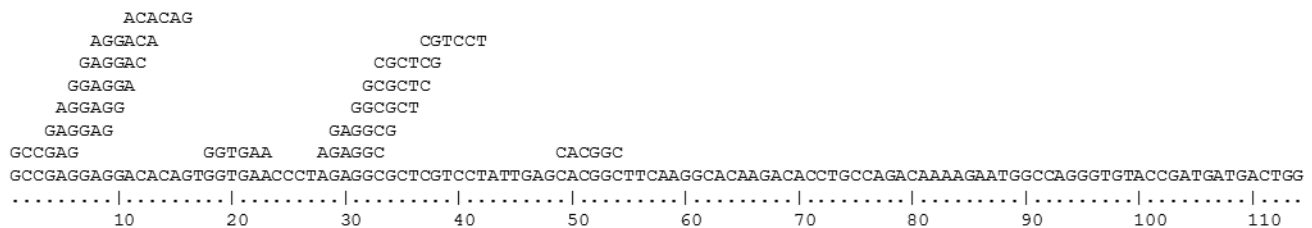


Supplementary Figure 2 – *fga*Δ19-56 genotyping and RT-PCR from liver samples (A) PCR-genotyping individual fish using the restriction enzyme HpaI. After HpaI cut, *fga*^{+/+}: 301 nt; *fga*^{+/Δ19-56}: 301 nt + 230 nt + 71 nt bands and *fga*^{Δ19-56/Δ19-56}: 230 nt + 71 nt bands. **(B)** RT-PCR of RNA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

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

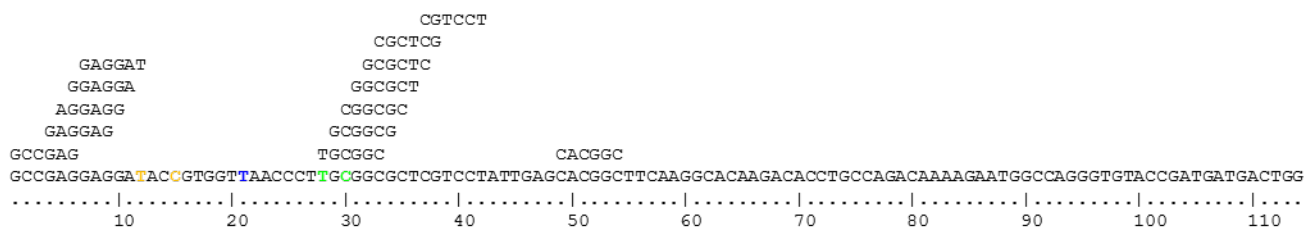
A

***fga* exon 2 – Predicted ESEs:**



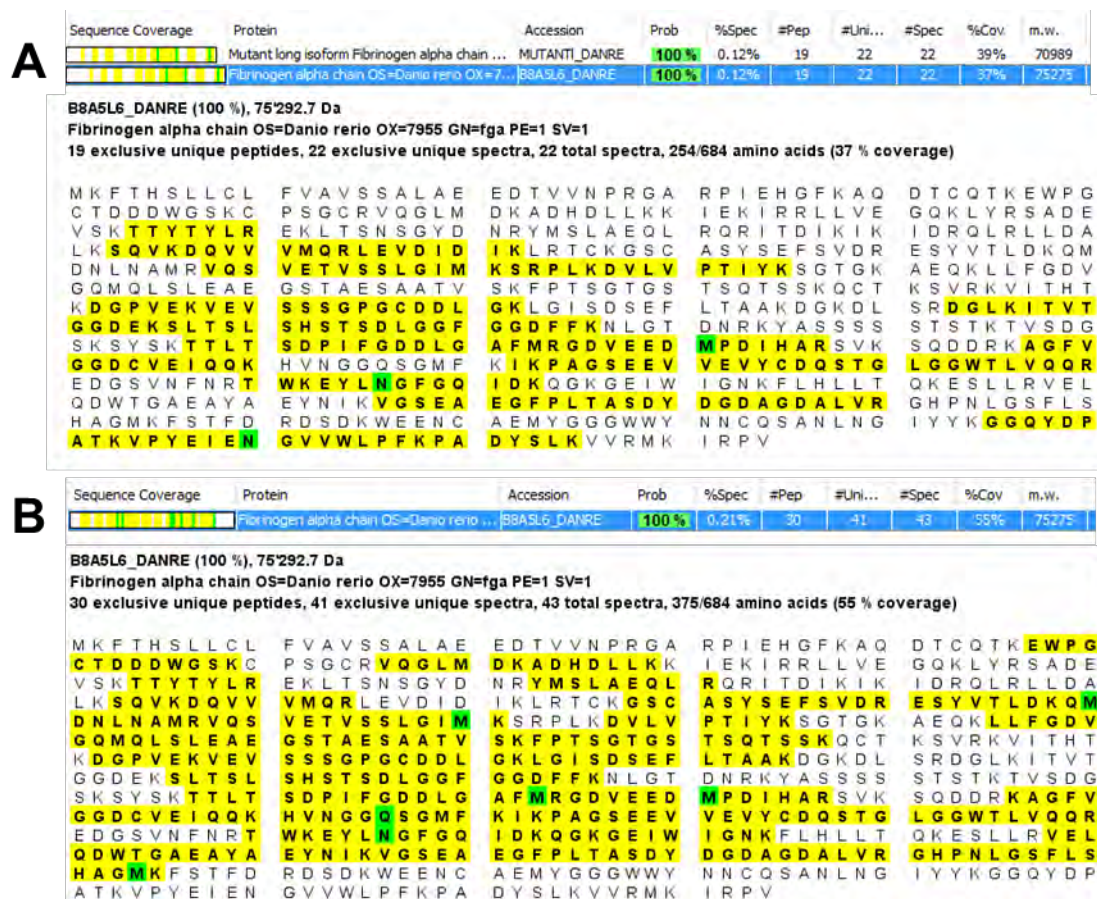
B

Edited *fga* exon 2 – Predicted ESEs:



Supplementary Figure 3 –Exon Splicing Enhancer Sequence prediction (ESES)

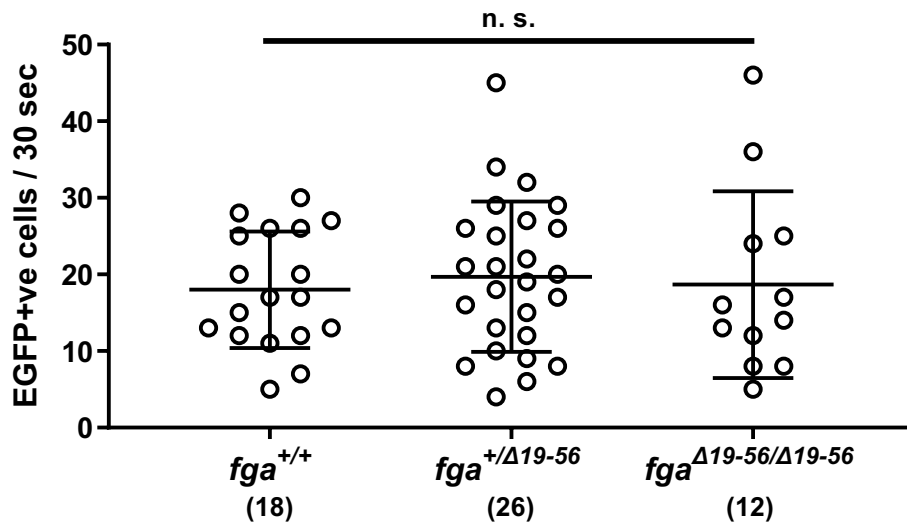
in *fga* exon 2 from wild-type and genome edited. A splicing element annotation tool (RESCUE-ESE Web Server - genes.mit.edu) was used to predict the Exonic Splicing Enhancer (ESE) sequences in *fga* exon 2 and in *fga* exon 2 with targeted genome editing. In *fga* exon 2, 15 ESE sequences were predicted. 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*^{+/Δ19-56} and *fga*^{Δ19-56/Δ19-56} larvae at 5dpf. Unpaired t-test: *fga*^{+/Δ19-56} vs. *fga*^{+/+} *P*= 0.5422; *fga*^{Δ19-56/Δ19-56} vs. *fga*^{+/+} *P*= 0.8546; *fga*^{+/Δ19-56} vs *Δ19-56* *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 A α E chain in early hemostasis

Abstract

Fibrinogen is composed of two copies of three types of chains: A α , B β 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 A α -chain (accounting for the 90% of the transcripts), and the extended A α E isoform which (accounting for the 1–2% of the transcripts). The A α E chain contains an extra C-terminal globular domain homologous to the C-terminal globular domain of fibrinogen B β and γ chains by 40%.

In a subclass of fibrinogen molecules, referred to as Fibrinogen 420 (Fib420), each of the two A α -chains is replaced by an A α E 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 A α E containing fibrinogen at larval stages.

We aimed to study the role of the A α E 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 A α E isoform due to a mutation in *fga* exon 6. We questioned whether the lack of A α E isoform delayed the TTO in the AB strain larvae. Our results showed that the A α E isoform rescued venous occlusion in fibrinogen deficient mutants or *fga* morpholino knock-down larvae, while the common A α -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 A α -chain or the A α E isoform. However, we did observe differences in erythrocyte recruitment at the injury site in larvae expressing the A α -chain or A α E isoform when studying venous thrombosis at 3dpf.

These observations demonstrate the role of A α E isoform on venous thrombosis during developmental hemostasis, the different contribution of the two isoforms (A α and A α E) on thrombosis (which seems to be linked to the availability of different cell types for clotting), and that the mutation that prevents A α E 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 A α -chains are replaced by A α E isoforms. The A α E isoforms differs from the common A α chains in the presence of an extra C-terminal globular domain, which shares homology with the C-terminal domain of B β 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 A α E 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 A α E 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 A α E chain in early hemostasis*

Cristina Freire^{*1}, Richard J. Fish^{*1}, Rui Vilar¹, Corinne Di Sanza¹, Steven J. Grzegorski², Catherine E. Richter², Jordan A. Shavit² and Marguerite Neerman-Arbez¹

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

²Division 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

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

Cristina Freire^{*1}, Richard J. Fish^{*1}, Rui Vilar¹, Corinne Di Sanza¹, Steven J. Grzegorski², Catherine E. Richter², Jordan A. Shavit² and Marguerite Neerman-Arbez¹

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

²Division 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 A α E

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 (A α E) is a genetic modifier of venous thrombosis in zebrafish.
- A functional role for the conserved A α E chain in early hemostasis is revealed.

Abstract

Plasma fibrinogen molecules comprise two copies of A α , B β and γ chains folded into a hexameric protein. A minor fibrinogen isoform with an extended A α chain (A α E) is more abundant in newborn human blood than in adults. Larval zebrafish produce predominantly A α E-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 A α E, due to a mutation in the A α E-specific coding region of *fga*. We investigated whether the lack of A α E explained the delayed AB TTO. Transgenic expression of A α E, but not A α , shortened the AB TTO to that of TU. A α E rescued venous occlusion in fibrinogen mutants or larvae with morpholino-targeted fibrinogen alpha chain mRNA, but A α 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 A α or A α E restored similar thrombocyte accumulation at the injury site. Our data demonstrate a genetic modifier of venous thrombosis, a role for fibrinogen A α E 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 $\text{A}\alpha$, $\text{B}\beta$ and γ polypeptides, combining to a molecular weight of about 340 kDa. Variability comes from post-translational modifications² and differential mRNA splicing giving rise to γ' ³ and $\text{A}\alpha\text{E}$ ^{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 XIII⁶ and thrombin.⁷ The $\text{A}\alpha\text{E}$ chain arises from splicing of a sixth exon in the $\text{A}\alpha$ chain mRNA. This encodes a C-terminal globular domain ($\alpha_{\text{E}}\text{C}$) with homology to the $\text{B}\beta$ and γ chain C-termini.⁵ $\text{A}\alpha\text{E}$ 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 $\alpha_{\text{E}}\text{C}$ domain is highly conserved⁸, 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 ago⁵, the role of the $\alpha_{\text{E}}\text{C}$ domain remains enigmatic, at least when compared to the understanding of the mechanistic¹⁰⁻¹² and clinical implications¹³ of the γ/γ' chain isoforms. Studies of the $\alpha_{\text{E}}\text{C}$ structure¹⁴, fibrinogen 420-containing fibrin clots¹⁵ and their dissolution¹⁶, have been reported. One proposed function for $\alpha_{\text{E}}\text{C}$ is as an additional binding site for leukocyte interactions with fibrinogen or fibrin via integrin receptors.¹⁷ The $\alpha_{\text{E}}\text{C}$ domain can be

seen as nodes bulging from fibrin fibers¹⁵, 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 A α and A α E isoforms¹⁸, 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 studies¹⁹ due to the conservation of coagulation proteins²⁰ 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 thrombocytes³², 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 A α E 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 A α E expression, leads to prolonged venous thrombosis times and low fibrinogen production. Without the deletion, TU strain larvae express predominantly A α E-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 A α or A α E highlighted the importance of the A α E 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 A α E chain, and differences in the abilities of fibrinogen with A α or A α E 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 μ S 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-MEM³⁷, 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 v4.3T⁴⁰ 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 SnpSift⁴¹ and custom python and R scripts.

Plasmids

Plasmids for expression of zebrafish fibrinogen A α or A α E under the control of a ubiquitin gene (*ubb*) promoter (subsequently labelled ubi) were prepared by Gateway cloning (Invitrogen). Middle entry clones for A α and A α E 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-A α and pME-A α E. 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:A α and pT2ubi:A α E. Our A α cDNA clone encodes the 456 amino acid zebrafish A α chain, the A α E cDNA the 684 amino acid zebrafish A α E chain. In the shared region of these polypeptides, spanning from methionine 1 to glycine 452, one amino acid differs. Our A α clone has a serine at position 286 and our A α E 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 A α , and

compared to known human mutations, this residue in zebrafish A α or A α E 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 MgSO₄, 0.6 mM Ca(NO₃)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 A α or A α E, injection mixes included 25 ng of pT2ubi:A α or pT2ubi:A α E 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 μ J, 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 $275.25 \mu\text{m}^2$ 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 previously²⁶, 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 work²⁶, 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_E C domain of the extended A α E isoform. The deletion is predicted to frameshift the A α E mRNA. Supporting this, we detected A α but not A α E 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 A α chain antibodies which were raised to an antigen common to A α and A α E. At 3dpf, the fibrinogen alpha chain seen in TU larvae was almost exclusively A α E. In AB larvae, the A α E chain was not detected, only a comparatively low amount of the A α chain. In TU x AB lysates both alpha chains were detected, but predominantly A α E (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 A α E (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 A α E chain is the major fibrinogen alpha chain in larval zebrafish, that crossing TU or TL strains with AB coincides with increased fibrinogen alpha (A α or A α E) and rescue of a delayed TTO phenotype, and the single nucleotide deletion in *fga* could be the recessive modifier of larval venous thrombosis.

Fibrinogen A α E, but not A α , 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 A α E chain. As fibrinogen-deficient zebrafish larvae fail to support laser-induced venous thrombosis²⁷ we reasoned that by re-introducing alpha chain expression in homozygous *fga* mutant embryos we could compare the relative activity of A α and A α E isoforms. Using micro-injection of a Tol2 transposon-based expression system⁴⁶ we expressed A α or A α E cDNA under the control of the ubi promoter⁴², 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 in²⁶). 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 al²⁷ in a similar *fga* mutant. Expression of fibrinogen A α 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 A α E 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).

A α E 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 A α E versus A α 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 A α and A α E chains in hemizygous *Tg(gata1:DsRed)* embryos from a transgenic parent mated with the TU

strain (Figure 4A). The *fga* MO lowers endogenous A α or A α E 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 A α 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 + A α). Expression of the A α E chain rescued occlusion in all *fga* MO + A α E 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 A α E versus A α 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 + A α E-injected larvae, mean fluorescence had not increased at the injury site after *fga* MO-mediated knock-down and barely changed in *fga* MO + A α -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 A α and A α E 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 + A α or *fga* MO + A α E (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 A α E isoform. This leads to low embryonic fibrinogen production and variable larval thrombotic responses that can be effectively rescued by fibrinogen A α E, but not A α , even when A α is expressed at levels resembling the A α E-containing fibrinogen in larvae lacking the deletion.

Fibrinogen A α or A α E 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 A α and A α E 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 A α or A α E 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 A α and A α E expression in *fga*^{-/-} increased thrombocyte accumulation compared to non-injected

fga^{-/-}, with A α E giving faster initial mean thrombocyte binding at the injury site compared to A α , and A α facilitating a slower buildup but larger mean thrombocyte aggregate area, compared to A α E (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 A α E 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, Germany⁴⁸, 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 mice⁴⁹.

The $\alpha_E C$ domain of fibrinogen A α E is conserved across vertebrates⁸, suggesting functional importance. It resembles the C-terminal globular domains of the fibrinogen B β and γ chains, in agreement with their common ancestry^{50,51}, but lacks the equivalent residues found in γC and βC involved in fibrin polymerization¹⁵. The early hemostasis we have studied in zebrafish larvae indicates that A α E is a more effective hemostatic alpha chain. This infers that the A α E chain is better adapted for fibrin formation during early development compared to A α , 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 reported¹⁷, but its importance in vivo is unclear. Our data support the hypothesis that fibrinogen with A α E, rather than A α , is involved in hemostasis when erythrocytes are the principle cellular component, in the near absence of circulating thrombocytes. This could implicate the fibrinogen $\alpha_E C$ 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 $\alpha_V \beta_3$ integrin⁵² and CD47⁵³. For erythrocyte retention in venous clots, where FXIII activity is important⁵⁴, it seems unlikely that $\alpha_E C$ would be critical because of its low abundance. While α -chain cross-linking is important⁵⁵, FXIII-mediated erythrocyte retention can be reconstituted with recombinant fibrinogen that presumably lacks $\alpha_E C$ 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 A α enabled vascular occlusion after injury in some AB larvae, with an extended average TTO compared to the TU strain. However, additional transgenic production of A α had little effect on the variable AB TTO while A α E 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 deficiencies⁵⁶⁻⁵⁸, but causality has not been proven. As fibrinogen 420 is only present as 1% of circulating adult fibrinogen, and only 3% in newborns⁹, we conclude that variation in the α _EC domain is unlikely to lead to a quantitative human fibrinogen disorder⁵⁹. However, if fibrinogen 420 is necessary for leukocyte interactions, its absence or mutation could have clinical consequences.

When a morpholino was used to lower A α or A α E expression (>90%) in embryos from a cross between transgenic adults and the TU strain, which can produce A α E, A α 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 A α E 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 A α E chain expression. The differential ability of A α and A α E chains to complement fibrinogen deficiency in zebrafish larvae supports a role for the α _EC domain in early blood coagulation, and the presence of A α E or A α 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.

Acknowledgments

This work was supported by Swiss National Science Foundation funding to (M.N.-A. #31003A_152633), National Institutes of Health grants R01 HL124232 and R01 HL125774 (J.A.S.), and T32 GM007863, T32 HL125242, and an American Heart Association Predoctoral Fellowship Award (S.J.G.).

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.

