THE DELINEATION OF THE FIBRINOGEN/TRIGGERING RECEPTOR IN MYELOID CELLS (TREM) LIKE TRANSCRIPT - (TLT) -1 MOLECULAR INTERACTION

by

SIOBHAN LAKEN BRANFIELD

A dissertation submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES

2024

Oakland University
Rochester, Michigan

Doctoral Advisory Committee:

Anthony Valance Washington, Ph.D., Chair
Elizabeth A Bonney, MD.
Sarah Hosch, Ph.D.
Randal J. Westrick, Ph.D.

Dedicated to George Clarence Branfield and Yolande Angela Branfield

Ephesians 6:1-3 "Honor your father and mother"- which is the first commandment with a promise-"so that it may go well with you and that you may enjoy long life on the earth." .

Mom and Dad, I know unconditional love because I was loved by you. The greatest gift you gave me was a firm foundation in Christ, from which all my values and principles have stemmed from, you introduced me to the purpose of life, in Christ, and for that I will forever be grateful.

Dad, you believed in me even when I did not believe in myself, you were always my number 1 fan and taught me to never settle, to be courageous and to speak up even when my voice shakes. I wish you were here to share this moment with me, but today I kneel and thank God for you, this Doctorate is in memory of you. Love always your baby girl, Siobhan.

My definition of a mother, a best friend, a confidant, an anchor, a rock and solid foundation. All those things you have been and are to me. Mom, you are the most humble, gentle, kind soul I have ever known. You put my needs before your own and sacrificed everything you had to put me through medical school, which gave me the foundation for this doctoral degree. I celebrate this milestone in your honor and am so grateful to do the next chapters of live with you by my side.

ACKNOWLEDGMENTS

I would like to acknowledge Dr. A. V Washington who took a chance on me when I had no experience in research and guided me to become the scientist I am today, this journey has been a joy, from the science to the brainstorming over coffee, it has been more than just a degree, it has been an environment that feels like home. I have been blessed and am eternality grateful to have had 5 years of your mentorship and guidance, my success reflects your mentorship.

A special thank you to my committee members for the immense contributions to this dissertation work (Elizabeth A Bonney, MD, Sarah Hosch, Ph.D. Randal J. Westrick, Ph.D.)

To my God parents, Caroline and Robert Pullen, thank you for giving me the opportunity to follow my dreams and being at my side along the way, I am so blessed and eternally grateful for you!

Saneliswa, God knew I needed you to walk at my side through this journey, thank you for walking this journey with me and being my emotional support buddies, I love you endlessly!

Phil, thank you for all your support and encouragement to get this done!

To my forever life partners – the siblings, (Sheraine, Sherlese, Shaun, and Michelle), my ENTIRE family and friends, thank you for doing life with me, this if for you

To my lab mates, past and present, the Washington laboratory became a second home for me, you welcomed me with open arms, and I have made lifelong friendships with you, I am so excited to continue doing life with you and celebrate your success.

Lastly, and most importantly, To God be the Glory, I am because you are! Jeremiah 29:11 For I know the plans I have for you," declares the Lord, "plans to prosper you and not to harm you, plans to give you hope and a future.

Siobhan Laken Branfield

PREFACE

Once known conventionally to regulate hemostasis and thrombosis, platelets have now been demonstrated to extend their function, as immune cells. Having multiple receptors on their surface, platelets can interact with different ligands (either cells or proteins) during inflammation and contribute to either mitigation or exacerbation of pathophysiological progression of disease. In the first chapter, this dissertation presents an introduction to platelet classical and immunological function, an introduction to the platelet ligand, fibrinogen, and an introduction to Triggering Receptor Expressed in Myeloid Cells -1 (TLT- 1), one of the receptors on platelets that binds fibrinogen. Subsequently, this dissertation discusses the molecular interaction between fibrinogen and TLT-1, aiming to locate specific binding sites for this interaction and validate the site(s).

Perhaps introducing the potential for a new platelet drug class targeting this interaction. This dissertation then discusses potential use of platelet specific ligands, in this case TLT-1 as a biomarker for disease severity and risk assessment for mortality, specifically in COVID-19 and Thromboembolic complications in Nephrotic Syndrome.

Finally, the dissertation uses atherosclerotic and hepatic regenerative murine models to determine whether TLT-1 plays a central role in regulating fibrinogen deposition during pathophysiology of disease and regenerative processes in the human body.

ABSTRACT

THE DELINEATION OF THE FIBRINOGEN/TRIGGERING RECEPTOR IN MYELOID CELLS (TREM) LIKE TRANSCRIPT - (TLT) -1 MOLECULAR INTERACTION

by

SIOBHAN LAKEN BRANFIELD

Advisor: Dr A V Washington

Triggering Receptor Expressed in Myeloid Cells -1 (TLT- 1) is a platelet specific receptor that facilitates platelet aggregation and plays a role in immunohemostasis. Previous studies identified fibrinogen as a ligand for TLT-1 but little has been reported on the molecular interaction or the role this TLT-1/Fibrinogen interaction has on pathophysiological progression of disease. This dissertation research presents basic research, with potential for translational application for this interaction, highlighting the versatile functionalization and application of TLT-1 as a contributor to clot formation, as a biomarker for disease progression and, extends to understand its regulation of fibrinogen deposition in pathophysiological models of disease.

Chapter 1 gives a literature overview on platelet function, its ligand fibrinogen and a review summary on TLT-1. Chapter 2 then presents a fundamental study on the molecular interaction between TLT-1 and Fibrinogen, introducing potential for the development of novel antiplatelet drugs against TLT-1. Chapter 3 introduces the role of TLT-1 in disease progression through fibrinogen deposition and the use of TLT-1 as a biomarker. Last,

Chapter 4 introduces a novel function for TLT-1, as a regulator of hepatic regeneration, facilitated by its ability to orchestrate fibrinogen deposition in regenerative liver. These applications are all realized by TLT-1, a platelet specific receptor, emphasizing the versatility of platelet function and potential for expansion of platelet specific targeted therapeutic intervention.

TABLE OF CONTENTS

TABLE OF CONTENTS—Continued

TABLE OF CONTENTS—Continued

TABLE OF CONTENTS—Continued

TABLE OF CONTENTS—Continued

TABLE OF CONTENTS—Continued

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES—Continued

LIST OF FIGURES—Continued

LIST OF ABBREVIATIONS

CVD	Cardiovascular Disease
TLT-1	Triggering Receptor Expressed in Myeloid Cells -1
ARDS/ALI	Acute Respiratory Distress Syndrome/ Acute Lung Injury
FDP	Fibrinogen Degradation Products
CAD	Coronary Artery Disease
CHF	Congestive Heart Failure
ACS	Acute Coronary Syndrome
PHx	Partial Hepatectomy
BLI	Biolayer Interferometry
AFM	Atomic Force Microscopy
SEM	Scanning Electron Microscopy
ICU	Intensive Care Unit
N/S	Nephrotic Syndrome

ROC	Receiver Operator Curve

CHAPTER 1

INTRODUCTION

The Classical and Immune Platelet

Platelets, derived from megakaryocytes, play a pivotal role in hemostasis, orchestrating the cessation of bleeding following vascular injury[1,2]. Platelets circulate in an inactive state until they encounter damaged endothelial cells or exposed subendothelial collagen, which promptly triggers a cascade of events leading to the formation of a hemostatic plug, essential to prevent excessive blood loss.