References

1. Doolittle RF. Step-by-step evolution of vertebrate blood coagulation. *Cold Spring Harb Symp Quant Biol.* 2009;74:35-40.
2. Doolittle RF. Fibrinogen and fibrin. *Annu Rev Biochem.* 1984;53:195-229.
3. Chung DW, Davie EW. gamma and gamma' chains of human fibrinogen are produced by alternative mRNA processing. *Biochemistry.* 1984;23(18):4232-4236.
4. Fu Y, Grieninger G. Fib420: a normal human variant of fibrinogen with two extended alpha chains. *Proc Natl Acad Sci U S A.* 1994;91(7):2625-2628.
5. Fu Y, Weissbach L, Plant PW, et al. Carboxy-terminal-extended variant of the human fibrinogen alpha subunit: a novel exon conferring marked homology to beta and gamma subunits. *Biochemistry.* 1992;31(48):11968-11972.
6. Siebenlist KR, Meh DA, Mosesson MW. Plasma factor XIII binds specifically to fibrinogen molecules containing gamma chains. *Biochemistry.* 1996;35(32):10448-10453.
7. Meh DA, Siebenlist KR, Mosesson MW. Identification and characterization of the thrombin binding sites on fibrin. *J Biol Chem.* 1996;271(38):23121-23125.
8. Fu Y, Cao Y, Hertzberg KM, Grieninger G. Fibrinogen alpha genes: conservation of bipartite transcripts and carboxy-terminal-extended alpha subunits in vertebrates. *Genomics.* 1995;30(1):71-76.
9. Grieninger G, Lu X, Cao Y, et al. Fib420, the novel fibrinogen subclass: newborn levels are higher than adult. *Blood.* 1997;90(7):2609-2614.
10. Cooper AV, Standeven KF, Ariens RA. Fibrinogen gamma-chain splice variant gamma' alters fibrin formation and structure. *Blood.* 2003;102(2):535-540.
11. Duval C, Ariens RAS. Fibrinogen splice variation and cross-linking: Effects on fibrin structure/function and role of fibrinogen gamma' as thrombomodulin II. *Matrix Biol.* 2017;60-61:8-15.
12. Omarova F, Uitte de Willige S, Simioni P, et al. Fibrinogen gamma' increases the sensitivity to activated protein C in normal and factor V Leiden plasma. *Blood.* 2014;124(9):1531-1538.
13. Macrae FL, Domingues MM, Casini A, Ariens RA. The (Patho)physiology of Fibrinogen gamma'. *Semin Thromb Hemost.* 2016;42(4):344-355.
14. Spraggon G, Applegate D, Everse SJ, et al. Crystal structure of a recombinant alphaEC domain from human fibrinogen-420. *Proc Natl Acad Sci U S A.* 1998;95(16):9099-9104.
15. Mosesson MW, DiOrio JP, Hernandez I, Hainfeld JF, Wall JS, Grieninger G. The ultrastructure of fibrinogen-420 and the fibrin-420 clot. *Biophys Chem.* 2004;112(2-3):209-214.
16. Applegate D, Steben LS, Hertzberg KM, Grieninger G. The alpha(E)C domain of human fibrinogen-420 is a stable and early plasmin cleavage product. *Blood.* 2000;95(7):2297-2303.
17. Lishko VK, Yakubenko VP, Hertzberg KM, Grieninger G, Ugarova TP. The alternatively spliced alpha(E)C domain of human fibrinogen-420 is a novel ligand for leukocyte integrins alpha(M)beta(2) and alpha(X)beta(2). *Blood.* 2001;98(8):2448-2455.
18. Fish RJ, Vorjohann S, Bena F, Fort A, Neerman-Arbez M. Developmental expression and organisation of fibrinogen genes in the zebrafish. *Thromb Haemost.* 2012;107(1):158-166.
19. Kretz CA, Weyand AC, Shavit JA. Modeling Disorders of Blood Coagulation in the Zebrafish. *Curr Pathobiol Rep.* 2015;3(2):155-161.

20. Hanumanthaiah R, Day K, Jagadeeswaran P. Comprehensive analysis of blood coagulation pathways in teleostei: evolution of coagulation factor genes and identification of zebrafish factor VIII. *Blood Cells Mol Dis*. 2002;29(1):57-68.
21. Jagadeeswaran P, Carrillo M, Radhakrishnan UP, Rajpurohit SK, Kim S. Laser-induced thrombosis in zebrafish. *Methods Cell Biol*. 2011;101:197-203.
22. Jagadeeswaran P, Kulkarni V, Carrillo M, Kim S. Zebrafish: from hematology to hydrology. *J Thromb Haemost*. 2007;5 Suppl 1:300-304.
23. Jagadeeswaran P, Liu YC, Sheehan JP. Analysis of hemostasis in the zebrafish. *Methods Cell Biol*. 1999;59:337-357.
24. Jagadeeswaran P, Paris R, Rao P. Laser-induced thrombosis in zebrafish larvae: a novel genetic screening method for thrombosis. *Methods Mol Med*. 2006;129:187-195.
25. Sundaramoorthi H, Panapakam R, Jagadeeswaran P. Zebrafish thrombocyte aggregation by whole blood aggregometry and flow cytometry. *Platelets*. 2015;26(7):613-619.
26. Fish RJ, Di Sanza C, Neerman-Arbez M. Targeted mutation of zebrafish *fga* models human congenital afibrinogenemia. *Blood*. 2014;123(14):2278-2281.
27. Hu Z, Lavik KI, Liu Y, et al. Loss of fibrinogen in zebrafish results in an asymptomatic embryonic hemostatic defect and synthetic lethality with thrombocytopenia. *J Thromb Haemost*. 2019;17(4):607-617.
28. Hu Z, Liu Y, Huarng MC, et al. Genome editing of factor X in zebrafish reveals unexpected tolerance of severe defects in the common pathway. *Blood*. 2017;130(5):666-676.
29. Iyer N, Tcheuyap VT, Schneider S, Marshall V, Jagadeeswaran P. Knockout of von Willebrand factor in Zebrafish by CRISPR/Cas9 mutagenesis. *Br J Haematol*. 2019;186(4):e76-e80.
30. Liu Y, Kretz CA, Maeder ML, et al. Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function. *Blood*. 2014;124(1):142-150.
31. Weyand AC, Grzegorski SJ, Rost MS, et al. Analysis of factor V in zebrafish demonstrates minimal levels needed for early hemostasis. *Blood Adv*. 2019;3(11):1670-1680.
32. Jagadeeswaran P, Sheehan JP, Craig FE, Troyer D. Identification and characterization of zebrafish thrombocytes. *Br J Haematol*. 1999;107(4):731-738.
33. Huarng MC, Shavit JA. Simple and rapid quantification of thrombocytes in zebrafish larvae. *Zebrafish*. 2015;12(3):238-242.
34. Kim S, Carrillo M, Radhakrishnan UP, Jagadeeswaran P. Role of zebrafish thrombocyte and non-thrombocyte microparticles in hemostasis. *Blood Cells Mol Dis*. 2012;48(3):188-196.
35. O'Connor MN, Salles, II, Cvejic A, et al. Functional genomics in zebrafish permits rapid characterization of novel platelet membrane proteins. *Blood*. 2009;113(19):4754-4762.
36. Thattaliyath B, Cykowski M, Jagadeeswaran P. Young thrombocytes initiate the formation of arterial thrombi in zebrafish. *Blood*. 2005;106(1):118-124.
37. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14):1754-1760.
38. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 2010;20(9):1297-1303.
39. DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet*. 2011;43(5):491-498.

40. Cingolani P, Platts A, Wang le L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly (Austin)*. 2012;6(2):80-92.
41. Cingolani P, Patel VM, Coon M, et al. Using *Drosophila melanogaster* as a Model for Genotoxic Chemical Mutational Studies with a New Program, SnpSift. *Front Genet*. 2012;3:35.
42. Mosimann C, Kaufman CK, Li P, Pugach EK, Tamplin OJ, Zon LI. Ubiquitous transgene expression and Cre-based recombination driven by the ubiquitin promoter in zebrafish. *Development*. 2011;138(1):169-177.
43. Ablain J, Durand EM, Yang S, Zhou Y, Zon LI. A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev Cell*. 2015;32(6):756-764.
44. Kwan KM, Fujimoto E, Grabher C, et al. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn*. 2007;236(11):3088-3099.
45. Rost MS, Grzegorski SJ, Shavit JA. Quantitative methods for studying hemostasis in zebrafish larvae. *Methods Cell Biol*. 2016;134:377-389.
46. Suster ML, Kikuta H, Urasaki A, Asakawa K, Kawakami K. Transgenesis in zebrafish with the tol2 transposon system. *Methods Mol Biol*. 2009;561:41-63.
47. Brown KH, Dobrinski KP, Lee AS, et al. Extensive genetic diversity and substructuring among zebrafish strains revealed through copy number variant analysis. *Proc Natl Acad Sci U S A*. 2012;109(2):529-534.
48. Haffter P, Granato M, Brand M, et al. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development*. 1996;123:1-36.
49. Tomberg K, Khoriaty R, Westrick RJ, et al. Spontaneous 8bp Deletion in *Nbeal2* Recapitulates the Gray Platelet Syndrome in Mice. *PLOS ONE*. 2016;11(3):e0150852.
50. Doolittle RF. The structure and evolution of vertebrate fibrinogen. *Ann N Y Acad Sci*. 1983;408:13-27.
51. Kant JA, Fornace AJ, Jr., Saxe D, Simon MI, McBride OW, Crabtree GR. Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion. *Proc Natl Acad Sci U S A*. 1985;82(8):2344-2348.
52. Guedes AF, Carvalho FA, Malho I, Lousada N, Sargento L, Santos NC. Atomic force microscopy as a tool to evaluate the risk of cardiovascular diseases in patients. *Nature Nanotechnology*. 2016;11(8):687-692.
53. De Oliveira S, Vitorino de Almeida V, Calado A, Rosario HS, Saldanha C. Integrin-associated protein (CD47) is a putative mediator for soluble fibrinogen interaction with human red blood cells membrane. *Biochim Biophys Acta*. 2012;1818(3):481-490.
54. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest*. 2014;124(8):3590-3600.
55. Byrnes JR, Duval C, Wang Y, et al. Factor XIIIa-dependent retention of red blood cells in clots is mediated by fibrin alpha-chain crosslinking. *Blood*. 2015;126(16):1940-1948.
56. Hanss M, Biot F. A database for human fibrinogen variants. *Ann N Y Acad Sci*. 2001;936:89-90.
57. Zhou J, Ding Q, Chen Y, et al. Clinical features and molecular basis of 102 Chinese patients with congenital dysfibrinogenemia. *Blood Cells Mol Dis*. 2015;55(4):308-315.

58. Mukaddam A, Kulkarni B, Jadli A, Ghosh K, Shetty S. Spectrum of mutations in Indian patients with fibrinogen disorders and its application in genetic diagnosis of the affected families. *Haemophilia*. 2015;21(6):e519-523.
59. Neerman-Arbez M, de Moerloose P, Casini A. Laboratory and Genetic Investigation of Mutations Accounting for Congenital Fibrinogen Disorders. *Semin Thromb Hemost*. 2016;42(4):356-365.

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 A α E, but not A α 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 A α or A α E 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 A α E than A α 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 A α or A α E. This was done in the presence or absence of A α or A α E 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 A α or A α E 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 A α or A α E 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 A α or A α E chains. Mutant *fga*^{-/-} embryos, lacking fibrinogen showed less average thrombocyte accumulation.