Primary clot formation, also known as primary hemostasis, is the initial stage of blood clotting that occurs in response to vascular injury. It involves a series of complex interactions between platelets, endothelial cells, and the subendothelial matrix including collagen and von Willebrand factor (vWF to the blood stream and initiate the formation of a temporary plug at the site of injury, thereby preventing excessive bleeding. Here's an overview of the key steps involved in primary clot formation. Endothelial cells lining the blood vessels become activated and release various mediators that promote platelet adhesion and activation[3].

Circulating platelets rapidly adhere to the exposed subendothelial matrix at the site of injury. This process is mediated by the interaction between platelet surface receptors, such as glycoprotein Ib-IX-V complex, and adhesive proteins, particularly vWF and collagen. Adherent platelets become activated upon contact with the subendothelial matrix and soluble agonists released from damaged tissues or activated

endothelial cells. Platelet activation leads to changes in platelet shape, granule secretion, and activation of intracellular signaling pathways, initiating the process of platelet aggregation[2,3].

Activated platelets release granule contents, including adenosine diphosphate (ADP) and thromboxane A2 (TxA2), which recruit additional platelets to the site of injury. ADP promotes platelet activation and aggregation by binding to specific receptors (P2Y12) on neighboring platelets, while TxA2 amplifies platelet activation and induces further aggregation by activating integrin αIIbβ3 receptors. This plug serves as a barrier to prevent further blood loss and provides a scaffold for the subsequent deposition of fibrin during secondary clot formation[2].

Following the formation of the initial platelet plug, platelet retraction occurs, mediated by the contraction of platelet cytoskeletons. This process helps to consolidate the clot and reduce the size of the vessel defect. Primary clot formation is a critical physiological process that occurs rapidly in response to vascular injury, involving platelet adhesion, activation, aggregation, and the formation of a temporary hemostatic plug. This initial plug provides temporary hemostasis while the coagulation cascade is activated to form a more stable and permanent clot[2–5].

Secondary Clot Formation.

In addition to platelet aggregation, the formation of a stable clot involves the conversion of fibrinogen to fibrin, mediated by thrombin. Activated platelets provide a surface for the assembly of the enzyme complexes necessary for thrombin generation, thus promoting fibrin formation. Fibrin strands interlace with the aggregated platelets, forming a meshwork that consolidates the platelet plug into a stable clot.

Secondary clot formation, also known as secondary hemostasis, is the process by which a stable and insoluble fibrin clot is formed to reinforce the initial platelet plug and achieve long-term hemostasis. It involves the activation of the coagulation cascade, which involves the conversion of soluble fibrinogen into insoluble fibrin strands[2].

The exposure of tissue factor (TF) at the site of vascular injury initiates secondary clot formation. TF is a membrane-bound glycoprotein expressed by subendothelial cells and is exposed to the bloodstream upon vascular injury. TF forms a complex with circulating factor VIIa, activating it to initiate the extrinsic pathway of the coagulation cascade. The TF-FVIIa complex catalyzes the conversion of factor X to factor Xa, a serine protease, in the presence of calcium ions. This step represents the initiation phase of the coagulation cascade.Factor Xa forms a complex with factor Va and phospholipids on the platelet surface, forming the prothrombinase complex. The prothrombinase complex converts prothrombin (factor II) into thrombin (factor IIa), a central enzyme in the coagulation cascade[6–8].

Thrombin plays a central role in secondary clot formation. It not only converts fibrinogen into fibrin but also activates platelets, amplifying platelet activation and aggregation. Thrombin also activates factors VIII, IX, XI, and XIII, contributing to the propagation of the coagulation cascade[7,9].

Thrombin cleaves fibrinopeptides A and B from fibrinogen, exposing binding sites on fibrin monomers. The fibrin monomers polymerize to form insoluble fibrin strands, which aggregate and cross-link through the action of factor XIIIa, a transglutaminase enzyme. This process results in the formation of a stable fibrin meshwork that reinforces the platelet plug and stabilizes the clot.

Following fibrin formation, clot retraction occurs, mediated by the contraction of platelets and fibrin strands. This process helps to consolidate the clot, reduce its size, and promote wound closure[10,11].

Simultaneously with clot formation, inhibitors of fibrinolysis, such as plasminogen activator inhibitor-1 (PAI-1), are released to prevent premature breakdown of the clot. This allows sufficient time for tissue repair and wound healing to occur. Secondary clot formation is a complex and tightly regulated process that involves the activation of the coagulation cascade, generation of thrombin, conversion of fibrinogen to fibrin, and formation of a stable fibrin clot. This process ensures the formation of a durable clot that effectively seals the site of vascular injury and maintains hemostasis until tissue repair is complete[12].

The Immune Platelet: Bridging Hemostasis and Immune Responses.

Platelets, long recognized for their role in hemostasis, have recently emerged as integral players in the immune system, participating in inflammation, infection control, and tissue repair. Unlike their traditional function, solely as clotting agents, immune platelets exhibit a multifaceted range of functions, significantly impacting the body's immune responses[13–15].

During an immune response, platelets can be activated by various stimuli, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) released during tissue injury or inflammation. Additionally, platelets can interact directly with immune cells, such as leukocytes and endothelial cells, through specific receptors, cytokines, or chemokines, triggering their activation[16].

While some signaling pathways involved in platelet activation overlap between hemostasis and immune responses, there are distinct differences. In hemostasis, platelet activation primarily occurs in response to vascular injury, involving pathways such as the GPVI collagen receptor and thromboxane A2 signaling. Conversely, during immune responses, platelet activation can be triggered by a broader range of stimuli, including Toll-like receptors (TLRs), interleukins, and chemokines, leading to the activation of distinct intracellular signaling cascades, such as nuclear factor kappa B (NF-κB) and phosphoinositide 3-kinase (PI3K) pathways[2,5,17].

Platelets interact dynamically with various immune cells, including neutrophils, monocytes, dendritic cells, and lymphocytes. Through surface receptors and soluble mediators, platelets modulate the recruitment, activation, and function of immune cells, influencing inflammation, immune surveillance, and host defense mechanisms. Platelets exhibit immune cell-like properties, contributing to innate and adaptive immune responses. Platelets express pattern recognition receptors (PRRs), allowing them to recognize and respond to microbial pathogens. They also secrete immune mediators, including cytokines, chemokines, and antimicrobial peptides, which regulate immune cell function and modulate inflammatory processes[18–20].

In immune responses, clot formation can be influenced by the presence of inflammatory cytokines, which alter the composition and structure of the clot. Platelets interact with fibrinogen to form a scaffold for clot formation, and the incorporation of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), can modify the clot's properties, affecting its stability and susceptibility to degradation[18–20].

Platelet Receptors, their activation and signaling.

Platelets express receptors on their surface and release receptors from their granules (upon activation) that are crucial for their functionality. Among these are the glycoprotein (GP) Ib-V-IX complex, GPVI, integrins such as αIIbβ3, and G-protein coupled receptors like the thromboxane A2 receptor (TP) and ADP receptors (P2Y1 and P2Y12). These receptors play pivotal roles in mediating platelet adhesion, activation, and aggregation upon encountering vascular injury[13,16,21].

Upon vascular injury, platelet activation is initiated by the exposure of subendothelial collagen. The initial interaction involves binding of the GP Ib-V-IX complex to von Willebrand factor (vWF), an adhesive glycoprotein released by endothelial cells upon injury. This interaction facilitates the anchoring of platelets to the damaged vessel wall. Subsequently, collagen binding to GPVI triggers intracellular signaling cascades, leading to platelet shape change, granule secretion, and exposure of phosphatidylserine on the platelet surface. The exposure of phosphatidylserine facilitates the binding of coagulation factors, notably thrombin, a key mediator in the coagulation cascade. Thrombin further activates platelets by cleaving protease-activated receptors (PARs) on their surface, amplifying the activation process[2].

Platelet Signaling

Platelet activation involves the phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) signaling pathways. Upon receptor activation, these pathways converge to induce granule secretion, integrin activation, and cytoskeletal reorganization, promoting platelet aggregation and clot formation. Phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and mitogen-

activated protein kinase (MAPK) are crucial signaling pathways involved in platelet activation and aggregation[2,5]. Here's a description of how each pathway contributes to these processes:

Phosphoinositide 3-Kinase (PI3K) Pathway

Upon platelet activation by thrombin, collagen, or ADP, receptors on the platelet surface trigger the activation of PI3K. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2), converting it into phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 serves as a docking site for various signaling molecules, recruiting them to the plasma membrane. Key downstream effectors of the PI3K pathway in platelets include Akt (protein kinase B) and phosphoinositide-dependent kinase-1 (PDK1). Activation of Akt and PDK1 promotes platelet activation by modulating various signaling pathways, including those involved in cytoskeletal rearrangement, granule secretion, and integrin activation[5].

Protein Kinase C (PKC) Pathway

Platelet activation triggers the activation of PKC, a family of serine/threonine kinases. Platelets express several isoforms of PKC, including conventional (α, β, γ), novel (δ, ε, η, θ), and atypical (ζ, λ/ι) isoforms, each with distinct roles in platelet function. Upon activation, PKC isoforms translocate from the cytosol to the plasma membrane, where they bind to phospholipids, such as diacylglycerol (DAG) and phosphatidylserine. Activated PKC phosphorylates various substrates involved in platelet activation and aggregation, including proteins regulating calcium mobilization, cytoskeletal organization, and granule secretion[22–24].

Mitogen-Activated Protein Kinase (MAPK) Pathway

Platelet activation triggers the activation of MAPK signaling cascades, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. Sequential activation of MAPK kinases (MAPKKs) phosphorylates and activates MAPKs, leading to the phosphorylation of downstream substrates. The MAPK pathway regulates various aspects of platelet function, including granule secretion, cytoskeletal reorganization, and integrin activation. MAPK signaling interacts with other signaling pathways, such as PI3K and PKC, forming a complex network that coordinates platelet activation and aggregation in response to diverse stimuli[25].

Overall, the PI3K, PKC, and MAPK pathways play critical roles in platelet activation and aggregation by regulating intracellular signaling cascades that modulate platelet function. Understanding the intricacies of these signaling pathways provides insights into the molecular mechanisms underlying hemostasis, thrombosis, and potential targets for therapeutic intervention in thrombotic disorders.

Adenosine diphosphate (ADP) signaling pathway

ADP released from activated platelets binds to P2Y1 and P2Y12 receptors, amplifying platelet activation via secondary signaling pathways and promoting further platelet recruitment and aggregation. ADP is released from activated platelets themselves or from other cells, such as damaged endothelial cells or red blood cells, at the site of vascular injury. Upon release, ADP binds to and activates the P2Y1 receptor on the platelet surface. The P2Y1 receptor is a G protein-coupled receptor (GPCR) linked to Gq proteins[2,17,21,26].

Activation of the P2Y1 receptor leads to the activation of Gq proteins associated with the receptor, which stimulates phospholipase C (PLC) enzymes, leading to the

hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers the release of calcium ions (Ca2+) from intracellular stores, primarily the dense tubular system (DTS) within platelets. This elevation in cytosolic calcium levels is crucial for platelet activation and aggregation. Increased intracellular calcium levels result in platelet shape change and the secretion of granule contents, including ADP and serotonin. This amplifies platelet activation and recruits' additional platelets to the site of injury[2].

Simultaneously, ADP binds to and activates the P2Y12 receptor, another GPCR on the platelet surface, although with higher affinity compared to P2Y1. The P2Y12 receptor is coupled to Gi proteins. Activation of the P2Y12 receptor inhibits adenylate cyclase activity via Gi proteins, leading to decreased levels of cyclic adenosine monophosphate (cAMP) within the platelet Reduced cAMP levels prevent the inhibition of platelet activation pathways mediated by cAMP-dependent protein kinase A (PKA). This enhances platelet activation and aggregation.

The synergistic effect of P2Y1-mediated calcium mobilization and P2Y12-mediated amplification of platelet activation ensures a robust and coordinated response to vascular injury, promoting effective hemostasis while minimizing excessive thrombus formation. Given their critical role in platelet function, the P2Y1 and P2Y12 receptors have emerged as important therapeutic targets in the management of thrombotic disorders. Antiplatelet agents, such as P2Y12 receptor antagonists (e.g., clopidogrel, ticagrelor), effectively inhibit platelet activation and aggregation, thereby reducing the risk of thrombotic events in patients with cardiovascular diseases[2,5,8,21].

Platelets are not merely passive participants in hemostasis but serve as active immune cells, intricately involved in the body's immune responses. Through differential signaling pathways, interactions with immune cells, secretion of immune mediators, and modulation of clot structure, immune platelets contribute significantly to inflammation, host defense, and tissue repair processes, highlighting their essential role at the intersection of hemostasis and immunity[2,26–28].

Platelet specific therapeutic interventions

Studies over the years that have elucidated platelet function have made great progress to use mechanisms to develop platelet specific therapeutic interventions. Antiplatelet drugs interfere with various steps of platelet activation and aggregation, ultimately reducing the risk of thrombus formation. They can target different receptors, enzymes, and signaling pathways involved in platelet function[26].

Common modes of action include:

- Inhibiting ADP receptors (P2Y12 antagonists)
- Blocking glycoprotein IIb/IIIa receptors, preventing fibrinogen binding and platelet aggregation
- Inhibiting cyclooxygenase (COX) and thromboxane A2 (TxA2) synthesis
- Interfering with phosphodiesterase activity, leading to increased cAMP levels and inhibition of platelet activation
- Targeting intracellular signaling pathways, such as PI3K, PKC, and MAPK, involved in platelet activation

Examples of Antiplatelet Drugs:

- Aspirin (Acetylsalicylic Acid): Inhibits COX, reducing the synthesis of thromboxane A2 and platelet aggregation.
- Clopidogrel: Irreversibly blocks the P2Y12 ADP receptor on platelets, inhibiting ADP-induced platelet activation and aggregation.
- Ticagrelor: Reversible P2Y12 antagonist that inhibits ADP-induced platelet activation and aggregation.
- Prasugrel: Irreversible P2Y12 antagonist similar to clopidogrel, but with more potent and consistent antiplatelet effects.
- Dipyridamole: Inhibits phosphodiesterase activity, leading to increased cAMP levels and inhibition of platelet activation.
- Cilostazol: Phosphodiesterase inhibitor that increases cAMP levels, inhibiting platelet aggregation and smooth muscle cell proliferation.
- Abciximab, Eptifibatide, Tirofiban: Glycoprotein IIb/IIIa receptor antagonists that block fibrinogen binding and platelet aggregation.
- Vorapaxar: Protease-activated receptor-1 (PAR-1) antagonist that inhibits thrombin-induced platelet activation and aggregation.

These drugs are used alone or in combination, depending on the clinical indication and patient characteristics, to prevent thrombotic events in conditions such as acute coronary syndrome, myocardial infarction, stroke, and peripheral artery disease. It's important to note that antiplatelet therapy carries a risk of bleeding, and the choice of drug and dosage must be carefully balanced with the patient's individual risk factors[29–31].