Figures

Figure 1

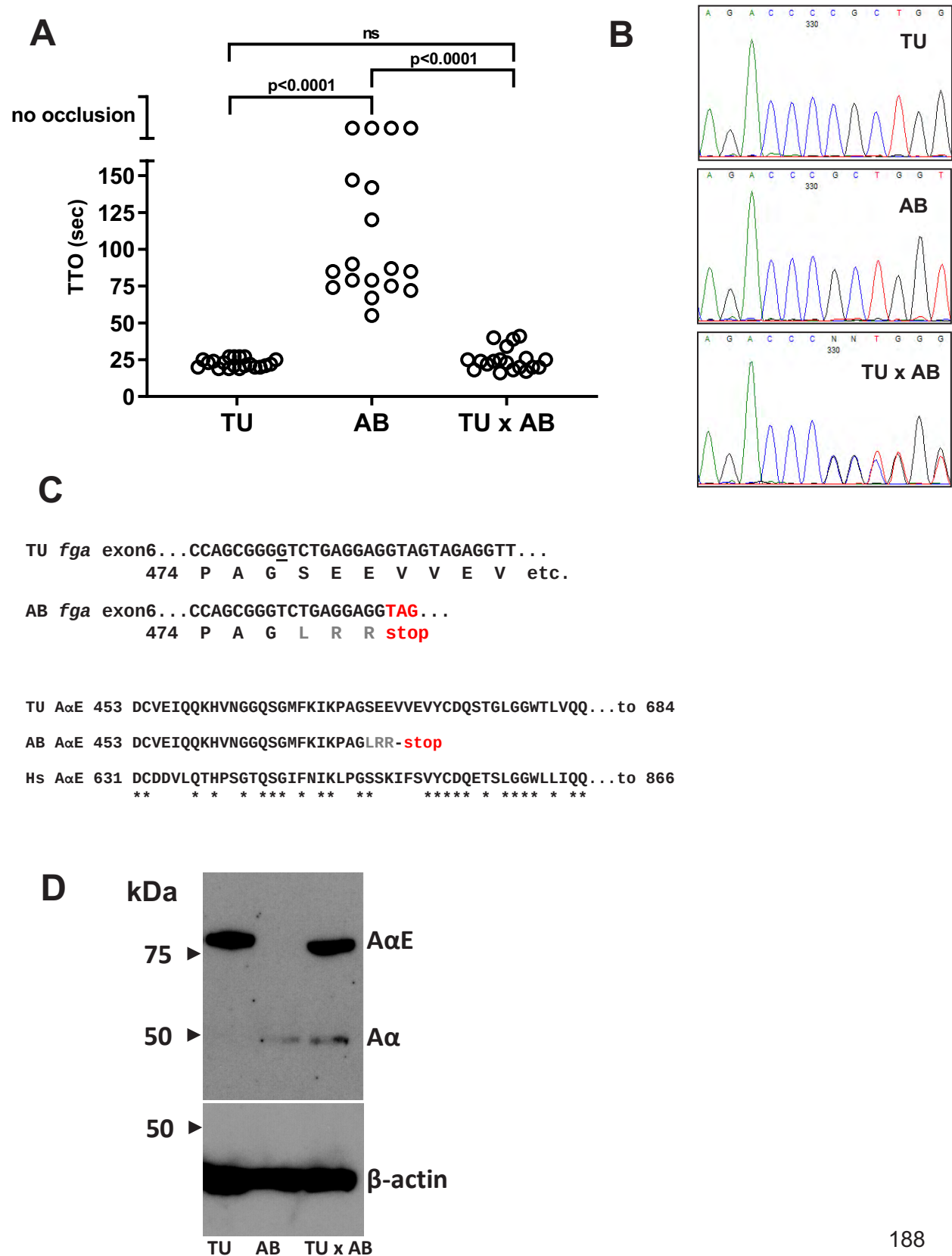


Figure 2

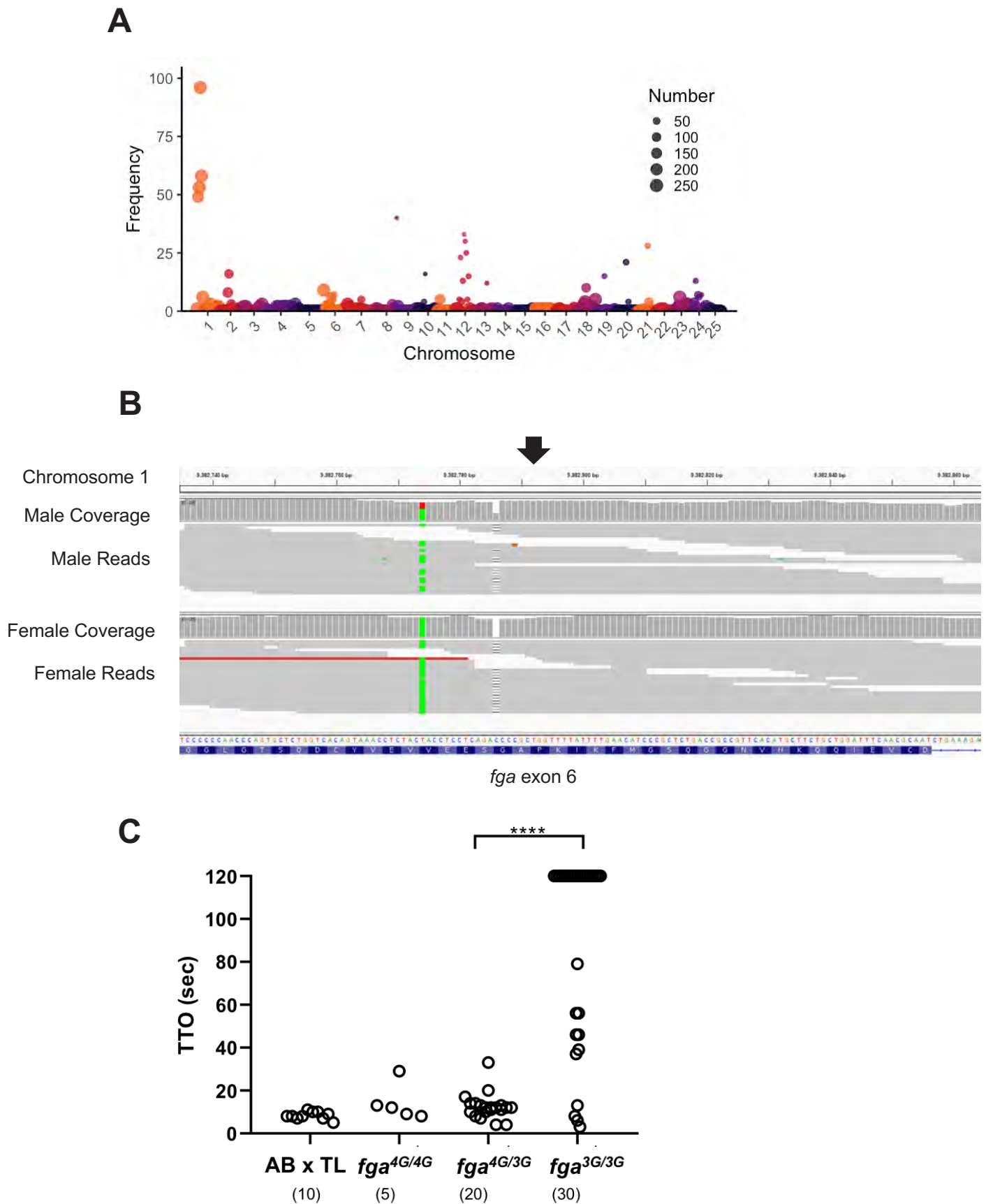


Figure 3

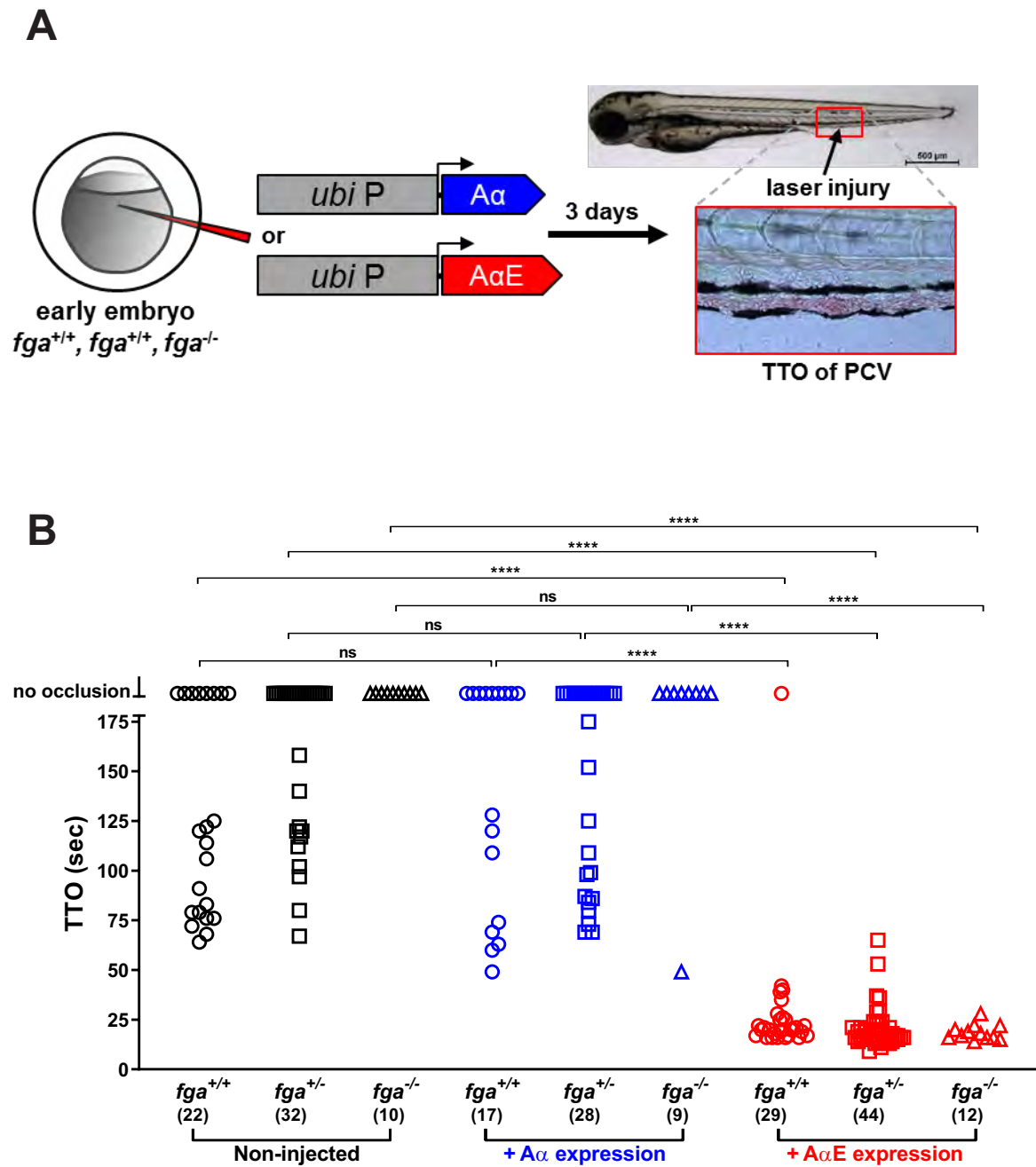


Figure 4

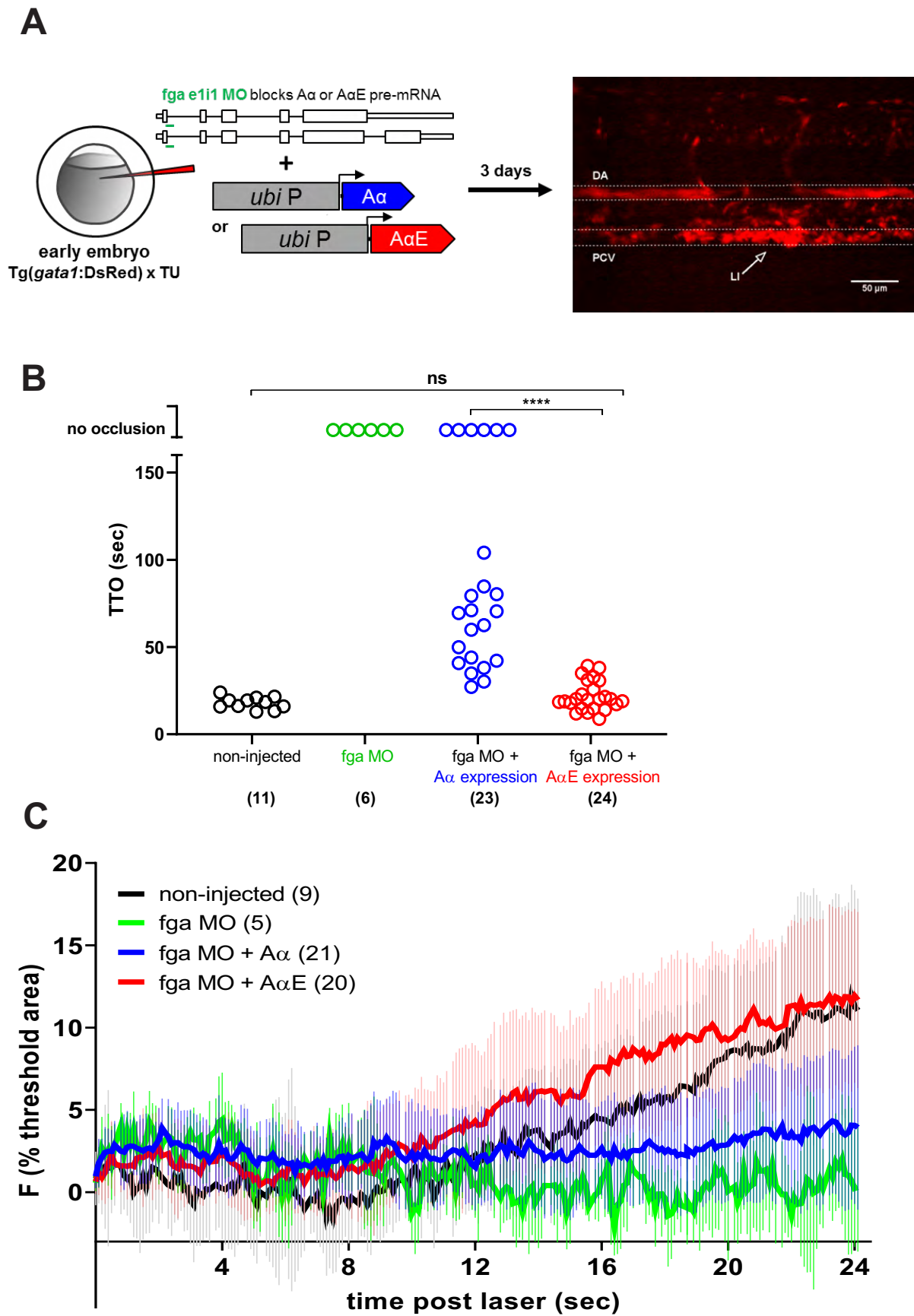
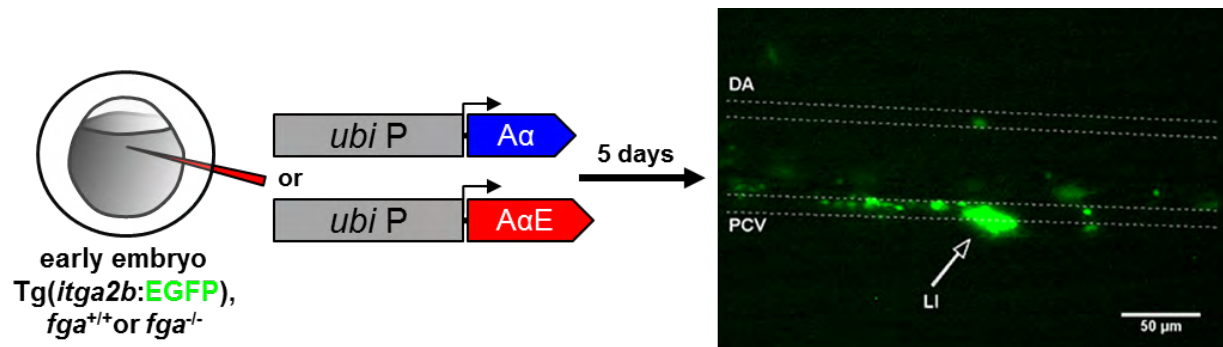
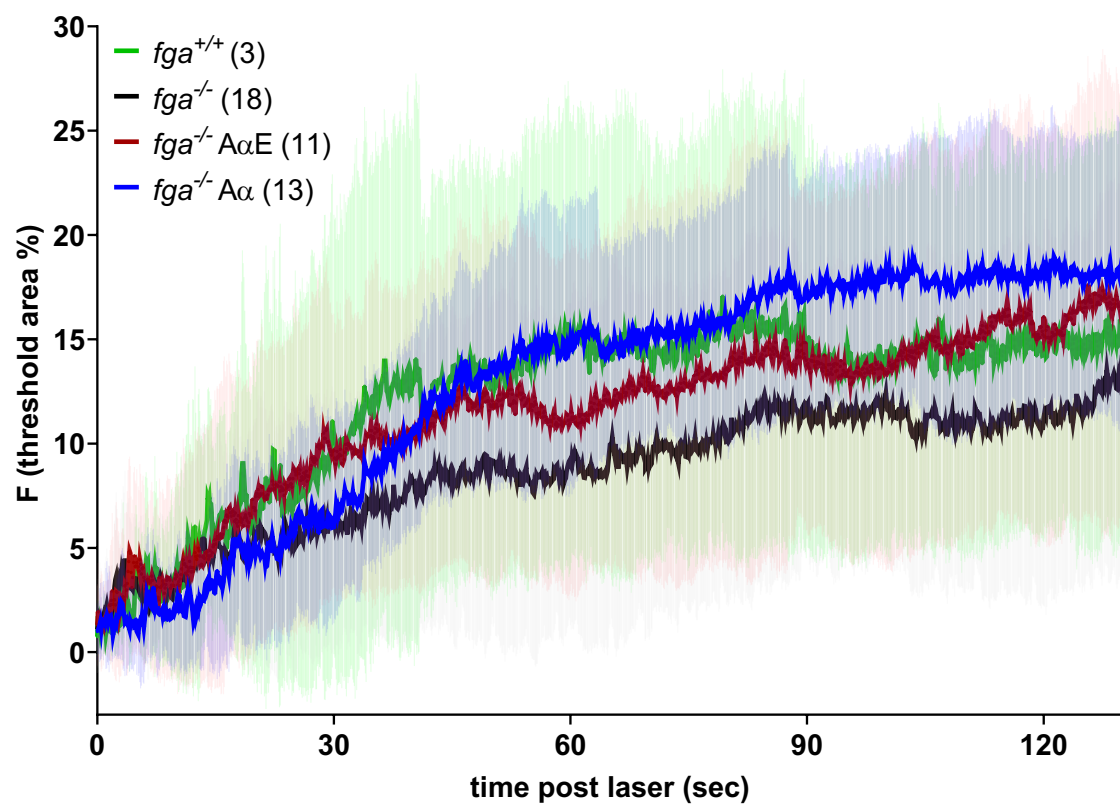


Figure 5

A



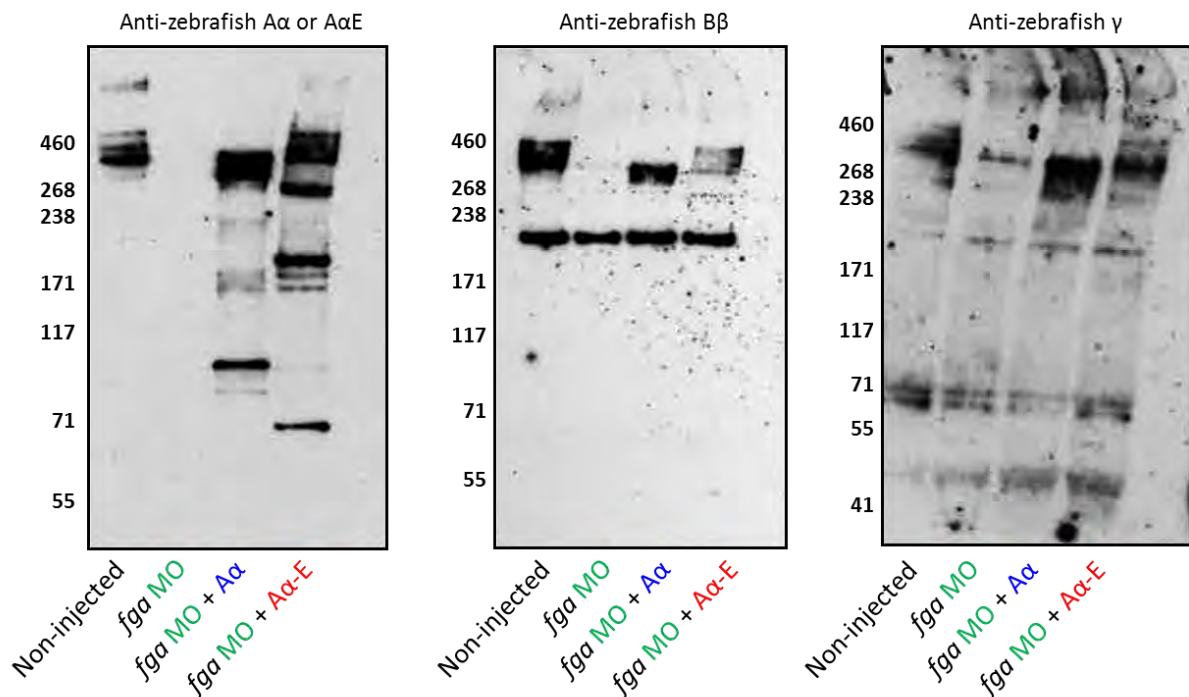
B



Supplementary Data

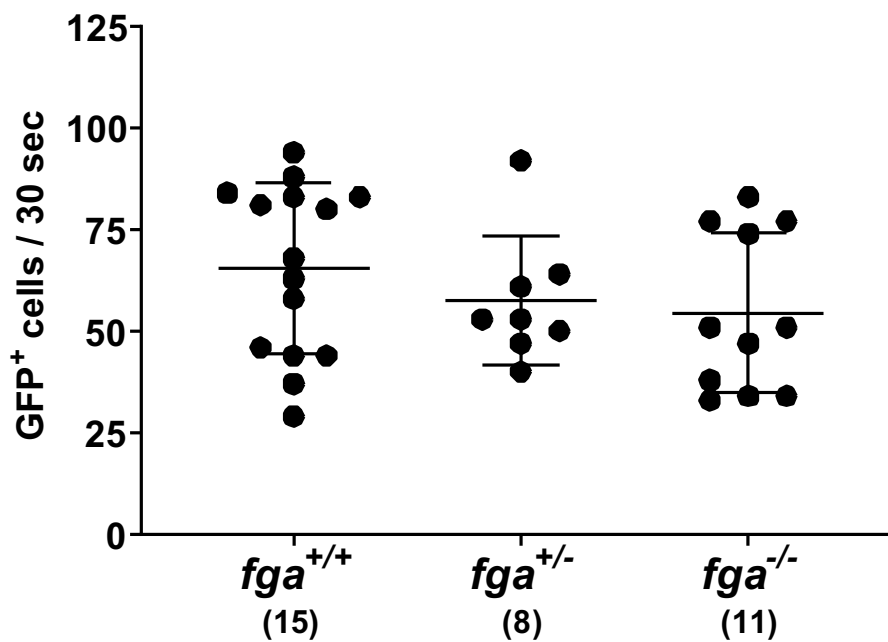
Supplementary Figure 1 - Immunoblots of fibrinogen production in zebrafish larvae after morpholino targeting of fibrinogen alpha chain mRNA and transgenic expression of fibrinogen A α or A α E

1 to 2 cell embryos were injected with an fga mRNA-targeting morpholino with and without expression of fibrinogen A α or A α E. 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 A α (or A α E), B β or γ chains. Without considering post-translational modifications, the



expected molecular weight for zebrafish fibrinogen (A α B β γ)₂ is approximately 295 kDa and 346 kDa with the A α E chains (A α EB β γ)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 A α or A α E 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 B β 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 A α 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 A α 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 A α 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 A α -chain translated from this exon-skipped transcript lacked amino acids 19 to 56 in the N-terminal of the fibrinogen A α 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* Δ 19–56, have an A α -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*^{+/ Δ 19–56}) to model the human *FGA* IVS2+3insCAT hypodysfibrinogenemia mutation.

When studying venous thrombosis in hypodysfibrinogenemic (*fga*^{+/ Δ 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 A α E 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 A α E 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 FeCl₃ injury of the carotid arteries.

Since hypodysfibrinogenemia and dysfibrinogenemia mutations lacking the thrombin cleavage site in the N-terminal end of the fibrinogen A α 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*^{+/ Δ 19-56} compared to wild type zebrafish, and the prolonged venous TTO shown in *fga* knock-down larvae with A α E + A α E R28C expression compared to *fga* knock-down larvae with A α E 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 A α -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 A α E 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 A α E 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 ($A\alpha$, $B\beta$ 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: $A\alpha$, $B\beta$ 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 $A\alpha$ -chain and $A\alpha E$ isoform between zebrafish and humans, we noted that the percentage of identity for $A\alpha E$ isoform was greater (32.53%) than that of the common $A\alpha$ -chain (18.46%) (Appendix, Figure A-3), suggesting that the common $A\alpha$ -chain has evolved more than the $A\alpha E$ isoform due to adaptation. Upon comparing the percentage of identity between the $A\alpha E$ C-terminal

domain of zebrafish and its human counterpart we observed an identity of 61.98% (Appendix, Figure A-4). This indicates that the A α E C-terminal domain is conserved to a great extent during vertebrate evolution. Since the A α E 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 B β 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 A α E isoform shows high amino acid sequence similarity with the C-terminal globular domain of the B β and γ chains [312] (Appendix Figure A-5 and Figure A-6). When comparing the C-terminal globular domains of the three A α E, B β 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 (A α E, B β 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 B β and γ chains may also be present in the A α E 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 A α E 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 A α E 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 A α E 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 A α E isoform is a genetic modifier of venous thrombosis in zebrafish.

We suggested that A α E 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 A α E C-terminal globular domain, generating an erythrocyte-rich thrombus after a vascular injury has occurred.

IV. A α E C-terminal domain as a ligand for erythrocyte binding

Several research groups have studied the role played by A α E 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 A α E 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 A α E 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 A α -chain [336] (the same motif that binds the fibrinogen A α -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 A α E 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 A α E isoform for erythrocytes in zebrafish larvae [335].

V. Mutations in the C-terminal domain of the A α -chain

Until this point, we have discussed the evolutionary conservation of Fib420, its implication in developmental hemostasis and the role of the A α E 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 A α E isoform, none were found. However, there are patients who present frameshift, nonsense, or point mutations leading to a truncation of the A α C-terminal domain or resulting in the absence of part of the A α C-terminal domain. For instance, the Fibrinogen Marburg mutation, which is a nonsense mutation that results in an A α 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 A α chain that lacks A α 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 A α chain that lacks A α 454-610 with deletion of the transglutaminase (TG) crosslinking site in the A α 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 A α E 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 A α E chains, like the AGD motif. Our goal for the future is to find out which residue(s) are involved in the binding of the A α E 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 A α E 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 A α E 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 A α E 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 A α containing fibrinogen form throughout the embryonic development onwards until adult stages) and the TU (which larvae produce predominantly A α E 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 B β and γ chains are known to participate in wound healing [313] [314]. Since the A α E C-terminal globular domains from Fib420 molecules show high homology with the C-terminal domain from B β 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 A α or A α E 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 efficacy 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].

REFERENCES

1. Hoffbrand, V. and P.A.H. Moss, *Essential Haematology*. 2011: Edition Wiley Blackwell
2. Gil, M.R., *Chapter 91 - Overview of the Coagulation System*. Transfusion Medicine and Hemostasis (Third Edition), 2019. **Clinical and Laboratory Aspects** p. 559-564.
3. Gale, A.J., *Continuing education course #2: current understanding of hemostasis*. Toxicol Pathol, 2011. **39**(1): p. 273-80.
4. Tanaka, K.A., N.S. Key, and J.H. Levy, *Blood coagulation: hemostasis and thrombin regulation*. Anesth Analg, 2009. **108**(5): p. 1433-46.
5. Cahill, P.A. and E.M. Redmond, *Vascular endothelium - Gatekeeper of vessel health*. Atherosclerosis, 2016. **248**: p. 97-109.
6. Yau, J.W., H. Teoh, and S. Verma, *Endothelial cell control of thrombosis*. BMC Cardiovasc Disord, 2015. **15**: p. 130.
7. Muller, K.A.H.A.J.M.W., *Vascular Function in Hemostasis*. Williams Hematology. **9e Eds**.
8. Yeung, J., W. Li, and M. Holinstat, *Platelet Signaling and Disease: Targeted Therapy for Thrombosis and Other Related Diseases*. Pharmacol Rev, 2018. **70**(3): p. 526-548.
9. Golebiewska, E.M. and A.W. Poole, *Platelet secretion: From haemostasis to wound healing and beyond*. Blood Rev, 2015. **29**(3): p. 153-62.
10. Broos, K., et al., *Platelets at work in primary hemostasis*. Blood Rev, 2011. **25**(4): p. 155-67.
11. Periyah, M.H., A.S. Halim, and A.Z. Mat Saad, *Mechanism Action of Platelets and Crucial Blood Coagulation Pathways in Hemostasis*. Int J Hematol Oncol Stem Cell Res, 2017. **11**(4): p. 319-327.
12. Montague, S.J., R.K. Andrews, and E.E. Gardiner, *Mechanisms of receptor shedding in platelets*. Blood, 2018. **132**(24): p. 2535-2545.
13. Ruggeri, Z.M. and G.L. Mendolicchio, *Adhesion mechanisms in platelet function*. Circ Res, 2007. **100**(12): p. 1673-85.
14. Durrant, T.N., M.T. van den Bosch, and I. Hers, *Integrin α IIb β 3 outside-in signaling*. Blood, 2017. **130**(14): p. 1607-1619.
15. Plow, E.F. and M.H. Ginsberg, *Cellular adhesion: GPIIb-IIIa as a prototypic adhesion receptor*. Prog Hemost Thromb, 1989. **9**: p. 117-56.
16. Savage, B., E. Saldivar, and Z.M. Ruggeri, *Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor*. Cell, 1996. **84**(2): p. 289-97.
17. Hantgan, R.R., et al., *Glycoprotein Ib, von Willebrand factor, and glycoprotein IIb:IIIa are all involved in platelet adhesion to fibrin in flowing whole blood*. Blood, 1990. **76**(2): p. 345-53.
18. Kauskot, A. and M.F. Hoylaerts, *Platelet receptors*. Handb Exp Pharmacol, 2012(210): p. 23-57.
19. Senis, Y.A., A. Mazharian, and J. Mori, *Src family kinases: at the forefront of platelet activation*. Blood, 2014. **124**(13): p. 2013-24.
20. Ossovskaya, V.S. and N.W. Bunnett, *Protease-activated receptors: contribution to physiology and disease*. Physiol Rev, 2004. **84**(2): p. 579-621.
21. Kroeze, W.K., D.J. Sheffler, and B.L. Roth, *G-protein-coupled receptors at a glance*. J Cell Sci, 2003. **116**(Pt 24): p. 4867-9.
22. Woulfe, D.S., *Platelet G protein-coupled receptors in hemostasis and thrombosis*. J Thromb Haemost, 2005. **3**(10): p. 2193-200.
23. Li, Z., et al., *Signaling during platelet adhesion and activation*. Arterioscler Thromb Vasc Biol, 2010. **30**(12): p. 2341-9.
24. Clemetson, K.J., *Platelets and primary haemostasis*. Thromb Res, 2012. **129**(3): p. 220-4.
25. Koupenova, M., et al., *Thrombosis and platelets: an update*. Eur Heart J, 2017. **38**(11): p. 785-791.
26. Sharda, A. and R. Flaumenhaft, *The life cycle of platelet granules*. F1000Res, 2018. **7**: p. 236.
27. Macfarlane, R.G., *An Enzyme Cascade in the Blood Clotting Mechanism, and Its Function as a Biochemical Amplifier*. Nature, 1964. **202**: p. 498-9.
28. Hoffman, M. and D.M. Monroe, 3rd, *A cell-based model of hemostasis*. Thromb Haemost, 2001. **85**(6): p. 958-65.