FIBRINOGEN

Fibrinogen structure and function

Fibrinogen, also known as Factor` I, is 340kDA in size and is a vital glycoprotein found in the plasma (within ranges of 200 to 400 mg/dL) that plays a crucial role in hemostasis, inflammation, and wound healing[1–3]. Its functions underscore its significance in maintaining vascular integrity and orchestrating immune responses. This part of the introductory chapter aims to provide an overview of fibrinogen, elucidating its definition, functions, production, regulation, and interactions with various ligands in different pathophysiological contexts.

Fibrinogen constitutes one of the key components of the coagulation cascade. Structurally, it comprises a hexameric complex composed of three pairs of polypeptide chains: alpha (α), beta (β), and gamma (γ). This structure facilitates fibrinogen polymerization into insoluble fibrin, forming a meshwork that stabilizes blood clots and prevents excessive bleeding.

Beyond its role in hemostasis, fibrinogen serves as a signaling molecule in inflammation, mediating leukocyte recruitment and tissue repair processes. Additionally, fibrinogen participates in angiogenesis, platelet aggregation, and modulation of cell adhesion, highlighting its versatile nature in physiology and pathophysiology.

Production and Regulation of Fibrinogen

Fibrinogen has a plasma half-life of three to four days and primarily originates from hepatocytes within the liver, where its synthesis is tightly regulated by several factors. Fibrinogen production is encoded by three genes Aα (*FGA*), Bβ (*FGB*), and γ (*FGG*) on chromosome 4, producing the chains that come together to form a hexameric complex with 644 (Aα), 491 (Bβ), and 437 (γ) amino acids in each chain, in duplicate, respectively[4]. Its production is mediated by transcriptional regulation of fibrinogen gene expression involves networks of transcription factors, including hepatocyte nuclear factor 1α (HNF-1α), CCAAT/enhancer-binding proteins (C/EBPs), and nuclear factor κB (NF-κB)[1,3,5–7].

Moreover, various cytokines and growth factors, such as interleukin-6 (IL-6) and transforming growth factor β (TGF-β), modulate fibrinogen production. Inflammatory stimuli, such as bacterial endotoxins and cytokines, can upregulate fibrinogen synthesis through activation of pro-inflammatory signaling pathways, including the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway and the mitogen-activated protein kinase (MAPK) pathway[7].

Upregulation of Fibrinogen Production in Inflammation

Inflammatory conditions are characterized by an upregulation in fibrinogen production, facilitating host defense mechanisms and tissue repair processes. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), induce fibrinogen expression by stimulating the transcriptional activity of NF-κB and activator protein 1 (AP-1) in hepatocytes[8].

Furthermore, the acute-phase response, a hallmark of inflammation, triggers a systemic surge in fibrinogen levels, mediated by IL-6 signaling through the JAK-STAT pathway. This response underscores the role of fibrinogen in mounting an effective immune response against invading pathogens and restoring tissue homeostasis.

Interaction with Ligands and Pathophysiological Implications

Fibrin(ogen) interacts with multiple ligands, including integrins, selectins, and proteases, thereby modulating various pathophysiological processes. Integrins, such as αIIbβ3 (a platelet specific receptor) and αvβ3, mediating platelet aggregation and adhesion to fibrinogen, facilitating thrombus formation at sites of vascular injury. Interestingly, fibrinogen also binds to TREM-Like Transcript-1 (TLT-1), a platelet specific membrane found in the alpha granules, and their interaction facilitates platelet aggregation.

Furthermore, fibrinogen interacts with leukocyte integrins, such as αMβ2 (Mac-1) and αLβ2 (LFA-1), promoting leukocyte adhesion and transmigration across the endothelium during inflammation. Additionally, fibrinogen binds to extracellular matrix components, such as fibronectin and collagen, modulating cell adhesion, migration, and tissue remodeling processes.

From Fibrinogen to Fibrin (Thrombin Cleavage)

After hemostasis, when a blood vessel is injured, thrombin (coagulation factor IIa) plays a crucial role in the conversion of fibrinogen into fibrin, forming a mesh that helps to seal the wound. Thrombin cleaves specific peptide bonds in fibrinogen, releasing fibrinopeptides A and B from A alpha and B beta chains, respectively[8–11]. This cleavage exposes binding sites on the fibrin molecules, allowing them to polymerize and

form long strands. These strands then cross-link with each other via interactions between specific amino acid residues, forming a stable fibrin network that reinforces the blood clot. In the post-cleavage structure, factor XIIIa facilitates fibrin molecule cross-linkage in branching network held together by covalent bonds[11]. This network provides mechanical strength to the clot, preventing further blood loss.

The stages of fibrinogen cleavage and fibrin formation are mostly irreversible, as the polymerization and cross-linking processes result in the formation of protofibrils and lateral aggregation, resulting in a stable clot. However, some steps, such as the activation of factor XIII and the degradation of fibrin by plasmin can be reversible to some extent, allowing for clot dissolution and tissue repair after the injury has healed. In the context of inflammation, thrombin generation leads to fibrinogen cleavage, contributing to the formation of a provisional matrix that facilitates tissue repair and immune cell recruitment at the site of injury.

Fibrinolysis

Fibrinolysis is the physiological process responsible for the dissolution of blood clots after they have fulfilled their primary function in hemostasis. It is crucial for maintaining vascular patency and preventing pathological thrombosis. Fibrinolysis involves the enzymatic degradation of fibrin, by the protease enzyme plasmin.

The fibrinolytic system comprises several components, including plasminogen, tissue plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-1). Plasminogen, an inactive precursor, is incorporated into the fibrin clot during its formation. Tissue plasminogen activator (t-PA) is released primarily from endothelial cells and binds to fibrin, where it catalyzes the conversion of plasminogen, the serine

protease, plasmin [3,12,13]. Plasmin in turn, cleaves fibrin molecules, breaking down the clot into soluble fibrin degradation products (FDPs). FDPs contain various domains of the fibrinogen molecule, including fibrinopeptides, fibrinogen α, β, and γ chains[3,12].

Pathological Fibrinolysis

Pathological fibrinolysis occurs when the fibrinolytic system is dysregulated, leading to excessive clot breakdown and potential bleeding complications. This can occur in various clinical scenarios, such as trauma, surgery, or certain medical conditions[12,14].

In hyperfibrinolysis there is excessive activation of the fibrinolytic system, resulting in accelerated clot breakdown. This can lead to the premature dissolution of clots before hemostasis is achieved, increasing the risk of bleeding and hemorrhage[15].

Pathological fibrinolysis can contribute to the development of coagulopathy, a condition characterized by abnormal clotting function. Excessive fibrinolysis can deplete clotting factors and platelets, impairing the body's ability to form stable clots and control bleeding.

Paradoxically, pathological fibrinolysis can also contribute to the development of thrombotic disorders. Excessive clot breakdown can release procoagulant factors and platelet activators, promoting the formation of new clots and increasing the risk of thrombosis[12,14].

The fibrinolytic process is tightly regulated to prevent excessive bleeding or inappropriate clot dissolution. Plasmin activity is controlled by inhibitors such as α2-antiplasmin and α2-macroglobulin, which limit its proteolytic action to the fibrin clot. Plasminogen activator inhibitor-1 (PAI-1) inhibits the activity of t-PA, thereby regulating the conversion of plasminogen to plasmin[3].

After clot breakdown, soluble FDPs are released into the circulation and subsequently cleared by the liver and kidneys. The gradual dissolution of the clot results in the restoration of blood flow and tissue perfusion to the affected area. Fibrinolysis is a dynamic process that occurs concurrently with clot formation and is essential for maintaining vascular homeostasis. Dysregulation of fibrinolysis can lead to thrombotic disorders, such as deep vein thrombosis (DVT) or pulmonary embolism (PE), or bleeding disorders, highlighting the importance of maintaining a delicate balance between clot formation and dissolution in health and disease.

A D-Dimers is a crosslinked dimer of the D-fragment of FDPs produced because of clot lysis during fibrinolysis. Measurement of FDPs, specifically D-Dimers in blood samples serves as a valuable diagnostic tool for evaluating fibrinolysis and assessing coagulation disorders. Elevated levels of FDPs indicate increased fibrinolytic activity and may be observed in various clinical conditions, including disseminated intravascular coagulation (DIC), thrombotic disorders, and liver disease. In DIC, widespread activation of the coagulation cascade leads to the formation of microthrombi throughout the vasculature, resulting in consumption of clotting factors and platelets. Concurrently, fibrinolysis is activated to degrade fibrin clots, leading to the release of FDPs into the circulation. Elevated FDP levels in DIC are associated with a poor prognosis and increased risk of bleeding complications.

In thrombotic disorders, such as deep vein thrombosis (DVT) or pulmonary embolism (PE), FDPs may be elevated due to fibrinolytic activity directed at dissolving thrombi[16,17]. Measurement of FDP levels provide valuable insights into fibrinolytic

activity and coagulation status in various clinical settings, aiding in diagnosis, prognosis, and management of coagulation disorders and thrombotic events.

In clinical practice, pathological fibrinolysis may require intervention to control bleeding or prevent thrombotic complications. This may involve the administration of antifibrinolytic agents, such as tranexamic acid or aminocaproic acid, to inhibit plasmin activity and stabilize clots. Understanding the mechanisms underlying fibrinolysis and its regulation is essential for managing both bleeding and thrombotic disorders in clinical practice.

Dysregulated Fibrinogen production and Treatment Implications

While physiological fibrinolysis is a vital process for maintaining vascular homeostasis, pathological fibrinolysis can lead to bleeding or thrombotic complications. Dysregulated fibrinogen production can arise from various pathologies, including liver disease, inflammatory disorders, and genetic mutations. In conditions like liver cirrhosis or hepatitis, impaired liver function hinders the synthesis of fibrinogen, leading to decreased levels in the bloodstream.

Conversely, inflammatory states such as sepsis or autoimmune diseases can trigger excessive fibrinogen production as part of the acute-phase response, contributing to hypercoagulability and thrombosis. Additionally, inherited disorders like afibrinogenemia or dysfibrinogenemia result in abnormal fibrinogen structure or function, predisposing individuals to bleeding or clotting complications.

Treatment implications for dysregulated fibrinogen production vary depending on the underlying cause. In liver disease, management focuses on addressing the hepatic dysfunction through measures like liver transplantation or supportive therapies to

optimize liver function. For inflammatory conditions, controlling the underlying inflammation with medications like corticosteroids or immunosuppressants may help normalize fibrinogen levels.

In cases of genetic abnormalities, specific interventions such as fibrinogen replacement therapy or anticoagulant medications may be necessary to mitigate bleeding or thrombotic risks. Overall, tailored treatment strategies targeting the underlying pathology are essential in managing dysregulated fibrinogen production effectively.

In conclusion, fibrinogen emerges as a central player in hemostasis, inflammation, and tissue repair, regulating physiological and pathological processes. Understanding the intricate regulation of fibrinogen production and its interactions with ligands holds immense therapeutic potential for mitigating thrombotic disorders, inflammatory diseases, and neoplastic processes. This dissertation aims to delve deeper into understanding the molecular mechanisms underlying fibrinogen's interaction with TREM-Like Transcript-1 (TLT-1) and its implications in health and disease.

Platelet Receptors for Fibrinogen Binding

Platelets, the crucial cellular components of blood, express a diverse array of receptors that facilitate their adhesion, activation, and aggregation during hemostasis and thrombosis. Among these receptors, one of the most important interactions is between platelets and fibrinogen, a key protein involved in clot formation. Platelet receptors that bind to fibrinogen play a central role in mediating platelet aggregation and stabilizing the formed clot.

The primary platelet receptor responsible for binding fibrinogen is integrin αIIbβ3, also known as glycoprotein IIb/IIIa (GPIIb/IIIa). Integrin αIIbβ3 is present in an inactive conformation on resting platelets and undergoes conformational changes upon platelet activation, exposing binding sites for fibrinogen. Fibrinogen is a dimeric glycoprotein with multiple binding sites, allowing it to cross-link platelets and promote platelet aggregation.

Upon platelet activation, integrin αIIbβ3 binds to fibrinogen, forming bridges between adjacent platelets and facilitating the formation of large platelet aggregates. This process, known as platelet-fibrinogen interaction, is essential for the stability and integrity of the hemostatic plug or thrombus. In addition to integrin αIIbβ3, other platelet receptors can also interact with fibrinogen to a lesser extent, including GPVI, α5β1, and αvβ3. These receptors contribute to fibrinogen binding and may play auxiliary roles in platelet activation and aggregation.

Understanding the mechanisms of platelet-fibrinogen interaction and the role of platelet receptors involved in this process is critical for developing therapeutic strategies to modulate platelet function in various cardiovascular diseases, including thrombosis, atherosclerosis, and myocardial infarction. Targeting platelet receptors that bind fibrinogen represents a promising approach for the prevention and treatment of thrombotic disorders.

Trem like transcript 1 (TLT-1), a member of the Triggering Receptor Expressed on Myeloid Cells (TREM) family, has emerged as a novel platelet receptor with a crucial role in mediating platelet function, particularly in the context of fibrinogen binding. TLT-1 is a type I transmembrane glycoprotein primarily expressed on platelets, megakaryocytes, and endothelial cells.

Recent studies have revealed that TLT-1 acts as a fibrinogen-binding receptor on platelets, playing a significant role in platelet adhesion, activation, and aggregation. TLT-1 contains an extracellular Ig-like domain that interacts with the γ-chain of fibrinogen, facilitating platelet-fibrinogen interaction and promoting platelet aggregation.

The engagement of TLT-1 with fibrinogen enhances platelet activation and stabilizes platelet aggregates, contributing to the formation and stability of thrombi under both physiological and pathological conditions. Additionally, TLT-1 may synergize with other platelet receptors, such as integrin αIIbβ3, to amplify platelet activation and thrombus formation.

Moreover, TLT-1 has been implicated in various physiological and pathological processes, including hemostasis, thrombosis, and inflammation. Dysregulation of TLT-1 expression or function may contribute to the pathogenesis of cardiovascular diseases,

such as myocardial infarction and stroke, highlighting its potential as a therapeutic target for the prevention and treatment of thrombotic disorders.

In conclusion, Trem like transcript 1 (TLT-1) serves as a novel fibrinogen-binding platelet receptor that plays a critical role in platelet function and thrombus formation. Further elucidation of the mechanisms underlying TLT-1-mediated platelet activation may offer insights into the development of novel therapeutic strategies for the management of thrombotic disorders.

The Enigmatic Nature of the Triggering Receptor Expressed in Myeloid Cells -1 (TLT- 1).