29. Smith, S.A., R.J. Travers, and J.H. Morrissey, *How it all starts: Initiation of the clotting cascade*. Crit Rev Biochem Mol Biol, 2015. **50**(4): p. 326-36.
30. Nemerson, Y. and D. Repke, *Tissue factor accelerates the activation of coagulation factor VII: the role of a bifunctional coagulation cofactor*. Thromb Res, 1985. **40**(3): p. 351-8.
31. Bom, V.J., et al., *Extrinsic activation of human blood coagulation factors IX and X*. Thromb Haemost, 1990. **63**(2): p. 224-30.
32. Komiyama, Y., A.H. Pedersen, and W. Kisiel, *Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor*. Biochemistry, 1990. **29**(40): p. 9418-25.
33. Nemerson, Y. and R. Gentry, *An ordered addition, essential activation model of the tissue factor pathway of coagulation: evidence for a conformational cage*. Biochemistry, 1986. **25**(14): p. 4020-33.
34. Tavoosi, N., et al., *Factor VII and protein C are phosphatidic acid-binding proteins*. Biochemistry, 2013. **52**(33): p. 5545-52.
35. Smith, S.A., *The cell-based model of coagulation*. J Vet Emerg Crit Care (San Antonio), 2009. **19**(1): p. 3-10.
36. Cesarman-Maus, G. and K.A. Hajjar, *Molecular mechanisms of fibrinolysis*. Br J Haematol, 2005. **129**(3): p. 307-21.
37. Kolodziejczyk, J. and M.B. Ponczek, *The role of fibrinogen, fibrin and fibrin(ogen) degradation products (FDPs) in tumor progression*. Contemp Oncol (Pozn), 2013. **17**(2): p. 113-9.
38. Henschen, A., et al., *Covalent structure of fibrinogen*. Ann N Y Acad Sci, 1983. **408**: p. 28-43.
39. Blomback, B., B. Hessel, and D. Hogg, *Disulfide bridges in nh2 -terminal part of human fibrinogen*. Thromb Res, 1976. **8**(5): p. 639-58.
40. Hoeprich, P.D., Jr. and R.F. Doolittle, *Dimeric half-molecules of human fibrinogen are joined through disulfide bonds in an antiparallel orientation*. Biochemistry, 1983. **22**(9): p. 2049-55.
41. Doolittle, R.F., *Fibrinogen and fibrin*. Annu Rev Biochem, 1984. **53**: p. 195-229.
42. Chung, D.W., J.E. Harris, and E.W. Davie, *Nucleotide sequences of the three genes coding for human fibrinogen*. Adv Exp Med Biol, 1990. **281**: p. 39-48.
43. Mosesson, M.W., K.R. Siebenlist, and D.A. Meh, *The structure and biological features of fibrinogen and fibrin*. Ann N Y Acad Sci, 2001. **936**: p. 11-30.
44. Weisel, J.W., *Fibrinogen and fibrin*. Adv Protein Chem, 2005. **70**: p. 247-99.
45. Casini, A., et al., *Diagnosis and classification of congenital fibrinogen disorders: communication from the SSC of the ISTH*. J Thromb Haemost, 2018. **16**(9): p. 1887-1890.
46. Fish, R.J. and M. Neerman-Arbez, *Fibrinogen gene regulation*. Thromb Haemost, 2012. **108**(3): p. 419-26.
47. Fu, Y., et al., *Carboxy-terminal-extended variant of the human fibrinogen alpha subunit: a novel exon conferring marked homology to beta and gamma subunits*. Biochemistry, 1992. **31**(48): p. 11968-72.
48. Fu, Y., et al., *Fibrinogen alpha genes: conservation of bipartite transcripts and carboxy-terminal-extended alpha subunits in vertebrates*. Genomics, 1995. **30**(1): p. 71-6.
49. Rein-Smith, C.M., N.W. Anderson, and D.H. Farrell, *Differential regulation of fibrinogen gamma chain splice isoforms by interleukin-6*. Thromb Res, 2013. **131**(1): p. 89-93.
50. Doolittle, R.F., *The structure and evolution of vertebrate fibrinogen*. Ann N Y Acad Sci, 1983. **408**: p. 13-27.
51. Kant, J.A., S.T. Lord, and G.R. Crabtree, *Partial mRNA sequences for human A alpha, B beta, and gamma fibrinogen chains: evolutionary and functional implications*. Proc Natl Acad Sci U S A, 1983. **80**(13): p. 3953-7.
52. Rixon, M.W., et al., *Characterization of a complementary deoxyribonucleic acid coding for the alpha chain of human fibrinogen*. Biochemistry, 1983. **22**(13): p. 3237-44.
53. Chung, D.W., et al., *Characterization of complementary deoxyribonucleic acid and genomic deoxyribonucleic acid for the beta chain of human fibrinogen*. Biochemistry, 1983. **22**(13): p. 3244-50.

54. Chung, D.W., W.Y. Chan, and E.W. Davie, *Characterization of a complementary deoxyribonucleic acid coding for the gamma chain of human fibrinogen*. *Biochemistry*, 1983. **22**(13): p. 3250-6.
55. Doolittle, R.F., B.A. Cottrell, and M. Riley, *Amino acid compositions of the subunit chains of lamprey fibrinogen. Evolutionary significance of some structural anomalies*. *Biochim Biophys Acta*, 1976. **453**(2): p. 439-52.
56. Crabtree, G.R., et al., *Evolution and structure of the fibrinogen genes. Random insertion of introns or selective loss?* *J Mol Biol*, 1985. **185**(1): p. 1-19.
57. Kant, J.A., et al., *Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion*. *Proc Natl Acad Sci U S A*, 1985. **82**(8): p. 2344-8.
58. Roy, S., O. Overton, and C. Redman, *Overexpression of any fibrinogen chain by Hep G2 cells specifically elevates the expression of the other two chains*. *J Biol Chem*, 1994. **269**(1): p. 691-5.
59. Fowlkes, D.M., et al., *Potential basis for regulation of the coordinately expressed fibrinogen genes: homology in the 5' flanking regions*. *Proc Natl Acad Sci U S A*, 1984. **81**(8): p. 2313-6.
60. Mizuguchi, J., et al., *Characterization of the 5'-flanking region of the gene for the gamma chain of human fibrinogen*. *J Biol Chem*, 1995. **270**(47): p. 28350-6.
61. Haidaris, P.J., *Induction of fibrinogen biosynthesis and secretion from cultured pulmonary epithelial cells*. *Blood*, 1997. **89**(3): p. 873-82.
62. Gruys, E., et al., *Acute phase reaction and acute phase proteins*. *J Zhejiang Univ Sci B*, 2005. **6**(11): p. 1045-56.
63. Castell, J.V., et al., *Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes*. *FEBS Lett*, 1989. **242**(2): p. 237-9.
64. Anderson, G.M., A.R. Shaw, and J.A. Shafer, *Functional characterization of promoter elements involved in regulation of human B beta-fibrinogen expression. Evidence for binding of novel activator and repressor proteins*. *J Biol Chem*, 1993. **268**(30): p. 22650-5.
65. Liu, Z. and G.M. Fuller, *Detection of a novel transcription factor for the A alpha fibrinogen gene in response to interleukin-6*. *J Biol Chem*, 1995. **270**(13): p. 7580-6.
66. Zhang, Z., N.L. Fuentes, and G.M. Fuller, *Characterization of the IL-6 responsive elements in the gamma fibrinogen gene promoter*. *J Biol Chem*, 1995. **270**(41): p. 24287-91.
67. Zhang, Z., et al., *STAT3 acts as a co-activator of glucocorticoid receptor signaling*. *J Biol Chem*, 1997. **272**(49): p. 30607-10.
68. Otto, J.M., H.E. Grenett, and G.M. Fuller, *The coordinated regulation of fibrinogen gene transcription by hepatocyte-stimulating factor and dexamethasone*. *J Cell Biol*, 1987. **105**(3): p. 1067-72.
69. Grieninger, G., et al., *Regulation of fibrinogen synthesis and secretion by the chicken hepatocyte*. *Ann N Y Acad Sci*, 1989. **557**: p. 257-70, discussion 270-1.
70. Fish, R.J. and M. Neerman-Arbez, *A novel regulatory element between the human FGA and FGG genes*. *Thromb Haemost*, 2012. **108**(3): p. 427-34.
71. Fort, A., et al., *A liver enhancer in the fibrinogen gene cluster*. *Blood*, 2011. **117**(1): p. 276-82.
72. Ghirlando, R. and G. Felsenfeld, *CTCF: making the right connections*. *Genes Dev*, 2016. **30**(8): p. 881-91.
73. Ong, C.T. and V.G. Corces, *CTCF: an architectural protein bridging genome topology and function*. *Nat Rev Genet*, 2014. **15**(4): p. 234-46.
74. Espitia Jaimes, C., R.J. Fish, and M. Neerman-Arbez, *Local chromatin interactions contribute to expression of the fibrinogen gene cluster*. *J Thromb Haemost*, 2018. **16**(10): p. 2070-2082.
75. Zhang, J.Z. and C.M. Redman, *Assembly and secretion of fibrinogen. Involvement of amino-terminal domains in dimer formation*. *J Biol Chem*, 1996. **271**(21): p. 12674-80.
76. Xu, W., D.W. Chung, and E.W. Davie, *The assembly of human fibrinogen. The role of the amino-terminal and coiled-coil regions of the three chains in the formation of the*

- alphagamma and betagamma heterodimers and alphabetagamma half-molecules.* J Biol Chem, 1996. **271**(44): p. 27948-53.
77. Huang, S., Z. Cao, and E.W. Davie, *The role of amino-terminal disulfide bonds in the structure and assembly of human fibrinogen.* Biochem Biophys Res Commun, 1993. **190**(2): p. 488-95.
 78. Gabriel, D.A., K. Muga, and E.M. Boothroyd, *The effect of fibrin structure on fibrinolysis.* J Biol Chem, 1992. **267**(34): p. 24259-63.
 79. Yu, S., et al., *Intracellular assembly of human fibrinogen.* J Biol Chem, 1983. **258**(22): p. 13407-10.
 80. S Yu, B.S., B Kudryk, and C M Redman, *Fibrinogen precursors. Order of assembly of fibrinogen chains.* J Biol Chem, 1984(259): p. 10574-10581.
 81. Hartwig, R. and K.J. Danishefsky, *Studies on the assembly and secretion of fibrinogen.* J Biol Chem, 1991. **266**(10): p. 6578-85.
 82. Redman, C.M. and H. Xia, *Fibrinogen biosynthesis. Assembly, intracellular degradation, and association with lipid synthesis and secretion.* Ann N Y Acad Sci, 2001. **936**: p. 480-95.
 83. Kudryk, B., et al., *Biosynthesis of dog fibrinogen. Characterization of nascent fibrinogen in the rough endoplasmic reticulum.* Eur J Biochem, 1982. **125**(3): p. 673-82.
 84. Roy, S., A. Sun, and C. Redman, *In vitro assembly of the component chains of fibrinogen requires endoplasmic reticulum factors.* J Biol Chem, 1996. **271**(40): p. 24544-50.
 85. Privalov, P.L. and L.V. Medved, *Domains in the fibrinogen molecule.* J Mol Biol, 1982. **159**(4): p. 665-83.
 86. Weisel, J.W. and R.I. Litvinov, *Fibrin Formation, Structure and Properties.* Subcell Biochem, 2017. **82**: p. 405-456.
 87. Medved, L., et al., *Recommendations for nomenclature on fibrinogen and fibrin.* J Thromb Haemost, 2009. **7**(2): p. 355-9.
 88. Mosesson, M.W., *Fibrinogen and fibrin structure and functions.* J Thromb Haemost, 2005. **3**(8): p. 1894-904.
 89. Pratt, K.P., et al., *The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal gamma chain fragment complexed with the peptide Gly-Pro-Arg-Pro.* Proc Natl Acad Sci U S A, 1997. **94**(14): p. 7176-81.
 90. Doolittle, R.F., *The conversion of fibrinogen to fibrin: A brief history of some key events.* Matrix Biol, 2017. **60-61**: p. 5-7.
 91. Blomback, B., et al., *A two-step fibrinogen--fibrin transition in blood coagulation.* Nature, 1978. **275**(5680): p. 501-5.
 92. Andrew, M., et al., *Development of the human coagulation system in the full-term infant.* Blood, 1987. **70**(1): p. 165-72.
 93. Pechik, I., et al., *Structural basis for sequential cleavage of fibrinopeptides upon fibrin assembly.* Biochemistry, 2006. **45**(11): p. 3588-97.
 94. Kattula, S., J.R. Byrnes, and A.S. Wolberg, *Fibrinogen and Fibrin in Hemostasis and Thrombosis.* Arterioscler Thromb Vasc Biol, 2017. **37**(3): p. e13-e21.
 95. Litvinov, R.I., et al., *Polymerization of fibrin: specificity, strength, and stability of knob-hole interactions studied at the single-molecule level.* Blood, 2005. **106**(9): p. 2944-51.
 96. Kononova, O., et al., *Molecular mechanisms, thermodynamics, and dissociation kinetics of knob-hole interactions in fibrin.* J Biol Chem, 2013. **288**(31): p. 22681-92.
 97. Litvinov, R.I., et al., *Polymerization of fibrin: Direct observation and quantification of individual B:b knob-hole interactions.* Blood, 2007. **109**(1): p. 130-8.
 98. Mosesson, M.W., et al., *Evidence for a second type of fibril branch point in fibrin polymer networks, the trimolecular junction.* Blood, 1993. **82**(5): p. 1517-21.
 99. Weisel, J.W. and R.I. Litvinov, *Mechanisms of fibrin polymerization and clinical implications.* Blood, 2013. **121**(10): p. 1712-9.
 100. Bagoly, Z., et al., *Factor XIII, clot structure, thrombosis.* Thromb Res, 2012. **129**(3): p. 382-7.
 101. Siebenlist, K.R., D.A. Meh, and M.W. Mosesson, *Protransglutaminase (factor XIII) mediated crosslinking of fibrinogen and fibrin.* Thromb Haemost, 2001. **86**(5): p. 1221-8.

102. Mosesson, M.W., et al., *The covalent structure of factor XIIIa crosslinked fibrinogen fibrils*. J Struct Biol, 1995. **115**(1): p. 88-101.
103. Chen, R. and R.F. Doolittle, *Identification of the polypeptide chains involved in the cross-linking of fibrin*. Proc Natl Acad Sci U S A, 1969. **63**(2): p. 420-7.
104. McKee, P.A., P. Mattock, and R.L. Hill, *Subunit structure of human fibrinogen, soluble fibrin, and cross-linked insoluble fibrin*. Proc Natl Acad Sci U S A, 1970. **66**(3): p. 738-44.
105. Kanaide, H. and J.R. Shainoff, *Cross-linking of fibrinogen and fibrin by fibrin-stabilizing factor (factor XIIIa)*. J Lab Clin Med, 1975. **85**(4): p. 574-97.
106. Doolittle, R.F., R. Chen, and F. Lau, *Hybrid fibrin: proof of the intermolecular nature of crosslinking units*. Biochem Biophys Res Commun, 1971. **44**(1): p. 94-100.
107. Matsuka, Y.V., et al., *Factor XIIIa-catalyzed cross-linking of recombinant alpha C fragments of human fibrinogen*. Biochemistry, 1996. **35**(18): p. 5810-6.
108. Sobel, J.H. and M.A. Gawinowicz, *Identification of the alpha chain lysine donor sites involved in factor XIIIa fibrin cross-linking*. J Biol Chem, 1996. **271**(32): p. 19288-97.
109. Shainoff, J.R., D.A. Urbanic, and P.M. DiBello, *Immunoelectrophoretic characterizations of the cross-linking of fibrinogen and fibrin by factor XIIIa and tissue transglutaminase. Identification of a rapid mode of hybrid alpha-/gamma-chain cross-linking that is promoted by the gamma-chain cross-linking*. J Biol Chem, 1991. **266**(10): p. 6429-37.
110. Aleman, M.M., et al., *Fibrinogen and red blood cells in venous thrombosis*. Thromb Res, 2014. **133 Suppl 1**: p. S38-40.
111. de Moerloose, P. and M. Neerman-Arbez, *Congenital fibrinogen disorders*. Semin Thromb Hemost, 2009. **35**(4): p. 356-66.
112. Wolberg, A.S., *Thrombin generation and fibrin clot structure*. Blood Rev, 2007. **21**(3): p. 131-42.
113. Blomback, B., et al., *Fibrin in human plasma: gel architectures governed by rate and nature of fibrinogen activation*. Thromb Res, 1994. **75**(5): p. 521-38.
114. Wolberg, A.S., et al., *Elevated prothrombin results in clots with an altered fiber structure: a possible mechanism of the increased thrombotic risk*. Blood, 2003. **101**(8): p. 3008-13.
115. Diamond, S.L., *Systems Analysis of Thrombus Formation*. Circ Res, 2016. **118**(9): p. 1348-62.
116. Zhang, Y.S., R. Oklu, and H. Albadawi, *Bioengineered in vitro models of thrombosis: methods and techniques*. Cardiovasc Diagn Ther, 2017. **7**(Suppl 3): p. S329-S335.
117. Furie, B. and B.C. Furie, *Thrombus formation in vivo*. J Clin Invest, 2005. **115**(12): p. 3355-62.
118. Furie, B. and B.C. Furie, *In vivo thrombus formation*. J Thromb Haemost, 2007. **5 Suppl 1**: p. 12-7.
119. Sachs, U.J. and B. Nieswandt, *In vivo thrombus formation in murine models*. Circ Res, 2007. **100**(7): p. 979-91.
120. Aleman, M.M., et al., *Differential contributions of monocyte- and platelet-derived microparticles towards thrombin generation and fibrin formation and stability*. J Thromb Haemost, 2011. **9**(11): p. 2251-61.
121. Scott, E.M., R.A. Ariens, and P.J. Grant, *Genetic and environmental determinants of fibrin structure and function: relevance to clinical disease*. Arterioscler Thromb Vasc Biol, 2004. **24**(9): p. 1558-66.
122. Farrell, D.H., et al., *Role of fibrinogen alpha and gamma chain sites in platelet aggregation*. Proc Natl Acad Sci U S A, 1992. **89**(22): p. 10729-32.
123. Bennett, J.S., *Structure and function of the platelet integrin alphaIIb beta3*. J Clin Invest, 2005. **115**(12): p. 3363-9.
124. Herrick, S., et al., *Fibrinogen*. Int J Biochem Cell Biol, 1999. **31**(7): p. 741-6.
125. Bennett, J.S., et al., *Interaction of fibrinogen with its platelet receptor. Differential effects of alpha and gamma chain fibrinogen peptides on the glycoprotein IIb-IIIa complex*. J Biol Chem, 1988. **263**(26): p. 12948-53.
126. Guedes, A.F., et al., *Sensing adhesion forces between erythrocytes and gamma' fibrinogen, modulating fibrin clot architecture and function*. Nanomedicine, 2018. **14**(3): p. 909-918.