Abstract

Receptors are important pharmacological targets on cells. The Triggering Receptor Expressed on Myeloid Cells (TREM) – Like Transcript – 1 is an abundant, yet little understood, platelet receptor. It is a single Ig domain containing receptor isolated in the a-granules of resting platelets and brought to the platelet surface upon activation. On platelets, the integrin aIIbb3 is the major receptor having roughly 80,000 copies. □II□□3 is a heterodimeric multidomain structure that mediates platelet aggregation through its interaction with the plasma protein fibrinogen. Anti-platelet drugs have successfully targeted aIIbb3 to control thrombosis. Like aIIbb3, TLT-1 also binds fibrinogen, making its role in platelet function somewhat obscure. In this review, we highlight the known structural features of TLT-1 and present the challenges of understanding TLT-1 function. In our analysis of the dynamics of the platelet surface after activation we propose a model in which TLT-1 supports □II□□3 function as a mechanoreceptor that may direct platelets toward immune function.

Introduction

Receptors are important targets for the pharmacological manipulation of cellular functions and disease. Even though platelets are considered, by many, as simple cells because of their lack of a nucleus, their functions are very complex. Platelets probe our vasculature, identify breaches, and restore vessel integrity[1,2]. In recent years, the role of platelets beyond hemostasis has taken center stage, especially in the field of innate immunology, where they have been shown to be key determinates in disease severity and outcomes[1,3]. As such, understanding the pathways that are regulated by platelet receptors is paramount, if we are to be able to manipulate the diseases in which their function is key.

In this review we will focus on the enigmatic platelet receptor Triggering Receptor Expressed in Myeloid cells (TREM) – Like Transcript -1 or TLT-1. TLT-1 is a paradigmatic type-1 single immunoglobulin domain membrane protein receptor, similar in structure to other immunoglobulin-like variable domains, particularly those of triggering receptor expressed on myeloid cells-1 (TREM-1), the polymeric immunoglobulin receptor (plgR), and the natural killer cell-activating receptor of 44kD (NKp44)[4–6]. TLT-1 was identified in a screen of the Celera database using the sequence of NKp46 as a probe. It was surprising when TLT-1 was identified as an abundant receptor unique to platelets[5,7]. Given its abundance on activated platelets, its localization in the platelet □-granules of resting platelets explains how it had remained undiscovered for so long; however now that it has been identified, TLT-1 holds its secrets tightly guarded.

Here, we will look at the structural aspects of TLT-1 in relation to the functional

and pathophysiological evidence that has been presented. From this we hope to derive a clearer picture of the niche that TLT-1 fills in platelet function and present a road map to uncovering the secrets to platelet function that it guards.

The TREM-Like Transcript-1 Structure

TLT-1 lies within the TREM gene cluster[6,8]. Cloning and expression studies have defined TLT-1 as a membrane bound protein consisting of 311 amino acids and a mass of 32,679 Da[4,5]. Topologically it is characterized by an extracellular domain (147 amino acids in length), a transmembrane domain (21 amino acids in length) and a cytoplasmic domain (127 amino acids in length) that is found exclusively in platelets and megakaryocytes (MKs)[9]

The immunoglobulin-like domain of hTLT-1 is typical of V-type immunoglobulin like folds. It consists of 105 amino acids forming 9 beta strands and it has conserved CDR loop regions (CDR1, CDR2 and CDR3)[4] .

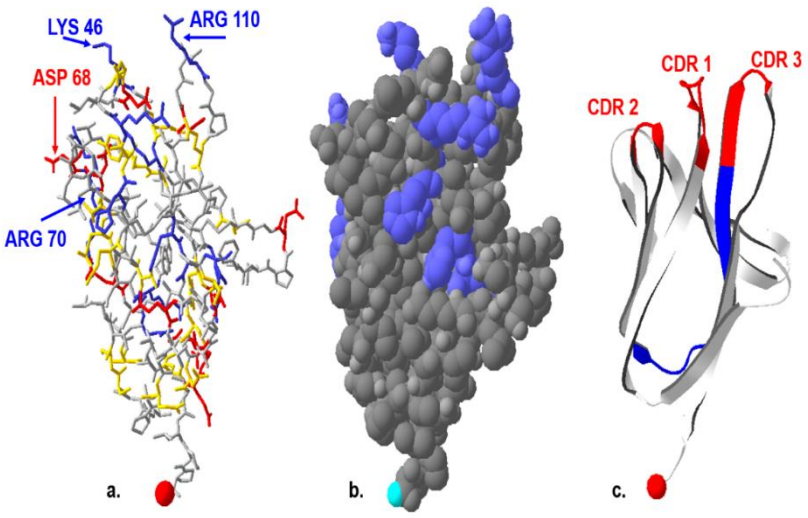


Figure 1.1: The crystal structure of the TLT-1 extracellular domain (aa 20 – 125)

The crystal structure of the TLT-1 extracellular domain (aa 20 – 125) a: stick model showing the negative residues in red, positive residues in blue, and key residue of the beta sheet structure in yellow. The C terminal region is represented by the red space filled proline. b: Space filled model showing the positively charged surface molecules (blue) over a relatively electrostatically neutral surface gray, the C-terminal region is represented by the light blue space filed proline. c: The ribbon structure showing the CDR loops1 & 2 in red and the regions representing the LR12 peptide in blue.

Several charged patches run across the molecular electrostatic surface of hTLT-1 (Figure 1.1)[4]. The hypervariable CDR regions are of particular interest because their tips contain an abundance of lysine and arginine residues, thus determining a predominantly positive charge in these regions. Some interesting structural features to highlight these CDR loops are the R41 & K46 extending from the center of CDR1, the D68 containing negative path of CDR2 proceeding the positive charge from R70, R110 extending from CDR3, and the short 4 residue loop of CRD3 containing hydrogen bonds that are not characteristic of B-strands (Figure 1.1 a & c). CRD3 is the most variable loop, giving the greatest conformational flexibility in comparison to similar ligands with immunoglobulin like domains such as TREM-1, and NKp44[10,11]. On the other hand, like NKp44 and plgR, hTLT-1 has two disulfide bonds connecting the β-strands :C to C', and B to F [11]. The disulfide bond between C38-C104 is conserved in human and murine TREM-1, NKp44 and plgR. The second disulfide bond between C52 and C59 in strand BC is conserved in NKp44 and plgR but not TREM-1(Figure 1.1) [4,10]. The immunoglobulin-like domain is attached to the membrane spanning helix by a 37-amino acid stalk containing a negatively charge patch of 5 consecutive glutamic acids.

The putative transmembrane segment of hTLT-1 is 20 amino acids long and lacks the positively charged amino acid that other TREM family members use to interact with a negatively charged side chain of the adaptor molecule DNAX activation protein (DAP)[12–14]. However, TLT-1 has been shown to be palmitoylated on amino acid cysteine 196[15], meaning that it should be found in lipid rafts with □□□b□□□and may influence aIIbb3 function directly.

TLT-1 conveys its versatility in the four isoforms that have been isolated to date[16]. In platelets, three isoforms have been reported: TLT-1 full length (TLT-1), TLT-1 splice variant (TLT-1sv); the soluble isoform that is released upon platelet activation (sTLT-1), and a form that contains a very short extracellular domain (TLT-1s; see Figure 1d)[4,7,17]. TLT-1sv has not been identified in mice and the specific function of this form is yet to be determined. The soluble isoform corresponds to the extracellular sequence of the protein with a distinct C-terminus containing an additional 2 cysteines that lead to sTLT-1 multimerization[18]. The fourth isoform (TLT-1s) has only been identified in pre-osteoclast[17]. TLT-1s has an alternative translational start sight and contains a very short extracellular domain consisting of the 37 amino acid stalk region, the transmembrane and the complete intracellular domain[17]. The full-length form of TLT-1 (37kDa) and isoform TLT-1sv (25k Da) both contain a transmembrane domain (TMD) but the TLT-1 full length form harbors an immunoreceptor tyrosine-based inhibition motif (ITIM). The cleaved form of TLT-1 which is also called soluble TLT-1 (sTLT-1;17kDa), is distinct from the soluble splice variant[5,6]. The exact cleavage site of cleaved form is not known but it lacks the extra cysteines allowing it to multimerize like the spliced soluble form.

The full-length form (311 amino acids) has only been found on platelets and megakaryocytes to date.

The Soluble Fragment

The soluble fragment of the TLT-1 extracellular domain (sTLT-1) is found in serum of humans and mice[4]. In humans there is a splice variant that multimerizes. However, based on sequencing reads from 16 volunteers, only represents an average 5% of the available TLT-1 transcript in platelets[19]. The majority of soluble fragment found in human serum, however, is believed to be derived from proteolytic cleavage of either the full length or TLT-1sv from the platelet surface[19]. In mice, sTLT-1 seems to be cleaved whole from the surface, releasing a single 25-kDa fragment that is not further proteolyzed. Soluble TLT-1 corresponds roughly to 100 and 110 amino acids of the extracellular domain, or approximately the complete immunoglobulin-like domain (105 residues total, 20 –125)[4]. The recombinant form has been demonstrated to enhance platelet aggregation in a dose-dependent manner[18]. Although the smaller forms of sTLT-1 may result from alternative transcription, release of recombinant human TLT-1 extracellular domain from HEK293 cells strongly suggests that the stalk region of TLT-1 is susceptible to hydrolysis[16]. Interestingly, sTLT-1 is the platelet's fourth most abundantly released molecule upon platelet activation[20] and has been detected in the serum but not in plasma of healthy mice and humans[18]. TLT-1 has been demonstrated to be a better marker of platelet activation than P-selectin in mice[21], while sTLT-1 has been shown to be a biomarker for disease severity in acute respiratory distress syndrome (ARDS) in humans[19].

The TLT-1 Ligand

Key to understanding the function of any receptor is the identification of its ligand. In our early studies we proposed fibrinogen as a ligand of TLT-1 and suggested that during platelet aggregation, TLT-1 cross-links extracellular fibrinogen, stabilizing higher-order platelet aggregates[18]. In this study lysates generated from purified human platelets were applied to AminoLink columns preloaded with either sTLT-1 or sTREM-1. This approach revealed specific binding of 3 proteins with molecular masses between 50 and 80kDa on a Coomassie stained gel. Mass spectroscopy identified these proteins as the α, β, and γ chains of fibrinogen. To confirm these findings, fibrinogen was also eluted from His-tagged TLT-1 but not TREM-1 bound to nickel columns and resolved by PAGE in either native or reduced conditions. Consistent with disulfide-linked multimers of fibrinogen, TLT-1 column specifically bound a high molecular weight complex that when reduced resolved into the same 3 bands detected with AminoLink columns. Immunoblotting with anti-fibrinogen confirmed the identity of these TLT-1–interacting proteins as fibrinogen. This was further supported by ELISA, confirming fibrinogen as a TLT-1 ligand. While the identification of fibrinogen as a ligand was expected to bring answers, it only generated larger questions.

TLT-1 / aIIbb3 / Fibrinogen

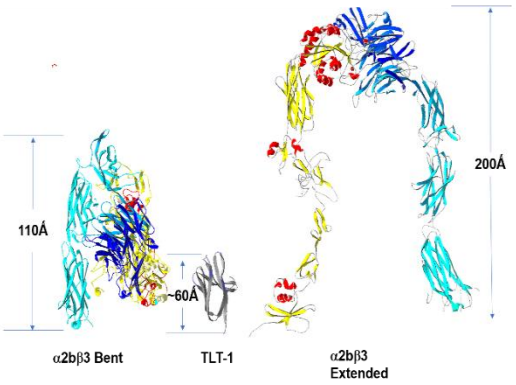


Figure 1.2: a2bb3 is the major platelet receptor. a2bb3 is a heterodimeric integrin receptor that binds fibrinogen. The a-subunit has 10xx amino acids and the b-subunit has 9xx amino acids. On the resting platelets current models suggest that a2bb3 maintains an inactive bent (left) confirmation and upon platelet activation it changes from the bent form reaching to 110 Å above the platelet surface to the extended form which towers to 200 Å above the platelet surface (right). TLT-1 (middle) is estimated to reach between 50 and 60 Å over the platelet surface.

There is broad agreement that platelet aggregation is generally dependent on fibrinogen binding to the glycoprotein (GP) alpha 2b beta 3a integrin (aIIbb3) receptor expressed on the platelet surface (Figure 1.2)[22]. Mutations, in aIIbb3 effectively reduce platelet aggregation and individuals lacking functional aIIbb3 are afflicted with a disease called Glanzmann's thrombasthenia[23], characterized by a severe bleeding disorder. Mice lacking either the aIIb or the b3 subunits of the integrin display a similar bleeding diathesis as patients with Glanzmann's thrombasthenia[23].

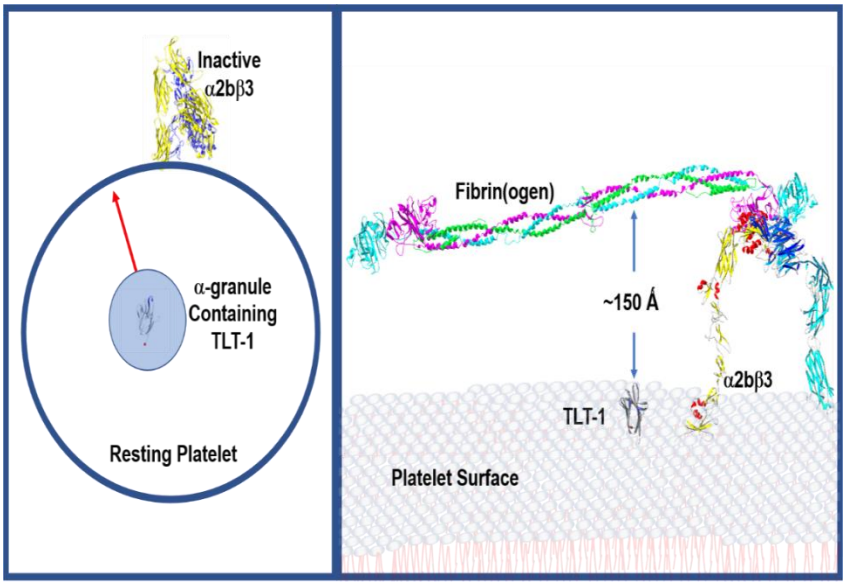


Figure 1.3: The platelet membrane landscape changes after platelet activation. The platelet membrane landscape changes after platelet activation. Left panel: shows the relationship of TLT-1 to fibrinogen in resting platelets. The greater part of a2bb3 is found in the bent formation on the surface of resting platelets. There is some a2bb3 stored in the granules (not shown for clarity). TLT-1 on the other hand is sequestered in the a-granules and brought to the surface upon activation. In this conformation, neither receptor bind fibrinogen. Right panel shows the surface of activated platelets. a2bb3 interacting with fibrin on the surface of a platelet showing TLT-1 for size comparison. Once on the platelet surface TLT-1 seems dwarfed in comparison to a2bb3. a2bb3 stands approximately 200 Å over the platelet surface where TLT-1 is between 50-60 Å including the stalk (not shown).

Accordingly, pharmacological agents, such as Integrilin (Eptifibatide acetate)[24,25] and Abciximab, directed toward[26] the aIIbb3/fibrinogen interaction site are effective at blocking thrombosis, thereby substantiating the importance of aIIbb3 to platelet/platelet interaction.