127. Sanchez-Cortes, J. and M. Mrksich, *The platelet integrin α IIb β 3 binds to the RGD and AGD motifs in fibrinogen*. Chem Biol, 2009. **16**(9): p. 990-1000.
128. Telen, M.J., *Red blood cell surface adhesion molecules: their possible roles in normal human physiology and disease*. Semin Hematol, 2000. **37**(2): p. 130-42.
129. Weisel, J.W. and R.I. Litvinov, *Red blood cells: the forgotten player in hemostasis and thrombosis*. J Thromb Haemost, 2019. **17**(2): p. 271-282.
130. Carvalho, F.A., et al., *Atomic force microscopy-based molecular recognition of a fibrinogen receptor on human erythrocytes*. ACS Nano, 2010. **4**(8): p. 4609-20.
131. Lominadze, D. and W.L. Dean, *Involvement of fibrinogen specific binding in erythrocyte aggregation*. FEBS Lett, 2002. **517**(1-3): p. 41-4.
132. Guedes, A.F., et al., *Atomic force microscopy as a tool to evaluate the risk of cardiovascular diseases in patients*. Nat Nanotechnol, 2016. **11**(8): p. 687-92.
133. Thiagarajan, P., A.J. Rippon, and D.H. Farrell, *Alternative adhesion sites in human fibrinogen for vascular endothelial cells*. Biochemistry, 1996. **35**(13): p. 4169-75.
134. Lishko, V.K., et al., *The alternatively spliced α (E)C domain of human fibrinogen-420 is a novel ligand for leukocyte integrins α (M) β (2) and α (X) β (2)*. Blood, 2001. **98**(8): p. 2448-55.
135. Mackman, N., *New insights into the mechanisms of venous thrombosis*. J Clin Invest, 2012. **122**(7): p. 2331-6.
136. Lishko, V.K., et al., *Multiple binding sites in fibrinogen for integrin α M β 2 (Mac-1)*. J Biol Chem, 2004. **279**(43): p. 44897-906.
137. Roland Haubner, R.G., Beate Diefenbach, Simon L. Goodman, Alfred Jonczyk, and Horst Kessler, *Structural and Functional Aspects of RGD-Containing Cyclic Pentapeptides as Highly Potent and Selective Integrin α V β 3 Antagonists*. Journal of the American Chemical Society, 1996.
138. Kapp, T.G., et al., *A Comprehensive Evaluation of the Activity and Selectivity Profile of Ligands for RGD-binding Integrins*. Sci Rep, 2017. **7**: p. 39805.
139. Yakovlev, S., et al., *Interaction of fibrin(ogen) with heparin: further characterization and localization of the heparin-binding site*. Biochemistry, 2003. **42**(25): p. 7709-16.
140. Makogonenko, E., et al., *Interaction of fibrin(ogen) with fibronectin: further characterization and localization of the fibronectin-binding site*. Biochemistry, 2002. **41**(25): p. 7907-13.
141. Barczyk, M., S. Carracedo, and D. Gullberg, *Integrins*. Cell Tissue Res, 2010. **339**(1): p. 269-80.
142. Humphries, J.D., A. Byron, and M.J. Humphries, *Integrin ligands at a glance*. J Cell Sci, 2006. **119**(Pt 19): p. 3901-3.
143. Fu, Y. and G. Grieninger, *Fib420: a normal human variant of fibrinogen with two extended α chains*. Proc Natl Acad Sci U S A, 1994. **91**(7): p. 2625-8.
144. Pan, Y. and R.F. Doolittle, *cDNA sequence of a second fibrinogen α chain in lamprey: an archetypal version alignable with full-length β and γ chains*. Proc Natl Acad Sci U S A, 1992. **89**(6): p. 2066-70.
145. Strong, D.D., et al., *Lamprey fibrinogen γ chain: cloning, cDNA sequencing, and general characterization*. Biochemistry, 1985. **24**(1): p. 92-101.
146. Spraggon, G., et al., *Crystal structure of a recombinant α EC domain from human fibrinogen-420*. Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9099-104.
147. Applegate, D., et al., *The α (E)C domain of human fibrinogen-420 is a stable and early plasmin cleavage product*. Blood, 2000. **95**(7): p. 2297-303.
148. Doolittle, R.F., *A detailed consideration of a principal domain of vertebrate fibrinogen and its relatives*. Protein Sci, 1992. **1**(12): p. 1563-77.
149. Mosesson, M.W., et al., *The ultrastructure of fibrinogen-420 and the fibrin-420 clot*. Biophys Chem, 2004. **112**(2-3): p. 209-14.
150. Grieninger, G., *Contribution of the α EC domain to the structure and function of fibrinogen-420*. Ann N Y Acad Sci, 2001. **936**: p. 44-64.

151. Manco-Johnson, M.J., *Development of hemostasis in the fetus*. Thromb Res, 2005. **115 Suppl 1**: p. 55-63.
152. Andrew, M., et al., *Maturation of the hemostatic system during childhood*. Blood, 1992. **80**(8): p. 1998-2005.
153. Gibson, B., *Neonatal haemostasis*. Arch Dis Child, 1989. **64**(4 Spec No): p. 503-6.
154. Grieninger, G., et al., *Fib420, the novel fibrinogen subclass: newborn levels are higher than adult*. Blood, 1997. **90**(7): p. 2609-14.
155. Dawson, N.A., C.F. Barr, and B.M. Alving, *Acquired dysfibrinogenemia. Paraneoplastic syndrome in renal cell carcinoma*. Am J Med, 1985. **78**(4): p. 682-6.
156. Joshua, V., et al., *Antibody responses to de novo identified citrullinated fibrinogen peptides in rheumatoid arthritis and visualization of the corresponding B cells*. Arthritis Res Ther, 2016. **18**(1): p. 284.
157. Francis, J.L. and D.J. Armstrong, *Acquired dysfibrinogenemia in liver disease*. J Clin Pathol, 1982. **35**(6): p. 667-72.
158. Aurousseau, M.H., et al., *[Transient dysfibrinogenemia and thrombocytopenia associated with recurrent acute pancreatitis in the course of isotretinoin therapy]*. Rev Med Interne, 1995. **16**(8): p. 622-5.
159. Chouhan, V.D., et al., *Simultaneous occurrence of human antibodies directed against fibrinogen, thrombin, and factor V following exposure to bovine thrombin: effects on blood coagulation, protein C activation and platelet function*. Thromb Haemost, 1997. **77**(2): p. 343-9.
160. Besser, M.W. and S.G. MacDonald, *Acquired hypofibrinogenemia: current perspectives*. J Blood Med, 2016. **7**: p. 217-225.
161. Casini, A., et al., *Mutational Epidemiology of Congenital Fibrinogen Disorders*. Thromb Haemost, 2018. **118**(11): p. 1867-1874.
162. Casini, A., et al., *Natural history of patients with congenital dysfibrinogenemia*. Blood, 2015. **125**(3): p. 553-61.
163. de Moerloose, P., A. Casini, and M. Neerman-Arbez, *Congenital fibrinogen disorders: an update*. Semin Thromb Hemost, 2013. **39**(6): p. 585-95.
164. Dorgalaleh, A., *Congenital Bleeding Disorders*. 2018: Springer International Publishing AG, part of Springer Nature
165. Acharya, S.S. and D.M. Dimichele, *Rare inherited disorders of fibrinogen*. Haemophilia, 2008. **14**(6): p. 1151-8.
166. Peyvandi, F., *Epidemiology and treatment of congenital fibrinogen deficiency*. Thromb Res, 2012. **130 Suppl 2**: p. S7-11.
167. Rabe F, S.E., *Ueber-faserstoffmangel im Blute bei einem Falle von Hämophilie*. Arch Intern Med, 1920.
168. Neerman-Arbez, M., et al., *Deletion of the fibrinogen [correction of fibrogen] alpha-chain gene (FGA) causes congenital afibrinogenemia*. J Clin Invest, 1999. **103**(2): p. 215-8.
169. Attanasio, C., A. David, and M. Neerman-Arbez, *Outcome of donor splice site mutations accounting for congenital afibrinogenemia reflects order of intron removal in the fibrinogen alpha gene (FGA)*. Blood, 2003. **101**(5): p. 1851-6.
170. Stenson, P.D., et al., *The Human Gene Mutation Database: towards a comprehensive repository of inherited mutation data for medical research, genetic diagnosis and next-generation sequencing studies*. Hum Genet, 2017. **136**(6): p. 665-677.
171. Bill, B.R., et al., *A primer for morpholino use in zebrafish*. Zebrafish, 2009. **6**(1): p. 69-77.
172. Neerman-Arbez, M. and P. de Moerloose, *Mutations in the fibrinogen gene cluster accounting for congenital afibrinogenemia: an update and report of 10 novel mutations*. Hum Mutat, 2007. **28**(6): p. 540-53.
173. Mosesson, M.W., *Antithrombin I. Inhibition of thrombin generation in plasma by fibrin formation*. Thromb Haemost, 2003. **89**(1): p. 9-12.

174. de Bosch, N.B., et al., *Inhibition of thrombin generation in plasma by fibrin formation (Antithrombin I)*. Thromb Haemost, 2002. **88**(2): p. 253-8.
175. Dupuy, E., et al., *Embolized ischemic lesions of toes in an afibrinogenemic patient: possible relevance to in vivo circulating thrombin*. Thromb Res, 2001. **102**(3): p. 211-9.
176. Remijn, J.A., et al., *Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions*. Thromb Haemost, 2001. **85**(4): p. 736-42.
177. Korte, W. and A. Feldges, *Increased prothrombin activation in a patient with congenital afibrinogenemia is reversible by fibrinogen substitution*. Clin Invest, 1994. **72**(5): p. 396-8.
178. E, R., *Die fibrinogenie*. Z Klin Med, 1935.
179. Brennan, S.O., A.P. Fellowes, and P.M. George, *Molecular mechanisms of hypo- and afibrinogenemia*. Ann N Y Acad Sci, 2001. **936**: p. 91-100.
180. Casini, A., et al., *FGB mutations leading to congenital quantitative fibrinogen deficiencies: an update and report of four novel mutations*. Thromb Res, 2014. **133**(5): p. 868-74.
181. Casini, A., et al., *Protein modelling to understand FGB mutations leading to congenital hypofibrinogenemia*. Haemophilia, 2017. **23**(4): p. 583-589.
182. Casini, A., et al., *Hypofibrinogenemia and liver disease: a new case of Aguadilla fibrinogen and review of the literature*. Haemophilia, 2015. **21**(6): p. 820-7.
183. Brennan, S.O., et al., *Fibrinogen brescia: hepatic endoplasmic reticulum storage and hypofibrinogenemia because of a gamma284 Gly-->Arg mutation*. Am J Pathol, 2000. **157**(1): p. 189-96.
184. Brennan, S.O., et al., *Novel fibrinogen gamma375 Arg-->Trp mutation (fibrinogen aguadilla) causes hepatic endoplasmic reticulum storage and hypofibrinogenemia*. Hepatology, 2002. **36**(3): p. 652-8.
185. Imperato, C. and A.G. Dettori, *[Congenital hypofibrinogenemia with fibrinoasthenia]*. Helv Paediatr Acta, 1958. **13**(4): p. 380-99.
186. Blomback, M., et al., *Fibrinogen Detroit--a molecular defect in the N-terminal disulphide knot of human fibrinogen?* Nature, 1968. **218**(5137): p. 134-7.
187. Galanakis, D.K., et al., *Fibrinogen Stony Brook, a heterozygous A alpha 16Arg----Cys dysfibrinogenemia. Evaluation of diminished platelet aggregation support and of enhanced inhibition of fibrin assembly*. J Clin Invest, 1989. **84**(1): p. 295-304.
188. Casini, A., et al., *Dysfibrinogenemia: from molecular anomalies to clinical manifestations and management*. J Thromb Haemost, 2015. **13**(6): p. 909-19.
189. Flood, V.H., H.A. Al-Mondhiry, and D.H. Farrell, *The fibrinogen Aalpha R16C mutation results in fibrinolytic resistance*. Br J Haematol, 2006. **134**(2): p. 220-6.
190. Ikeda, M., et al., *Novel heterozygous dysfibrinogenemia, Sumida (AalphaC472S), showed markedly impaired lateral aggregation of protofibrils and mildly lower functional fibrinogen levels*. Thromb Res, 2015. **135**(4): p. 710-7.
191. Koopman, J., et al., *Molecular basis for fibrinogen Dusart (A alpha 554 Arg-->Cys) and its association with abnormal fibrin polymerization and thrombophilia*. J Clin Invest, 1993. **91**(4): p. 1637-43.
192. Tiscia, G.L. and M. Margaglione, *Human Fibrinogen: Molecular and Genetic Aspects of Congenital Disorders*. Int J Mol Sci, 2018. **19**(6).
193. Casini, A., P. de Moerloose, and M. Neerman-Arbez, *Clinical Features and Management of Congenital Fibrinogen Deficiencies*. Semin Thromb Hemost, 2016. **42**(4): p. 366-74.
194. Uemichi, T., et al., *A frame shift mutation in the fibrinogen A alpha chain gene in a kindred with renal amyloidosis*. Blood, 1996. **87**(10): p. 4197-203.
195. Gillmore, J.D., et al., *Hereditary fibrinogen A alpha-chain amyloidosis: clinical phenotype and role of liver transplantation*. Blood, 2010. **115**(21): p. 4313; author reply 4314-5.
196. Asselta, R., et al., *Molecular characterization of 7 patients affected by dys- or hypo-dysfibrinogenemia: Identification of a novel mutation in the fibrinogen Bbeta chain causing a gain of glycosylation*. Thromb Res, 2015. **136**(1): p. 168-74.

197. Casini, A., et al., *Genetics, diagnosis and clinical features of congenital hypodysfibrinogenemia: a systematic literature review and report of a novel mutation*. J Thromb Haemost, 2017. **15**(5): p. 876-888.
198. Haneishi, A., et al., *Recombinant variant fibrinogens substituted at residues gamma326Cys and gamma339Cys demonstrated markedly impaired secretion of assembled fibrinogen*. Thromb Res, 2009. **124**(3): p. 368-72.
199. Zhang, J.Z. and C. Redman, *Fibrinogen assembly and secretion. Role of intrachain disulfide loops*. J Biol Chem, 1996. **271**(47): p. 30083-8.
200. Vorjohann, S., et al., *Hypodysfibrinogenaemia due to production of mutant fibrinogen alpha-chains lacking fibrinopeptide A and polymerisation knob 'A'*. Thromb Haemost, 2010. **104**(5): p. 990-7.
201. Giles, F.J. and M. Albitar, *Plasma-based testing as a new paradigm for clinical testing in hematologic diseases*. Expert Rev Mol Diagn, 2007. **7**(5): p. 615-23.
202. Walker HK, H.W., Hurst JW, *Clinical Methods: The History, Physical, and Laboratory Examinations*. 3rd edition. Boston: Butterworths, 1990.
203. Winter, W.E., S.D. Flax, and N.S. Harris, *Coagulation Testing in the Core Laboratory*. Lab Med, 2017. **48**(4): p. 295-313.
204. Triplett, D.A., *Coagulation and bleeding disorders: review and update*. Clin Chem, 2000. **46**(8 Pt 2): p. 1260-9.
205. Arbustini, E., N. Narula, and A.M. D'Armini, *Fibrinogen: a circulating factor in search of its genetic architecture*. Circulation, 2013. **128**(12): p. 1276-80.
206. Fibrinogen Studies, C., et al., *Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis*. JAMA, 2005. **294**(14): p. 1799-809.
207. Lowe, G.D., *Fibrinogen assays for cardiovascular risk assessment*. Clin Chem, 2010. **56**(5): p. 693-5.
208. Lowe, G.D., *Fibrinogen and cardiovascular disease: historical introduction*. Eur Heart J, 1995. **16 Suppl A**: p. 2-5.
209. Sabater-Lleal, M., et al., *Multiethnic meta-analysis of genome-wide association studies in >100 000 subjects identifies 23 fibrinogen-associated Loci but no strong evidence of a causal association between circulating fibrinogen and cardiovascular disease*. Circulation, 2013. **128**(12): p. 1310-24.
210. Lovely, R.S., et al., *Association of gammaA/gamma' fibrinogen levels and coronary artery disease*. Thromb Haemost, 2002. **88**(1): p. 26-31.
211. Mannila, M.N., et al., *Elevated plasma fibrinogen gamma' concentration is associated with myocardial infarction: effects of variation in fibrinogen genes and environmental factors*. J Thromb Haemost, 2007. **5**(4): p. 766-73.
212. Cheung, E.Y., et al., *Fibrinogen gamma' in ischemic stroke: a case-control study*. Stroke, 2008. **39**(3): p. 1033-5.
213. Siebenlist, K.R., et al., *Studies on the basis for the properties of fibrin produced from fibrinogen-containing gamma' chains*. Blood, 2005. **106**(8): p. 2730-6.
214. Wolberg, A.S., *Primed to Understand Fibrinogen in Cardiovascular Disease*. Arterioscler Thromb Vasc Biol, 2016. **36**(1): p. 4-6.
215. Luyendyk, J.P., J.G. Schoenecker, and M.J. Flick, *The multifaceted role of fibrinogen in tissue injury and inflammation*. Blood, 2019. **133**(6): p. 511-520.
216. Davalos, D. and K. Akassoglou, *Fibrinogen as a key regulator of inflammation in disease*. Semin Immunopathol, 2012. **34**(1): p. 43-62.
217. Vilar, R., et al., *Fibrin(ogen) in human disease: both friend and foe*. Haematologica, 2020. **105**(2): p. 284-296.
218. Degen, J.L., *Hemostatic factors and inflammatory disease*. Thromb Haemost, 1999. **82**(2): p. 858-64.

219. Jennewein C., T.N., Zacharowski K, *The Inflammatory Potential of Fibrin(ogen) and its Degradation Products*. Springer, 2011. **Annual Update in Intensive Care and Emergency Medicine**
220. Ziliotto, N., et al., *Coagulation Pathways in Neurological Diseases: Multiple Sclerosis*. Front Neurol, 2019. **10**: p. 409.
221. Lin, Y., et al., *Clinical significance of plasma D-dimer and fibrinogen in digestive cancer: A systematic review and meta-analysis*. Eur J Surg Oncol, 2018. **44**(10): p. 1494-1503.
222. Hogan, K.A., H. Weiler, and S.T. Lord, *Mouse models in coagulation*. Thromb Haemost, 2002. **87**(4): p. 563-74.
223. Suh, T.T., et al., *Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice*. Genes Dev, 1995. **9**(16): p. 2020-33.
224. Drew, A.F., et al., *Wound-healing defects in mice lacking fibrinogen*. Blood, 2001. **97**(12): p. 3691-8.
225. Prasad, J.M., et al., *Mice expressing a mutant form of fibrinogen that cannot support fibrin formation exhibit compromised antimicrobial host defense*. Blood, 2015. **126**(17): p. 2047-58.
226. Holmback, K., et al., *Impaired platelet aggregation and sustained bleeding in mice lacking the fibrinogen motif bound by integrin alpha IIb beta 3*. EMBO J, 1996. **15**(21): p. 5760-71.
227. Hogan, K.A., et al., *Synthesis of a mouse model of the dysfibrinogen Vlissingen/Frankfurt IV*. Ann N Y Acad Sci, 2001. **936**: p. 117-21.
228. Hogan, K.A., et al., *Recombinant fibrinogen Vlissingen/Frankfurt IV. The deletion of residues 319 and 320 from the gamma chain of firbinogen alters calcium binding, fibrin polymerization, cross-linking, and platelet aggregation*. J Biol Chem, 2000. **275**(23): p. 17778-85.
229. Gullledge, A.A., et al., *A novel transgenic mouse model of hyperfibrinogenemia*. Thromb Haemost, 2001. **86**(2): p. 511-6.
230. Flick, M.J., et al., *Leukocyte engagement of fibrin(ogen) via the integrin receptor alphaMbeta2/Mac-1 is critical for host inflammatory response in vivo*. J Clin Invest, 2004. **113**(11): p. 1596-606.
231. Flick, M.J., et al., *Fibrin(ogen) exacerbates inflammatory joint disease through a mechanism linked to the integrin alphaMbeta2 binding motif*. J Clin Invest, 2007. **117**(11): p. 3224-35.
232. Adams, R.A., et al., *The fibrin-derived gamma377-395 peptide inhibits microglia activation and suppresses relapsing paralysis in central nervous system autoimmune disease*. J Exp Med, 2007. **204**(3): p. 571-82.
233. Vidal, B., et al., *Fibrinogen drives dystrophic muscle fibrosis via a TGFbeta/alternative macrophage activation pathway*. Genes Dev, 2008. **22**(13): p. 1747-52.
234. Wilberding, J.A., et al., *Development of pulmonary fibrosis in fibrinogen-deficient mice*. Ann N Y Acad Sci, 2001. **936**: p. 542-8.
235. Mullarky, I.K., et al., *Infection-stimulated fibrin deposition controls hemorrhage and limits hepatic bacterial growth during listeriosis*. Infect Immun, 2005. **73**(7): p. 3888-95.
236. C.Cooley, B., *Murine models of thrombosis*. Thrombosis Research, 2012: p. Pages S62-S64.
237. Diaz, J.A., et al., *Critical review of mouse models of venous thrombosis*. Arterioscler Thromb Vasc Biol, 2012. **32**(3): p. 556-62.
238. Diaz, J.A., et al., *Choosing a Mouse Model of Venous Thrombosis*. Arterioscler Thromb Vasc Biol, 2019. **39**(3): p. 311-318.
239. Jagadeeswaran, P., et al., *Animal Models of Thrombosis From Zebrafish to Nonhuman Primates: Use in the Elucidation of New Pathologic Pathways and the Development of Antithrombotic Drugs*. Circ Res, 2016. **118**(9): p. 1363-79.
240. Mackman, N., *Mouse models in haemostasis and thrombosis*. Thromb Haemost, 2004. **92**(3): p. 440-3.
241. Streisinger, G., et al., *Production of clones of homozygous diploid zebra fish (Brachydanio rerio)*. Nature, 1981. **291**(5813): p. 293-6.

242. Shahbazi, S. and R. Mahdian, *Factor VII Gene Defects: Review of Functional Studies and Their Clinical Implications*. Iran Biomed J, 2019. **23**(3): p. 165-74.
243. Fantacini, D.M., et al., *The F309S mutation increases factor VIII secretion in human cell line*. Rev Bras Hematol Hemoter, 2016. **38**(2): p. 135-40.
244. Mashayekhi, A., et al., *In vitro expression of mutant factor VII proteins and characterization of their clinical significance*. Mol Med Rep, 2018. **17**(2): p. 2738-2742.
245. Balestra, D. and A. Branchini, *Molecular Mechanisms and Determinants of Innovative Correction Approaches in Coagulation Factor Deficiencies*. Int J Mol Sci, 2019. **20**(12).
246. Santoriello, C. and L.I. Zon, *Hooked! Modeling human disease in zebrafish*. J Clin Invest, 2012. **122**(7): p. 2337-43.
247. Jagadeeswaran, P., et al., *Zebrafish: a genetic model for hemostasis and thrombosis*. J Thromb Haemost, 2005. **3**(1): p. 46-53.
248. Weyand, A.C. and J.A. Shavit, *Zebrafish as a model system for the study of hemostasis and thrombosis*. Curr Opin Hematol, 2014. **21**(5): p. 418-22.
249. Davidson, C.J., et al., *Molecular evolution of the vertebrate blood coagulation network*. Thromb Haemost, 2003. **89**(3): p. 420-8.
250. Davidson, C.J., E.G. Tuddenham, and J.H. McVey, *450 million years of hemostasis*. J Thromb Haemost, 2003. **1**(7): p. 1487-94.
251. Hanumanthaiah, R., K. Day, and P. Jagadeeswaran, *Comprehensive analysis of blood coagulation pathways in teleostei: evolution of coagulation factor genes and identification of zebrafish factor VIII*. Blood Cells Mol Dis, 2002. **29**(1): p. 57-68.
252. Kretz, C.A., A.C. Weyand, and J.A. Shavit, *Modeling Disorders of Blood Coagulation in the Zebrafish*. Curr Pathobiol Rep, 2015. **3**(2): p. 155-161.
253. Jiang, Y. and R.F. Doolittle, *The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes*. Proc Natl Acad Sci U S A, 2003. **100**(13): p. 7527-32.
254. Gore, A.V., et al., *Vascular development in the zebrafish*. Cold Spring Harb Perspect Med, 2012. **2**(5): p. a006684.
255. Carradice, D. and G.J. Lieschke, *Zebrafish in hematology: sushi or science?* Blood, 2008. **111**(7): p. 3331-42.
256. Howe, K., et al., *The zebrafish reference genome sequence and its relationship to the human genome*. Nature, 2013. **496**(7446): p. 498-503.
257. Bedell, V.M., et al., *In vivo genome editing using a high-efficiency TALEN system*. Nature, 2012. **491**(7422): p. 114-8.
258. Irion, U., J. Krauss, and C. Nusslein-Volhard, *Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system*. Development, 2014. **141**(24): p. 4827-30.
259. Prykhodzhiy, S.V., et al., *Optimized knock-in of point mutations in zebrafish using CRISPR/Cas9*. Nucleic Acids Res, 2018. **46**(17): p. e102.
260. Fish, R.J., C. Di Sanza, and M. Neerman-Arbez, *Targeted mutation of zebrafish fga models human congenital afibrinogenemia*. Blood, 2014. **123**(14): p. 2278-81.
261. Liu, Y., et al., *Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function*. Blood, 2014. **124**(1): p. 142-50.
262. Hu, Z., et al., *Genome editing of factor X in zebrafish reveals unexpected tolerance of severe defects in the common pathway*. Blood, 2017. **130**(5): p. 666-676.
263. Weyand, A.C., et al., *Analysis of factor V in zebrafish demonstrates minimal levels needed for early hemostasis*. Blood Adv, 2019. **3**(11): p. 1670-1680.
264. Kimura, Y., et al., *Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering*. Sci Rep, 2014. **4**: p. 6545.
265. Ma, D., et al., *The identification and characterization of zebrafish hematopoietic stem cells*. Blood, 2011. **118**(2): p. 289-97.

266. Traver, D., et al., *Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants*. Nat Immunol, 2003. **4**(12): p. 1238-46.
267. Lin, H.F., et al., *Analysis of thrombocyte development in CD41-GFP transgenic zebrafish*. Blood, 2005. **106**(12): p. 3803-10.
268. Khandekar, G., S. Kim, and P. Jagadeeswaran, *Zebrafish thrombocytes: functions and origins*. Adv Hematol, 2012. **2012**: p. 857058.
269. Ellertsdottir, E., et al., *Developmental role of zebrafish protease-activated receptor 1 (PAR1) in the cardio-vascular system*. PLoS One, 2012. **7**(7): p. e42131.
270. Watkins, S.C., et al., *High resolution imaging of vascular function in zebrafish*. PLoS One, 2012. **7**(8): p. e44018.
271. Rost, M.S., et al., *Nfe2 is dispensable for early but required for adult thrombocyte formation and function in zebrafish*. Blood Adv, 2018. **2**(23): p. 3418-3427.
272. Lenard, A., et al., *Using zebrafish to model erythroid lineage toxicity and regeneration*. Haematologica, 2016. **101**(5): p. e164-7.
273. Hoffman, M., *Animal models of bleeding and tissue repair*. Haemophilia, 2008. **14 Suppl 3**: p. 62-7.
274. Rosen, E.D., et al., *Laser-induced noninvasive vascular injury models in mice generate platelet- and coagulation-dependent thrombi*. Am J Pathol, 2001. **158**(5): p. 1613-22.
275. Williams, C.M. and A.W. Poole, *Using zebrafish (Danio rerio) to assess gene function in thrombus formation*. Methods Mol Biol, 2012. **788**: p. 305-19.
276. Gregory, M., R. Hanumanthaiah, and P. Jagadeeswaran, *Genetic analysis of hemostasis and thrombosis using vascular occlusion*. Blood Cells Mol Dis, 2002. **29**(3): p. 286-95.
277. K.RajpurohitSeongcheolKim, P.P.R., *Chapter 9 - Laser-Induced Thrombosis in Zebrafish*. Methods in Cell Biology, 2011. **Volume 101**: p. Pages 197-203.
278. Rost, M.S., S.J. Grzegorski, and J.A. Shavit, *Quantitative methods for studying hemostasis in zebrafish larvae*. Methods Cell Biol, 2016. **134**: p. 377-89.
279. Jagadeeswaran, P., et al., *Laser-induced thrombosis in zebrafish*. Methods Cell Biol, 2011. **101**: p. 197-203.
280. Ni, H., et al., *Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen*. J Clin Invest, 2000. **106**(3): p. 385-92.
281. Hu, Z., et al., *Loss of fibrinogen in zebrafish results in an asymptomatic embryonic hemostatic defect and synthetic lethality with thrombocytopenia*. J Thromb Haemost, 2019. **17**(4): p. 607-617.
282. Peyvandi, F., et al., *Incidence of bleeding symptoms in 100 patients with inherited afibrinogenemia or hypofibrinogenemia*. J Thromb Haemost, 2006. **4**(7): p. 1634-7.
283. Mukaddam, A., et al., *Paradoxical bleeding and thrombosis in a patient with afibrinogenemia and fibrinogen Mumbai mutation*. Am J Clin Pathol, 2015. **143**(5): p. 755-7.
284. Nagler, M., et al., *Thromboembolism in patients with congenital afibrinogenemia. Long-term observational data and systematic review*. Thromb Haemost, 2016. **116**(4): p. 722-32.
285. Korte, W., et al., *Thrombosis in Inherited Fibrinogen Disorders*. Transfus Med Hemother, 2017. **44**(2): p. 70-76.
286. Plow, E.F. and G.A. Marguerie, *Induction of the fibrinogen receptor on human platelets by epinephrine and the combination of epinephrine and ADP*. J Biol Chem, 1980. **255**(22): p. 10971-7.
287. Liu, Q., et al., *The AGDV residues on the gamma chain carboxyl terminus of platelet-bound fibrinogen are needed for platelet aggregation*. Biochim Biophys Acta, 1997. **1343**(2): p. 316-26.
288. Bonnefoy, A., et al., *Efficiency of platelet adhesion to fibrinogen depends on both cell activation and flow*. Biophys J, 2000. **78**(6): p. 2834-43.
289. Swieringa, F., et al., *Integrating platelet and coagulation activation in fibrin clot formation*. Res Pract Thromb Haemost, 2018. **2**(3): p. 450-460.

290. Jackson, S.P., *The growing complexity of platelet aggregation*. Blood, 2007. **109**(12): p. 5087-95.
291. Flood, V.H., et al., *Incorporation of fibrin molecules containing fibrinopeptide A alters clot ultrastructure and decreases permeability*. Br J Haematol, 2007. **138**(1): p. 117-24.
292. Hanss, M. and F. Biot, *A database for human fibrinogen variants*. Ann N Y Acad Sci, 2001. **936**: p. 89-90.
293. Blomback, B., et al., *Fibrin gels as biological filters and interfaces*. Biorheology, 1984. **21**(1-2): p. 93-104.
294. Galanakis, D., S. Spitzer, and I. Scharrer, *Unusual A alpha 16Arg-->Cys dysfibrinogenemic family: absence of normal A alpha-chains in fibrinogen from two of four heterozygous siblings*. Blood Coagul Fibrinolysis, 1993. **4**(1): p. 67-71.
295. Okumura, N., et al., *B:b interactions are essential for polymerization of variant fibrinogens with impaired holes 'a'*. J Thromb Haemost, 2007. **5**(12): p. 2352-9.
296. Campbell, R.A., et al., *Contributions of extravascular and intravascular cells to fibrin network formation, structure, and stability*. Blood, 2009. **114**(23): p. 4886-96.
297. Wolberg, A.S., *Plasma and cellular contributions to fibrin network formation, structure and stability*. Haemophilia, 2010. **16 Suppl 3**: p. 7-12.
298. Wolberg, A.S., *Determinants of fibrin formation, structure, and function*. Curr Opin Hematol, 2012. **19**(5): p. 349-56.
299. Weisel, J.W., *Structure of fibrin: impact on clot stability*. J Thromb Haemost, 2007. **5 Suppl 1**: p. 116-24.
300. Hoffman, M., *Alterations of fibrinogen structure in human disease*. Cardiovasc Hematol Agents Med Chem, 2008. **6**(3): p. 206-11.
301. Gersh, K.C., K.E. Edmondson, and J.W. Weisel, *Flow rate and fibrin fiber alignment*. J Thromb Haemost, 2010. **8**(12): p. 2826-8.
302. Undas, A. and R.A. Ariens, *Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases*. Arterioscler Thromb Vasc Biol, 2011. **31**(12): p. e88-99.
303. Litvinov, R.I., et al., *The Platelet Integrin alphaIIb beta3 Differentially Interacts with Fibrin Versus Fibrinogen*. J Biol Chem, 2016. **291**(15): p. 7858-67.
304. Clark, R.A., *Fibrin and wound healing*. Ann N Y Acad Sci, 2001. **936**: p. 355-67.
305. Whelan, D., N.M. Caplice, and A.J. Clover, *Fibrin as a delivery system in wound healing tissue engineering applications*. J Control Release, 2014. **196**: p. 1-8.
306. Doolittle, R.F., *Step-by-step evolution of vertebrate blood coagulation*. Cold Spring Harb Symp Quant Biol, 2009. **74**: p. 35-40.
307. Doolittle, R.F., *The evolution of vertebrate fibrinogen*. Fed Proc, 1976. **35**(10): p. 2145-9.
308. Doolittle, R.F., *The structure and evolution of vertebrate fibrinogen: a comparison of the lamprey and mammalian proteins*. Adv Exp Med Biol, 1990. **281**: p. 25-37.
309. Peterson, M.E., et al., *Evolutionary constraints on structural similarity in orthologs and paralogs*. Protein Sci, 2009. **18**(6): p. 1306-15.
310. Forslund, K., I. Pekkari, and E.L. Sonnhammer, *Domain architecture conservation in orthologs*. BMC Bioinformatics, 2011. **12**: p. 326.
311. Gabaldon, T. and E.V. Koonin, *Functional and evolutionary implications of gene orthology*. Nat Rev Genet, 2013. **14**(5): p. 360-6.
312. GRIENINGER, G., *Contribution of the α EC Domain to the Structure and Function of Fibrinogen-420*. Annals of the New York Academy of Sciences, 25 January 2006. **Volume 936**(Issue 1).
313. Zuliani-Alvarez, L. and K.S. Midwood, *Fibrinogen-Related Proteins in Tissue Repair: How a Unique Domain with a Common Structure Controls Diverse Aspects of Wound Healing*. Adv Wound Care (New Rochelle), 2015. **4**(5): p. 273-285.
314. Novoa, B. and A. Figueras, *Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases*. Adv Exp Med Biol, 2012. **946**: p. 253-75.

315. Monagle, P., V. Ignjatovic, and H. Savoia, *Hemostasis in neonates and children: pitfalls and dilemmas*. Blood Rev, 2010. **24**(2): p. 63-8.
316. Appel, I.M., et al., *Age dependency of coagulation parameters during childhood and puberty*. J Thromb Haemost, 2012. **10**(11): p. 2254-63.
317. Andrew, M., et al., *Development of the human coagulation system in the healthy premature infant*. Blood, 1988. **72**(5): p. 1651-7.
318. Hanmod, S.S., et al., *Neonatal Hemostatic Disorders: Issues and Challenges*. Semin Thromb Hemost, 2016. **42**(7): p. 741-751.
319. Saxonhouse, M.A. and M.J. Manco-Johnson, *The evaluation and management of neonatal coagulation disorders*. Semin Perinatol, 2009. **33**(1): p. 52-65.
320. Will, A., *Neonatal haemostasis and the management of neonatal thrombosis*. Br J Haematol, 2015. **169**(3): p. 324-32.
321. Reverdiau-Moalic, P., et al., *Evolution of blood coagulation activators and inhibitors in the healthy human fetus*. Blood, 1996. **88**(3): p. 900-6.
322. Nuss, M.M.-J.a.R., *Hemostasis in the Neonate*. NeoReviews, 2000: p. 1 (10) e191-e195.
323. Manco-Johnson, M.J., *Hemostasis in the Neonate*. NeoReviews, 2008: p. 9 (3) e119-e123.
324. Matthew A.Saxonhouse, M.J.M.-J., *The Evaluation and Management of Neonatal Coagulation Disorders*
Seminars in perinatology, 2009. **Volume 33**(Issue 1): p. 52-65.
325. Andrew, M.E., et al., *Thromboembolic disease and antithrombotic therapy in newborns*. Hematology Am Soc Hematol Educ Program, 2001: p. 358-74.
326. Andrew, M.M., Monagle, P.T. & Brooker, R.T. , *Thromboembolic Complications during Infancy and Childhood*. . BC Decker, Hamilton, pp. 5-47. 2000.
327. Lappin, D.P.K.S.L., *Physiology, Fetal Hemoglobin* 2019: StatPearls.
328. Sankaran, V.G. and S.H. Orkin, *The switch from fetal to adult hemoglobin*. Cold Spring Harb Perspect Med, 2013. **3**(1): p. a011643.
329. Favaloro, E.J. and G. Lippi, *Translational aspects of developmental hemostasis: infants and children are not miniature adults and even adults may be different*. Ann Transl Med, 2017. **5**(10): p. 212.
330. Margraf, A., C. Nussbaum, and M. Sperandio, *Ontogeny of platelet function*. Blood Adv, 2019. **3**(4): p. 692-703.
331. Tang, H., et al., *Fibrinogen has chaperone-like activity*. Biochem Biophys Res Commun, 2009. **378**(3): p. 662-7.
332. Marton, Z., et al., *Red blood cell aggregation measurements in whole blood and in fibrinogen solutions by different methods*. Clin Hemorheol Microcirc, 2001. **24**(2): p. 75-83.
333. Guedes, A.F., et al., *Fibrinogen-erythrocyte binding and hemorheology measurements in the assessment of essential arterial hypertension patients*. Nanoscale, 2019. **11**(6): p. 2757-2766.
334. Brust, M., et al., *The plasma protein fibrinogen stabilizes clusters of red blood cells in microcapillary flows*. Sci Rep, 2014. **4**: p. 4348.
335. Carvalho, F.A., et al., *Variations on fibrinogen-erythrocyte interactions during cell aging*. PLoS One, 2011. **6**(3): p. e18167.
336. Carvalho, F.A., et al., *The 95RGD97 sequence on the Aalpha chain of fibrinogen is essential for binding to its erythrocyte receptor*. Int J Nanomedicine, 2018. **13**: p. 1985-1992.
337. Kononova, O., et al., *Mechanistic Basis for the Binding of RGD- and AGDV-Peptides to the Platelet Integrin alphaIIb beta3*. Biochemistry, 2017. **56**(13): p. 1932-1942.
338. Adamson, K., et al., *Fibrinogen Motif Discriminates Platelet and Cell Capture in Peptide-Modified Gold Micropore Arrays*. Langmuir, 2018. **34**(2): p. 715-725.
339. Lin, F.Y., et al., *beta-Subunit Binding Is Sufficient for Ligands to Open the Integrin alphaIIb beta3 Headpiece*. J Biol Chem, 2016. **291**(9): p. 4537-46.

340. Lu, X., et al., *The effect of the single substitution of arginine within the RGD tripeptide motif of a modified neurotoxin dendroaspin on its activity of platelet aggregation and cell adhesion.* Cell Commun Adhes, 2006. **13**(3): p. 171-83.
341. Koopman, J., et al., *Fibrinogen Marburg: a homozygous case of dysfibrinogenemia, lacking amino acids A alpha 461-610 (Lys 461 AAA-->stop TAA).* Blood, 1992. **80**(8): p. 1972-9.
342. Soria, J., et al., *Fibrinogen alphaC domain: Its importance in physiopathology.* Res Pract Thromb Haemost, 2019. **3**(2): p. 173-183.
343. Furlan, M., et al., *A frameshift mutation in Exon V of the A alpha-chain gene leading to truncated A alpha-chains in the homozygous dysfibrinogen Milano III.* J Biol Chem, 1994. **269**(52): p. 33129-34.
344. Collen, A., et al., *Aberrant fibrin formation and cross-linking of fibrinogen Nieuwegein, a variant with a shortened Aalpha-chain, alters endothelial capillary tube formation.* Blood, 2001. **97**(4): p. 973-80.
345. Sullivan, C. and C.H. Kim, *Zebrafish as a model for infectious disease and immune function.* Fish Shellfish Immunol, 2008. **25**(4): p. 341-50.
346. Neely, M.N., J.D. Pfeifer, and M. Caparon, *Streptococcus-zebrafish model of bacterial pathogenesis.* Infect Immun, 2002. **70**(7): p. 3904-14.
347. Lam, S.H., et al., *Morphologic transformation of the thymus in developing zebrafish.* Dev Dyn, 2002. **225**(1): p. 87-94.
348. Traver, D., et al., *The zebrafish as a model organism to study development of the immune system.* Adv Immunol, 2003. **81**: p. 253-330.
349. Meijer, A.H. and H.P. Spaik, *Host-pathogen interactions made transparent with the zebrafish model.* Curr Drug Targets, 2011. **12**(7): p. 1000-17.
350. van der Sar, A.M., et al., *Zebrafish embryos as a model host for the real time analysis of Salmonella typhimurium infections.* Cell Microbiol, 2003. **5**(9): p. 601-11.
351. O'Toole, R., et al., *Visualisation of zebrafish infection by GFP-labelled Vibrio anguillarum.* Microb Pathog, 2004. **37**(1): p. 41-6.
352. Torraca, V., et al., *Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model.* Dis Model Mech, 2014. **7**(7): p. 785-97.
353. Davis, J.M., et al., *Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos.* Immunity, 2002. **17**(6): p. 693-702.
354. Mathias, J.R., et al., *Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish.* J Leukoc Biol, 2006. **80**(6): p. 1281-8.
355. Renshaw, S.A., et al., *A transgenic zebrafish model of neutrophilic inflammation.* Blood, 2006. **108**(13): p. 3976-8.
356. Rissone, A. and S.M. Burgess, *Rare Genetic Blood Disease Modeling in Zebrafish.* Front Genet, 2018. **9**: p. 348.
357. Howe, D.G., et al., *The Zebrafish Model Organism Database: new support for human disease models, mutation details, gene expression phenotypes and searching.* Nucleic Acids Res, 2017. **45**(D1): p. D758-D768.
358. Bradford, Y.M., et al., *Zebrafish Models of Human Disease: Gaining Insight into Human Disease at ZFIN.* ILAR J, 2017. **58**(1): p. 4-16.
359. Jagadeeswaran, P., et al., *Identification and characterization of zebrafish thrombocytes.* Br J Haematol, 1999. **107**(4): p. 731-8.
360. Brownlie, A., et al., *Characterization of embryonic globin genes of the zebrafish.* Dev Biol, 2003. **255**(1): p. 48-61.
361. Kulkeaw, K. and D. Sugiyama, *Zebrafish erythropoiesis and the utility of fish as models of anemia.* Stem Cell Res Ther, 2012. **3**(6): p. 55.
362. Davidson, A.J. and L.I. Zon, *The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis.* Oncogene, 2004. **23**(43): p. 7233-46.
363. Amatruda, J.F. and L.I. Zon, *Dissecting hematopoiesis and disease using the zebrafish.* Dev Biol, 1999. **216**(1): p. 1-15.

364. Childs, S., et al., *Zebrafish dracula encodes ferrochelatase and its mutation provides a model for erythropoietic protoporphyria*. *Curr Biol*, 2000. **10**(16): p. 1001-4.
365. Ransom, D.G., et al., *Characterization of zebrafish mutants with defects in embryonic hematopoiesis*. *Development*, 1996. **123**: p. 311-9.
366. Shafizadeh, E., et al., *Characterization of zebrafish merlot/chablis as non-mammalian vertebrate models for severe congenital anemia due to protein 4.1 deficiency*. *Development*, 2002. **129**(18): p. 4359-70.
367. Davis, E.E., S. Frangakis, and N. Katsanis, *Interpreting human genetic variation with in vivo zebrafish assays*. *Biochim Biophys Acta*, 2014. **1842**(10): p. 1960-1970.
368. Blum, M., et al., *Morpholinos: Antisense and Sensibility*. *Dev Cell*, 2015. **35**(2): p. 145-9.
369. Khan, S.H., *Genome-Editing Technologies: Concept, Pros, and Cons of Various Genome-Editing Techniques and Bioethical Concerns for Clinical Application*. *Mol Ther Nucleic Acids*, 2019. **16**: p. 326-334.
370. Niederriter, A.R., et al., *In vivo modeling of the morbid human genome using Danio rerio*. *J Vis Exp*, 2013(78): p. e50338.
371. Holtzman, N.G., et al., *Learning to Fish with Genetics: A Primer on the Vertebrate Model Danio rerio*. *Genetics*, 2016. **203**(3): p. 1069-89.
372. You, L., et al., *Advancements and Obstacles of CRISPR-Cas9 Technology in Translational Research*. *Mol Ther Methods Clin Dev*, 2019. **13**: p. 359-370.
373. Adli, M., *The CRISPR tool kit for genome editing and beyond*. *Nat Commun*, 2018. **9**(1): p. 1911.
374. Lino, C.A., et al., *Delivering CRISPR: a review of the challenges and approaches*. *Drug Deliv*, 2018. **25**(1): p. 1234-1257.
375. Woods, I.G., et al., *A comparative map of the zebrafish genome*. *Genome Res*, 2000. **10**(12): p. 1903-14.
376. Fish, R.J., et al., *Developmental expression and organisation of fibrinogen genes in the zebrafish*. *Thromb Haemost*, 2012. **107**(1): p. 158-66.
377. Ponczek, M.B., D. Gailani, and R.F. Doolittle, *Evolution of the contact phase of vertebrate blood coagulation*. *J Thromb Haemost*, 2008. **6**(11): p. 1876-83.
378. Parsons, M.J., et al., *Zebrafish mutants identify an essential role for laminins in notochord formation*. *Development*, 2002. **129**(13): p. 3137-46.
379. Lang, M.R., et al., *Hemostasis in Danio rerio: is the zebrafish a useful model for platelet research?* *J Thromb Haemost*, 2010. **8**(6): p. 1159-69.
380. Jagadeeswaran, P., *Zebrafish: a tool to study hemostasis and thrombosis*. *Curr Opin Hematol*, 2005. **12**(2): p. 149-52.

APPENDIX

Appendix A: Multiple sequence alignment

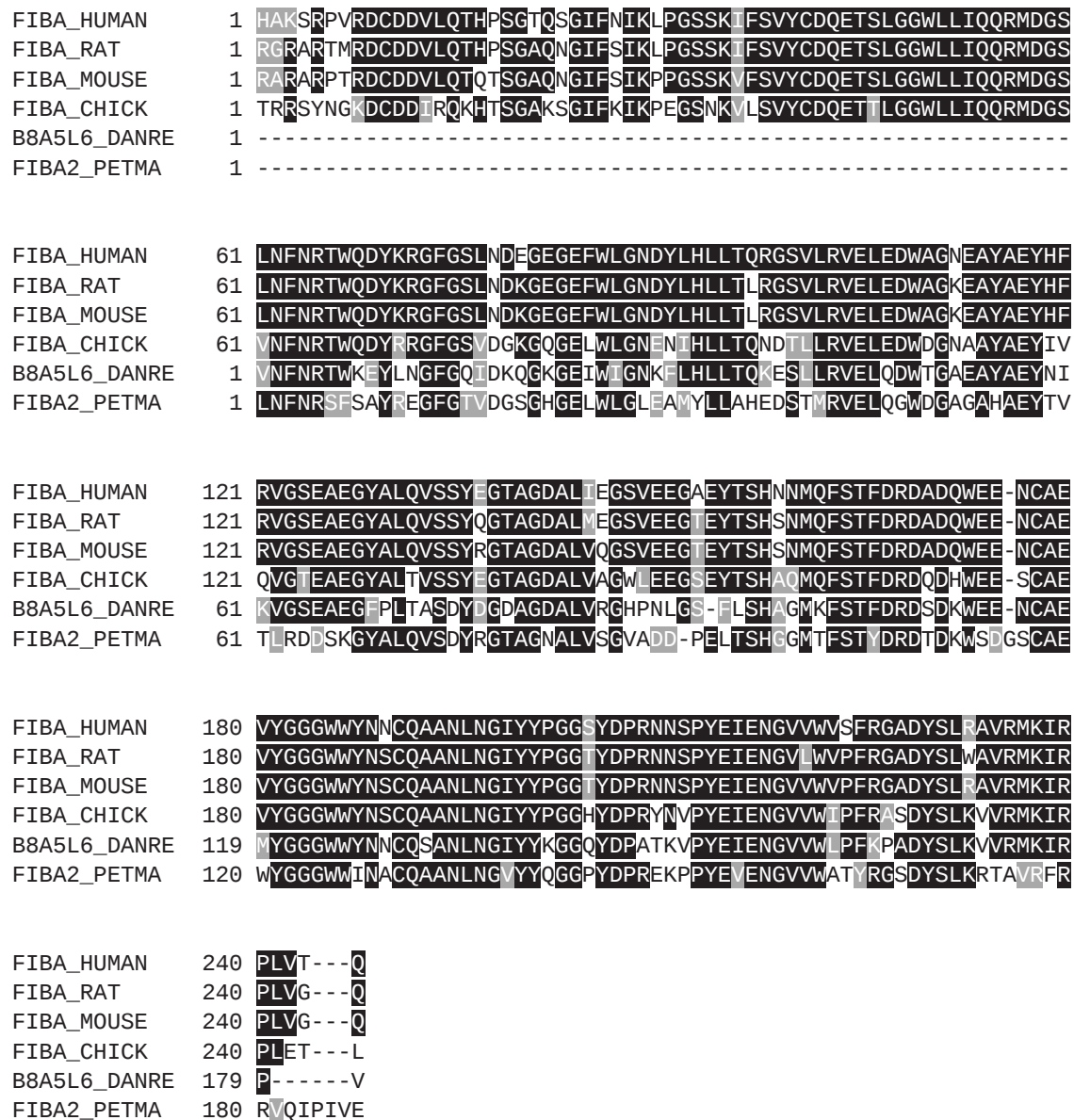


Figure A-1. Multiple protein sequence alignment of AaE C-terminal domains from several vertebrate species (human, rat, mouse, chicken, zebrafish (*Danio rerio*) and *Petromyzon marinus*) was generated using T-Coffee server and then the figure was formatted using the BoxShade server.

(a)

FIBB_HUMAN	1	NIPVVS	GKECEE	IIRKGG	ETSEMY	LIQPD	SSVKPY	RVYCD	MNTENG	GGWTV	IQNRQ	DGSVD					
Q6NVE1_DANRE	1	P	IPVVS	GKECED	IIRKGG	EDSQMY	IIRPD	PLGTPY	KVFC	DQTSK	NGGWV	IQNRMDGSVD					
FIBB_HUMAN	61	FGRKWD	PYKQ	GFGNVA	TNTD	GK	NYCGL	PGEYWL	GNDKIS	QLTR	MGPTELL	IEMEDW	KGDK				
Q6NVE1_DANRE	61	FGRRWDD	YRGR	GFGNIA	FDV	-GK	GHCTP	GGEYWL	GNDRIS	QLSK	MGATELL	IEMEDW	SGSK				
FIBB_HUMAN	121	VKAHY	GGFTV	QNEANK	YQISV	NKYR	GTAGN	ALMD	GASQLM	GENRT	MTHNGM	FFSTY	DRD				
Q6NVE1_DANRE	120	VYAQY	EQFSM	QGEAS	NYILG	VGRYS	GTAGN	TFLE	GATELF	GENRT	MTHNGM	FFSTY	DRD				
FIBB_HUMAN	181	NDGW	LTS	DP	RKQ	CSKED	GGGW	WYNR	CHAA	NPNG	RYWGG	QYTD	MAKHGT	DDGV	VWMNW	K	
Q6NVE1_DANRE	180	NDKW	IPG	DPS	KQ	CSKED	GGGW	WYNR	CH	SCNP	NGRY	WGGAY	TKY	MAKHGT	DDGV	VWMNW	K
FIBB_HUMAN	241	GSWYS	MRK	SMK	KIRP	FFP	QQ										
Q6NVE1_DANRE	240	GSWYS	LKT	ISM	KIRP	YFK	QK										

(b)

FIBG_HUMAN	1	QI	H	D	I	T	G	K	D	C	Q	D	I	A	N	K	G	A	K	Q	S	G	L	Y	F	I	K	P	L	K	A	N	Q	Q	F	L	V	Y	C	E	I	D	S	G	S	N	G	W	T	V	F	Q	K	R	L	D	G	S	V	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
Q7ZVG7_DANRE	1	E	I	Q	T	V	T	G	K	D	C	Q	D	I	A	N	K	G	K	V	S	G	L	Y	Y	V	K	P	A	R	A	E	A	F	L	V	Y	C	E	I	D	S	F	G	R	G	W	T	V	L	Q	R	R	R	D	G	S	V	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
FIBG_HUMAN	61	F	K	K	N	W	I	Q	Y	K	E	G	F	G	H	L	S	P	T	G	T	E	F	W	L	G	N	E	K	I	H	L	I	S	T	Q	S	A	I	P	Y	A	L	R	V	E	L	D	W	N	G	R	T	S	T	A	D	Y	A																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
Q7ZVG7_DANRE	61	F	S	K	N	W	I	Q	Y	K	E	G	F	G	Y	L	S	P	D	D	R	T	E	F	W	L	G	N	E	K	I	H	L	S	V	Q	S	S	V	P	Y	V	L	R	I	E	M	V	D	W	E	G	N	K	K	Y	A	D	Y	A																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
FIBG_HUMAN	121	M	F	K	V	G	P	E	A	D	K	Y	R	L	T	Y	A	Y	F	A	G	G	D	A	G	D	A	F	D	G	F	D	G	D	P	S	D	K	F	T	S	H	N	G	M	Q	F	S	T	W	D	N	D	N	D	K	F	E																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			
Q7ZVG7_DANRE	121	T	F	K	L	G	P	E	V	D	A	Y	R	L	T	Y	A	Y	F	F	G	G	D	A	G	D	A	F	D	G	F	D	G	D	P	S	D	K	F	Y	T	S	H	N	G	M	Q	F	S	T	V	D	R	D	N	D	K	F	Q																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
FIBG_HUMAN	181	G	N	C	A	E	Q	D	G	S	G	W	M	N	K	C	H	A	G	H	L	N	G	V	Y	Y	Q	G	G	T	Y	S	K	A	S	T	P	N	G	Y	D	N	G	I	I	W	A	T	W	K	T	R	W	Y	S	M	K	K	T	T																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
Q7ZVG7_DANRE	181	G	H	C	A	Q	Q	D	G	S	G	W	M	N	R	C	H	A	A	H	L	N	G	K	Y	Y	Q	G	G	K	Y	T	E	K	D	A	E	S	G	Y	D	N	G	I	I	W	A	T	W	H	S	R	W	Y	S	L	K	E	T	T																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
FIBG_HUMAN	241	M	K	I	I	P	F	N	R	L	T	I	G	E	G	Q	Q	H	L	G	G	A	K	Q	A	-	-	-	G	D	V																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														</

Figure A-2. Multiple protein sequence alignment of the C-terminal domains from B β (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 B β 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-FSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSAACKDSWPFCSD
E9QCD1_DANRE	1	MKFTHSLLCLFVAVSSA-----LAEDTVVNPRGARPIEHGFKAQDTCQTKWPFGCTDD
FIBA_HUMAN	60	WNYKCPSGCRMKGLIDEVNQDFTNRINKLKNLSIFEYQKNNKDSHSLTTNIMEILRGDFSS
E9QCD1_DANRE	56	WGSKCPSGCRVQGLMDKADHDLLKKIEKIRRLIVEGQKLYRSADEVSKTTYTYLREKLTS
FIBA_HUMAN	120	ANNRDNNTYNRVSEDLRSRIEVLKRVIEKQHIQLLQKNVFAQLVDMKRLEVDIDIKIRS
E9QCD1_DANRE	116	NSGYDNRYMSLAELRQRITDIKIKIDRQLRLDALKSQVKDQVVMQRLVVDIDIKLRT
FIBA_HUMAN	180	CRGSCSRALAREVDLKDVEDQKQLEQVIAKDLPSRDRQHPLIKMKPVPD-LVPGNFK
E9QCD1_DANRE	176	CKGSCASYSEFSVDRESVYTLDKQMDNLNAMRVQSVETVSSLGIMKSRPKDVLVPTIYK
FIBA_HUMAN	239	SQLOKVPPEWKALTDMPQMRMELERPGGNEITRGGSYGTGSETESPRNPSSAGSWNSG
E9QCD1_DANRE	236	SGTGKAEQK-LLFGDVGQMQLSLEAEGSTAESAATVS-----
FIBA_HUMAN	299	SSGPGSTGNRNPSSSGTGGTATWKPGSSGPGSTGSWNSGSSGTGSTGNQNPSPRPGSTG
E9QCD1_DANRE	272	-----KFP-----TSGTGST-----
FIBA_HUMAN	359	TWNPGSSERGSAGHWTSESSVSGSTGQWHSESGSFRPDSPGSGNARPNPDWGTFFEEVSG
E9QCD1_DANRE	282	-----
FIBA_HUMAN	419	NVSPGTRREYHTEKLVTSKGDKELRGKEKVTSGSTTTTRSCSKTVTKTVIG-PDGHKE
E9QCD1_DANRE	282	-----SQTSSKQCTKSVRKVTHTKDGPV-
FIBA_HUMAN	478	VTKEVVTSEDGSDCPEAMDGLTSGIGTLDGFRHRHPDEAAFFDFASTGKTFPGFFSPML
E9QCD1_DANRE	306	--EKVEVSSSGPGCDD--LGKL-GT-----SSEFLTAAKDGKDLRS-----
FIBA_HUMAN	538	GEFVSEITSRGSESGTFTNTKESSSHHPGIAE--FPSR-----GK
E9QCD1_DANRE	343	-DGLKITVTGGDEKS-LTSLSHSTSDLGFGCGDFFKNLGTDNRYASSSSSTSTKTVSDG
FIBA_HUMAN	576	SSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEADHETHSTKRGHAKSRPVRG-IHT
E9QCD1_DANRE	401	SKSYSKTTLTSD-----PIFGDGLGAFMRGVVEEDMPDIHARSVKSQDDRKA
FIBA_HUMAN	635	SPLGKPSLSP
E9QCD1_DANRE	448	GFVGGGTLE-

(b)

FIBA_HUMAN	1	M-FSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSAACKDSWPFCSD
B8A5L6_DANRE	1	MKFTHSLLCLFVAVSSAL-----AEEDTVVNPRGARPIEHGFKAQDTCQTKWPFGCTDD
FIBA_HUMAN	60	WNYKCPSGCRMKGLIDEVNQDFTNRINKLKNLSIFEYQKNNKDSHSLTTNIMEILRGDFSS
B8A5L6_DANRE	56	WGSKCPSGCRVQGLMDKADHDLLKKIEKIRRLIVEGQKLYRSADEVSKTTYTYLREKLTS
FIBA_HUMAN	120	ANNRDNNTYNRVSEDLRSRIEVLKRVIEKQHIQLLQKNVFAQLVDMKRLEVDIDIKIRS
B8A5L6_DANRE	116	NSGYDNRYMSLAELRQRITDIKIKIDRQLRLDALKSQVKDQVVMQRLVVDIDIKLRT
FIBA_HUMAN	180	CRGSCSRALAREVDLKDVEDQKQLEQVIAKDLPSRDRQHPLIKMKPVPD-LVPGNFK
B8A5L6_DANRE	176	CKGSCASYSEFSVDRESVYTLDKQMDNLNAMRVQSVETVSSLGIMKSRPKDVLVPTIYK
FIBA_HUMAN	239	SQLOKVPPEWKALTDMPQMRMELERPGGNEITRGGSYGTGSETESPRNPSSAGSWNSG
B8A5L6_DANRE	236	SGTGKAEQK-LLFGDVGQMQLSLEAEGSTAESAATVS-----
FIBA_HUMAN	299	SSGPGSTGNRNPSSSGTGGTATWKPGSSGPGSTGSWNSGSSGTGSTGNQNPSPRPGSTG
B8A5L6_DANRE	272	-----KFP-----SGTGST-----

FIBA_HUMAN	359	TWNP GSS ERGSAGHWTSESSVSGSTGQWHSESGSFRPDSPGSGNARNPNPDWGTFFEEVSG
B8A5L6_DANRE	282	-----
FIBA_HUMAN	419	NVSPGTRREYHTEKLVTSKGDKELR TGKEKVTSGSTTTTRFSCSKTVTKTVIGP-DGHKE
B8A5L6_DANRE	282	-----SQTSSKQCTKSVRKVLTHTKDGPV-
FIBA_HUMAN	478	VTKEVVTSEDGSDCP EAMD LGT LSGIGTLDGFRHRHPDEAAFFD TASTGKTFPGFFSPML
B8A5L6_DANRE	306	--EKVEVSSSGPGCD DLGKLG----I-----SSEFLTA AKDGKDL SR-----
FIBA_HUMAN	538	GEFVSETE SRGSESGTFTNTKESSSHHPGI-----AEFP SRG
B8A5L6_DANRE	343	-DGLKI TVTGGDEKS-LTSLSHSTSDLGFGG DFFKNLGT DNRKYASSSSSTSTKTVSDG
FIBA_HUMAN	575	KSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSE-----ADHEGTHSTKRGHAKSR-
B8A5L6_DANRE	401	-SKSYSKTTLTS-----DPIFGD DLGAFMRGDVEEDMPDIHARSVKSQDDRK
FIBA_HUMAN	628	---PVRDCDDVLQTHPSGTQSGTFNKLPGSSKTF SVYCDQETSLGGWLLTQQRMDGSLN
B8A5L6_DANRE	447	AGFVGGDCVETQQKHVNGGQSGMFKIKPAGSEEVVEVYCDQSTGLGGWTLVQQRDGSVN
FIBA_HUMAN	685	FNRTWQDYKRGFGSLNDEGEGEFWLGNDY LHL LTQRGSVLRVEL EDWAGNEAYAEYHFRV
B8A5L6_DANRE	507	FNRTWK EYLNFGFGQIDKQKGGEIWI GNKFLHL LTQKESVLRVELQDWTGAEAYAEYNIKV
FIBA_HUMAN	745	GSEAEGALQVSSYEGTAGDALIEGSVEEGAEYTS HNNMQFSTFDRDADQWEENCAEYVG
B8A5L6_DANRE	567	GSEAEGPLTASDYDGDAGDALVRGHPNLGS-FLSHAGMKFSTFDRDSDKWEENCAEYVG
FIBA_HUMAN	805	GGWYNNCQAANLNGIYYPGGSYDPRNNSPYEIENG VVWV SFRGADYSLRAVRMKIRPLV
B8A5L6_DANRE	626	GGWYNNCQSANLNGIYYKGGQYDPATKVPYEIENG VVWV PFKPADYSLKVRMKIRPV-
FIBA_HUMAN	865	TQ
B8A5L6_DANRE		--

Figure A-3. Multiple protein sequence alignment of A α (a) and A α E (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 A α fibrinogen chain between humans and zebrafish is 18.457%. (b) The percentage of identity of the A α E fibrinogen chain between humans and zebrafish is: 32.534%.

FIBA_HUMAN	1	HAKSRPVRDCDDVLQTHPSGTQSGIFNKLPGSSKIFS	VYCDQETS	LGGWLLTQQRMDGS
B8A5L6_DANRE	1	RKAGFVGGDCVEIQQKHVNGGQSGMFKIKPAGSEEVVE	VYCDQSTGLGGWTLVQQREDGS	
FIBA_HUMAN	61	LNFNRTWQDYKRGFGSLNDEGEGEFWLGNDY	LHLLTQRGSLRVELEDWAGNEAYAEYHF	
B8A5L6_DANRE	61	VNFNRTWKEYLNGFGQIDKQGGKEIWIGNKFLHLLTQKES	LLRVELQDWTGAEAYAEYNI	
FIBA_HUMAN	121	RVGSEAEGYALQVSSYEGTAGDALIEGSVEEGAEYTS	HNNMQFSTFDRDADQWEENCAEV	
B8A5L6_DANRE	121	KVGSEAEGFPLTASDYDGDAGDALVRGHPNLGS-FLSHAGMK	FSTFDRDSDKWEENCAEM	
FIBA_HUMAN	181	YGGGWYNNCQAANLNGIYYPGGSYDPRNNSPYEIE	NGVVM/SFRGADYSLRAVRMKIRP	
B8A5L6_DANRE	180	YGGGWYNNCQSANLNGIYYKGGQYDPATKV	PYEIEINGVVM/LPEKPADYSLKAVRMKIRP	
FIBA_HUMAN	241	LVTQ		
B8A5L6_DANRE	240	V---		

Figure A-4. Multiple protein sequence alignment of the AaE 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  HAKSRPVDCDDVLQTHPSGTQSGIFNIKLPGSSKIFSVYCDQETSLGGWLLIQORMDGS
FIBB_HUMAN    1  NIPVVSGKECEEIIRK--GGETSEMYLIQPDSSVKPYRVYCDMNTENGWTVIQNRQDGS

FIBA_HUMAN    61  LNFNRTWQDYKRGFGLND--EG-----EGEFWLGN DYLHLLTQRGS-VLRVELEDWAG
FIBB_HUMAN    59  VDFGRKWDYPYKQGFGLNATNTDGKNYCGLPGEYWLGN DKISQLTRMGPTELLTEMEDWKG

FIBA_HUMAN    112 NEAYAEYH-FRVGSEAEGYALQVSSYEGTAGDALIEGSVE--EGAEYTSNNMQFSTFD
FIBB_HUMAN    119 DKVKAHYGGFTVQNEANKYQLSVNKYRGTAGNALMDGASQLMGENRTMTIHNGMFFSTYD

FIBA_HUMAN    168 RDADQWE-----ENCAEVYGGGWYNNCQAANLNGIYYPGGSYDPRNNSPYEIENG VVWV
FIBB_HUMAN    179 RDNDGWLTS DPRKQCSKEDGGGWYNNRCHAANPNGRYYWGGQYT-WDMAKHGTDG VVWM

FIBA_HUMAN    223 SFRGADYSLRVRMKIRPLVT-Q
FIBB_HUMAN    238 NWKGSWYSMRKMSMKIRPFPPQQ

```

(b)

```

FIBA_HUMAN    1  HAKSRPVDCDDVLQTHPSGTQSGIFNIKLPGSSKIFSVYCDQETSLGGWLLIQORMDGS
FIBG_HUMAN    1  QIHDITGKDCQDIAN--KGAKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDGS

FIBA_HUMAN    61  LNFNRTWQDYKRGFGLNDEGEFEWLGN DYLHLLTQRG---SVLRVELEDWAGNEAYAE
FIBG_HUMAN    59  VDFKKNWIQYKEGFGHLSPTGTTEFWLGNEKIHLISTQSAIPYALRVELEDWNGRTSTAD

FIBA_HUMAN    118 Y-HFRVGSEAEGYALQVSSYEG-TAGDALI---EGSVEEGAEYTSNNMQFSTFDRDADQ
FIBG_HUMAN    119 YAMFKVGPEADKYRLTYAYFAGGDAGDAFDGDFGDDPSDKFFTSNNGMQFSTWDNDNDK

FIBA_HUMAN    173 WEENCAEVYGGGWYNNCQAANLNGIYYPGGSYDPRNNSPYEIENG VVWVSFRGADYSLR
FIBG_HUMAN    179 FEGNCAEQDGS GWMNKKCHACHLNGVYYQG GTYS-KASTPNGYDNGI IWATWKTRWYSMK

FIBA_HUMAN    223 AVRMRKIRPLVTQ-----
FIBG_HUMAN    238 KTTMKIIPFNRLTIGEGQQHHLGGAKQAGDV

```

(c)

```

FIBB_HUMAN    1  NIPVVSGKECEEIIRKGGGETSEMYLIQPDSSVKPYRVYCDMNTENGWTVIQNRQDGSVD
FIBG_HUMAN    1  QIHDITGKDCQDIANKGAKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDGSVD

FIBB_HUMAN    61  FGRKWDYPYKQGFGLNATNTDGKNYCGLPGEYWLGN DKISQLTRM--GPTELLTEMEDWKG
FIBG_HUMAN    61  FKKNWIQYKEGFGHLSPT-----G-TTEFWLGNEKIHLISTQSAIPYALRVELEDWNG

FIBB_HUMAN    119 DKVKAHYGGFTVQNEANKYQLSVNKYRG-TAGNALMDGASQ-LMGENRTMTIHNGMFFST
FIBG_HUMAN    113 RTSTADYAMFKVGPEADKYRLTYAYFAGGDAGDA-FDGDFGDDPSDKFFTSNNGMQFST

FIBB_HUMAN    177 YDRDNDGWLTS DPRKQCSKEDGGGWYNNRCHAANPNGRYYWGGQYTWDMAKHGTDG VVW
FIBG_HUMAN    172 WDNDNDKFE-----EGNCAEQDGS GWMNKKCHACHLNGVYYQG GTYSKASTPNGYDNGI IW

FIBB_HUMAN    237 MNWKGSWYSMRKMSMKIRPFPPQQ-----
FIBG_HUMAN    227 ATWKTRWYSMKKTTMKIIPFNRLTIGEGQQHHLGGAKQAGDV

```

Figure A-5. Multiple protein sequence alignment of the C-terminal globular domain from: (a) A α E and B β human fibrinogen chains, (b) A α E and γ human fibrinogen chains; and (c) B β 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	PIPVVSGKECEDIIIRKG--GEDSQMYIIRPDPLGTPYKVFCDQTSKNGGWLTQNRMDS
B8A5L6_DANRE	61	VNFNRTWKEYLNGFGQID-----KQKGGEIWIQNKFLHLLTQ-KESLLRVELQDWTGA
Q6NYE1_DANRE	59	VDFGRWDDYRRGFGNIAFDVGKGHCQTPGEYWLGNDRISQLSKMGATELLVEMEDWSGS
B8A5L6_DANRE	113	EAYAEYN- IKVGSEAEGFPLTASDYDGDAGDALVRGHPNLG---SFLSHAGMKFSTFDR
Q6NYE1_DANRE	119	KVYAQYEQFSMQGEASNYILGVGRYSGTAGNTFLEGATELFGENRTMTIHNMGMFSTYDR
B8A5L6_DANRE	168	DSDKWE-----ENCAEMYGGGWYNNCQSANLNGIYYKGGQYDPATKVPYEIENGVVWLP
Q6NYE1_DANRE	179	DNDKWIIPGDPSKQCSKEDGGGWYNNRCHSCNPNGRYWGGAYTKY-MAKHGTDGIVWMN
B8A5L6_DANRE	223	FKPADYSLKVVVRMKIRPV----
Q6NYE1_DANRE	238	WKGSWYSLKTIISMKIRPYFKQK

(b)

B8A5L6_DANRE	1	RKAGFVGGDCVEIQQKHVNGGQSGMFKIKPAGSEEVVEVYCDQSTGLGGWTLVQQREDGS
Q7ZVG7_DANRE	1	EIQTVTGKDCQDIANK--GGKVSGLYYVKPARAPEAFLVYCEIDSFGRGWTVLQRRRDGS
B8A5L6_DANRE	61	VNFNRTWKEYLNGFGQIDKQKGGEIWIQNKFLHLLTQKES---LLRVELQDWTGAAYAE
Q7ZVG7_DANRE	59	VDFSKNWIQYKEGFGYLSPPDRTEFWLGNKTIHLLSVQSSVPYVLRLEMVDWEGNKKYAD
B8A5L6_DANRE	118	YN- IKVGSEAEGFPLTASDY- DGDAGDALV---RGHPNLGSFLSHAGMKFSTFDRSDK
Q7ZVG7_DANRE	119	YATFKLGPEVDAYRLTYAYYFGGDAGDAFDGYDFGDDPSDKFYTSHNGMQFSTVDRDNDK
B8A5L6_DANRE	172	WEENCAEMYGGGWYNNCQSANLNGIYYKGGQYDPATKVPYEIENGVVWLPFKPADYSLK
Q7ZVG7_DANRE	179	FQGHCAQQDGSWWNNRCHAAHLNGKYYQGCKYTEK-DAESGYDNGIHWATWHSRWYSLK
B8A5L6_DANRE	232	VVRMKIRPV-----
Q7ZVG7_DANRE	238	ETTMKIIPINRIGATGGQESGSKQFGGIGDI

(c)

Q6NYE1_DANRE	1	PIPVVSGKECEDIIIRKGGEDSQMYIIRPDPLGTPYKVFCDQTSKNGGWLTQNRMDSVD
Q7ZVG7_DANRE	1	EIQTVTGKDCQDIANKGGKVSGLYYVKPARAPEAFLVYCEIDSFGRGWTVLQRRRDGSVD
Q6NYE1_DANRE	61	FGRRWDDYRRGFGNIAFDVGKGHCQTPGEYWLGNDRISQLSKMGATE--LLVEMEDWSGS
Q7ZVG7_DANRE	61	FSKNWIQYKEGFGYLSPPDR-----TEFWLGNKTIHLLSVQSSVPYVLRLEMVDWEGN
Q6NYE1_DANRE	119	KVYAQYEQFSMQGEASNYILGVGRYS-GTAGNTFLEGATELFGE---NRTMTIHNMGMF
Q7ZVG7_DANRE	114	KKYADYATFKLGPEVDAYRLTYAYYFGGDAGDAFD-GY--DFGDDPSDKFYTSHNGMQFS
Q6NYE1_DANRE	175	TYDRDNDKIIPGDPSKQCSKEDGGGWYNNRCHSCNPNGRYWGGAYTKYMAKHGTDDGI
Q7ZVG7_DANRE	171	TVDRDNDKE-----QGHCAQQDGSWWNNRCHAAHLNGKYYQGCKYTEKDAESGYDNGII
Q6NYE1_DANRE	235	WMNWKGSWYSLKTIISMKIRPYFKQK-----
Q7ZVG7_DANRE	226	WATWHSRWYSLKETTMKIIPINRIGATGGQESGSKQFGGIGDI

Figure A-6. Multiple protein sequence alignment of the C-terminal globular domain from: (a) A α E and B β zebrafish fibrinogen chains, (b) A α E and γ zebrafish fibrinogen chains; and (c) B β 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	HA	KSRPVRDCDDVLQTHPSGTQSGIFN	KLP	GSSKIFS	VYCDQETSLGGWLLIQ	QRM	DGS
FIBB_HUMAN	1	NIPV	SGKECEEIIR--	KG	GETSEMYLIQPDSSVKPYRVYCDMNT	ENG	GWTVIQ	NRQDGS
FIBG_HUMAN	1	QIH	DITGKDCQDIAN--	KG	KQSGLYFIKPLKANQQFLVYCEIDGSG	NGWTVFQ	KRL	DGS
FIBA_HUMAN	61	LN	FNRTWQDYKRGFGSLNDE--	G	-----	EGEF	WLGNDYLHLLTQ	RG---SVLRVELEDW
FIBB_HUMAN	59	VDF	GRKWD	PKQGF	GNVATNTDGKNYCGLPGEY	WLG	NDKISQLTRMG	P--TELLEMEDW
FIBG_HUMAN	59	VDF	KKNWIQYKEGFGHLSP	T--G	-----	TTE	FWLGNEKIHLL	ISTQSAIPYALRVELEDW
FIBA_HUMAN	110	AG	NEAYAEY-HFRV	GSEAGYALQVSSYEG-TAG	DALIE---	GSV---	EEGAEY	TSHNNM
FIBB_HUMAN	117	KGD	KVKAHYCGFTVQNEANKYQISV	NKYRG-TAG	NALMD---	GAS	QLMG	ENRTMTIHNGM
FIBG_HUMAN	111	NG	RSTADYAMFKVGPEADKYRLTYAY	FAG	GDAGDAFDG	DFD	GDD---	PSDKFETSHNGM
FIBA_HUMAN	162	QF	STFDRDADQW----	EEN	CAEVYGGGWWYNNCQAANLNGIYY	P	GGSYDPRNNS	SPYEIE
FIBB_HUMAN	173	FF	STYDRDNDGWLTS	DPKQC	SKE	DGGG	WWYNRCHAANPN	GRYYWGGQYT-WDMAKHGTD
FIBG_HUMAN	168	QF	STWDNDNDKF----	EG	NCAEQDGS	GWWMNKCHAGHLNGVYY	QGGTYS-	KASTPNGYD
FIBA_HUMAN	217	NG	VVWVSFRGADYSLRAVR	MKIRPLVTQ	-----			
FIBB_HUMAN	232	DG	VVWNNWKG	SWSMRKMSMKIRPF	FPQQ	-----		
FIBG_HUMAN	222	NG	IITWATWKTRWYS	MKKTMTKIIP	FNRLTIGEGQ	QHHLG	GAQ	QAGDV
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	RK	AGFVGGDCVEIQ	QKHVNGGQSGMFKIKPAGSE	EVVE	VYCDQSTGLGGWTLVQ	QREDGS
Q6NYE1_DANRE	1	PI	PVSGKECEDIIRK	G--GED	SQMYIIRPDPLGTPYKVFCDQTSK	NGGWLTQ	NRMDGS
Q7ZVG7_DANRE	1	EIQ	TVTGKDCQDIANKG--	GKV	SGLYYKPARAPEAFLVYCEIDSF	GRGWTVLQ	RRRDGS
B8A5L6_DANRE	61	VN	FNRTWK	EYLN	GFGQIDK-----	QKGEI	WIGNKFLHLLTQKES---LLRVELQDWT
Q6NYE1_DANRE	59	VDF	GRRWDDYRRGFGNI	AFDVGKGHCQTPGEY	WLG	NDRISQLSKMGAT--	ELLVEMEDWS
Q7ZVG7_DANRE	59	VDF	SKNWIQYKEGFGY	SP-----	DDRT	EFWLGNEKIHLLSVQSSVPYVLR	TEMVDWE
B8A5L6_DANRE	111	GA	EAYAEYN-IKVG	SEAE	GFLTASDY-DGDAGDALVRGH---	PNLGSFLSHAG	MKFST
Q6NYE1_DANRE	117	GSK	VYAQYEQFSMQGEASNYIL	GVGRY-SGTAGNTFLEGATELFG	ENRTMTIHNGMM	FST	
Q7ZVG7_DANRE	112	GN	KKYADYATFKLGPEVD	AYRLTYAYYFGGDAGDAFDG	YDFGDDPSDKFY	TSHNGMQFST	
B8A5L6_DANRE	165	FDR	SDKME-----	ENCA	EMYGGGWWYNNCQSANLNGIYYKGGQY	DPATKVPY	EIENGVV
Q6NYE1_DANRE	176	YDR	DNDKWIPGDPSKQCSKE	DGGG	WWYNRCHSCNPNGRYYWGGAYTKY-MAKHGTD	DGIV	
Q7ZVG7_DANRE	172	VDR	DNDKF-----	QGH	CAQQDGS	GWWMNRC	AAHLNGKYYQGGKYTEK-DAESGYDNGII
B8A5L6_DANRE	220	WL	PKPADYSLKVV	RMKIRPV	-----		
Q6NYE1_DANRE	235	WM	NWKG	SWSLKTISM	KIRPYFKQK	-----	
Q7ZVG7_DANRE	226	WAT	WHSRWYSLKET	MTMKIIP	INRIGATGGQESGSKQFGGIGDI		
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) A α E, B β and γ human fibrinogen chains; and (b) A α E, B β 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.

```

FIBA_HUMAN 1 HAKSRPVRCDDVLQTHPSGTQSGIFNKLPGSSKIFSVYCDQETSLGGWLLIQQRMDGS
FIBB_HUMAN 1 NIPVVSQKECEEIIR--KGGETSEMYLIQPDSSVKPYRVYCDMNTENINQ
FIBG_HUMAN 1 QIHDITGKDCQDIAN--KGAQKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDGS

FIBA_HUMAN 61 LNFNRTWQDYKRGFGSLNDE--G-----EGEFWLGN DY L HLLTQRG---SVLRVELEDW
FIBB_HUMAN 59 VDFGRKWD PYKQGFGNVATNTDGKNYCGLPGEYWLGN DKISQLTRMGP--TELLTEMEDW
FIBG_HUMAN 59 VDFKKNW IQYKEGFGHLSPT--G-----TTEFWLGNEKIHLISTQSAIPYALRVELEDW

FIBA_HUMAN 110 AGNEAYAEY-HFRVGSEAEGYALQVSSYEG-TALIE---GSV---EEGAEYTSHNNM
FIBB_HUMAN 117 KGDKVKAHYCGFTVQNEANKYQISVNKYRG-TAGNALMD---GASQLMGENTMTIHNMG
FIBG_HUMAN 111 NGRTSTADYAMFKVGPEADKYRLTYAYFAGGDGFDGDD---PSDKFTTSHNGM

FIBA_HUMAN 162 QFSTFDRDADQW-----EENCAEVYGGGWYNNCQAA NLNGIYYPGGSYDPRNNSPYEIE
FIBB_HUMAN 173 FFSTYDRDNDGWLTS DPRKQCSKEDGGGWYNNRCHAANPNGRYYWGGQYT-WDMAKHGTD
FIBG_HUMAN 168 QFSTWDNDNDKFE-----EGNCAEQDGS GWMN KCHAGHLNGIYYQGGTYS-KASTPNGYD

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

```

Figure A-8. Multiple protein sequence alignment of the C-terminal globular domain from A α E, B β 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 A α E chain that are known to bind leukocytes through the integrin $\alpha_M\beta_2$.

B8A5L6_DANRE	1	RKAGFVGGDCVEIQQKHVNGGQSGMFKIKPAGSEEVVEVYCDQSTGLGGWTLVQQREDGS
Q6NYE1_DANRE	1	PIPVVSGKECEDIIRKG--GEDSQMYIIRPDPLGTPYKVFCDQTSKNGV I N M
Q7ZVG7_DANRE	1	EIQTVTGKDCQDIANKG--GKVSGLYYVKPARAPEAFLVYCEIDSFGRGWTVLQRRRDGS
B8A5L6_DANRE	61	VNFNRTWKEYLNGFGQIDK-----QGKGEIWIIGNKFLHLLTQKES---LLRVELQDWT
Q6NYE1_DANRE	59	VDFGRRWDYRRGFGNIAFDVVGKGHCQTPGEYWLGNDRISQLSKMGAT--ELLVEMEDWS
Q7ZVG7_DANRE	59	VDFSKNWIQYKEGFGYLS-----DDRTEFWLGNEKIHLLSVQSSVPYVLRTEMVDWE
B8A5L6_DANRE	111	GAEAYAEN- IKVGSEAEGFPLTASDY-DGDAGDALVRGH---PNLGSELSHAGMKFST
Q6NYE1_DANRE	117	GSKVYAQYEQFSMQGEASNYILGVGRY-SGTAGNTFLEGATELFGENRTMTIHNGMMFST
Q7ZVG7_DANRE	112	GKKKYADYATFKLGPEVDAYRLTYAYYFGGDAGDAFDGYDFGDDPSDKFYTSHNGMQFST
B8A5L6_DANRE	165	FDRSDKWE-----ENCAEMYGGGWYNNCQSANLNGIYYKGGQYDPATKVPYEIENGVV
Q6NYE1_DANRE	176	YDRDNDKWIPGDPKQCSKEDGGGWYNNRCHSCNPNGRYWGGAYTKY-MAKHGTDDGIV
Q7ZVG7_DANRE	172	VDRDNDKF-----QGHCAQQDGSQWMMNRCHAAHLNGKYYQGGKYTEK-DAESGYDNGII
B8A5L6_DANRE	220	WLPFKPADYSLKVVVRMKIRPV-----
Q6NYE1_DANRE	235	WMNWKGS TIS YFKQK-----
Q7ZVG7_DANRE	226	WATWHSRWYSLKETTMKTIPIINRIGATGGQESGSKQFGGIGDI

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