However, just like aIIbb3, TLT-1 binds fibrinogen[18,19], and herein lies the challenge. On resting platelets, aIIbb3 assumes an inactive conformation with greatly reduced ability to bind fibrinogen[27]. TLT-1, on the other hand is stored in the □-granules, sequestered from fibrinogen[9] (Figure 1.2 bottom). As such, neither receptor has the predilection to bind fibrinogen.

Activation, however, changes the platelet landscape and presents a seemingly inhospitable environment for TLT-1/fibrinogen interactions (Figure 1.2 bottom). aIIbb3 is the most abundant integrin on the platelet surface with approximately 80,000 complexes on the surface of each platelet[28] Activation changes aIIbb3 from the low affinity bent conformation to the high affinity extended conformation (Figure 1.2) [27] It is well established that aIIbb3 is able to bind both fibrinogen and fibrin, and monomeric fibrin displays a higher probability of interacting with aIIbb3 and a greater binding strength (kD) than fibrinogen (<1nM vs 70 -100nM)[29,30]. At present the binding specificity of TLT-1 to fibrin vs fibrinogen have not been determined. Measurement of fibrinogen deposition after LPS challenge in the lungs of mice suggest that TLT-1 may be more specific to fibrinogen than fibrin because there is reduced fibrinogen deposition in the TLT-1 null (*treml1*$^{-/-}$) when compared to the wild type[19]. However, clearer insights will come once the exact molecular interactions between the two molecules are known.

Platelet activation also brings TLT-1 to the platelet surface (Figure 1.2 bottom)[9]. Strength of activation seems to determine the amount of surface expressed TLT-1. Activation with 5 mM ADP yielded an average of ~50,000 copies of TLT-1 on the platelet surface. Using plate-bound fibrinogen, sTLT-1 interacts with fibrinogen with a kD of 50 nM[31]. The size difference between aIIbb3 and TLT-1 may also be a factor. The

height difference between extracellular domains of aIIbb3 and TLT-1 is ~3:1. The extracellular domain of TLT-1 is estimated to be about 50 - 60 angstroms compared to aIIbb3 which towers approximately 200 angstroms over the platelet surface[32] (Figure 1.2 top). If you can envision the singer Shakira (1.57 M height) pitted against Michael Jordan (1.98 M height) in a basketball game; would Shakira ever see the ball? Similarly, the question arises, "at what point does TLT-1 interact with fibrinogen.

The in vitro and in vivo studies of TLT-1 further support the complexity of this scenario. To date there are no known TLT-1 bleeding disorders. While antibodies to TLT-1 can be used to inhibit platelet aggregation in vitro[33,34], there is a very narrow range of activator concentrations in which antibodies are effective. Moreover, the *treml1*$^{-/-}$ mouse does not display an overt bleeding phenotype. We have demonstrated reduced aggregation with ADP or collagen[18] and reduced clotting in the pulmonary embolism model[35] and inhibited platelet aggregation using anti - TLT-1 antibodies[33,36]. Taken together, these studies demonstrate the subtlety of TLT-1 role in classical hemostasis.

All things considered, it is difficult to understand why there are two high-density fibrinogen receptors on platelets and even more difficult to delineate what the contribution of each one has to platelet function. When do they overlap and co-regulate platelet function, and when is there strict regulation of fibrinogen binding by one or the other?

In 2015 it was demonstrated that GpVI, the major signaling molecule on platelets for collagen, binds fibrin and stimulates activation pathways[37,38]. Interestingly, GpVI couples to ITAM containing FcRg. Early models of TLT-1 function suggested that the ITIM of TLT-1 may regulate GpVI/FcRg ITAM signaling. However, this model was

soon abandoned once TLT-1 was shown contribute to activation pathways. The fact that TLT-1 and GpVI bind fibrinogen and physiologically they both have a demonstrated role in immune-derived bleeding, which opens the possibility that these two fibrinogen binding receptors have either additive or synergistic affects during the inflammatory response. Fibrinogen has binding sites for the integrin MAC-1 which mediates transmigration of leukocytes such as neutrophils[39,40]. In a murine model, the presence of TLT-1 on platelets is associated with increased fibrinogen deposition. Interestingly, aIIbb3 is not considered necessary for immune-derived bleeding. Opening the possibility that platelets coordinate neutrophil transmigration and regulation of immune derived bleeding using GpVI, fibrinogen, and TLT-1 independently of aIIbb3.

Insights to role for TLT-1 in hemostasis came from studies using the reverse arthus reaction. In the reverse arthus reaction, immune complexes develop sub dermally and lead to complement deposition and neutrophil infiltration. In normal mice it leaves a bruise, in thrombocytopenic mice it leads to overt bleeding. In these studies, by Goerge et al[41] the b3 mice did not bleed, suggesting that the aIIbb3/fibrinogen interaction was not important for hemostasis derived from immune triggers. *Treml1*$^{-/-}$ mice show a distinct petechia when compared to their wild type counterparts[35] suggesting that the TLT-1/fibrinogen interaction may regulate immune functions of platelets.

Pathophysiological Role of TLT-1

In sepsis and more recently ARDS studies, we have demonstrated a role for TLT-1 in the regulation of immune-mediated hemostasis. In studies using lipopolysaccharide (LPS) to induce sepsis in mice, TLT-1 null mice (*treml1*$^{-/-}$) have a significantly greater rate of mortality than their wild-type counterparts. Studies using the Shwartzman

reaction, which correlates with the severity of sepsis and mimics the mechanisms of disseminated intravascular coagulation (DIC) in a localized lesion, demonstrate that the *treml1*$^{-\backslash-}$ mice bleed from the inflammatory insult, whereas the wild-type mice do not[18] A longitudinal study allowed a comparison of plasma sTLT-1 with D-dimer levels in patients diagnosed with severe sepsis, interestingly, sTLT-1 levels correlated with the presence of DIC better than D-dimers, suggesting sTLT-1 as a new candidate biomarker to test for DIC[18]. These results implicate TLT-1 in the regulation of immune-derived hemostasis.

Over the last decade, a novel concept that platelets are critical regulators of the lung inflammatory processes has been emerging[42–44]. In a study of ARDS patients, Morales-Ortiz et. al demonstrated that plasma levels of sTLT-1 of ARDS patients were significantly elevated compared to those in a control group. In a subsequent study using a cohort of 799 ARDS patients, we demonstrated the utility of sTLT-1 as a specific platelet marker in the development of ARDS due to sepsis. It was found that very low baseline platelet counts (<80 000/μL) and elevated plasma sTLT-1 concentrations (>1200 pg/mL) are predictors of mortality and poor prognosis[19]. This work was further supported in a murine model where LPS was used to induce acute lung injury. In these studies, TLT-1 was shown to be important for controlling bleeding into the alveolar sacs[19]. *Treml1*$^{-/-}$ mice demonstrate substantial bleeding in the lungs after LPS treatment whereas their wild-type counterparts did not. It was demonstrated that in the *treml1*$^{-/-}$ mice, that there was a delay of neutrophil transmigration with a subsequent influx of three times the neutrophils into the alveoli compared to wild type mice. Consistent with a TLT-1 interaction with fibrinogen, *treml1*$^{-/-}$ mice displayed

significantly reduced fibrinogen deposition and increased tissue damage in the lung compared to wile type mice. The addition of intravenous sTLT-1 in these studies increased the fibrinogen deposition and reduced tissue damage.

Neutrophil extravasation from the vessels is a common feature of the arthus, sepsis, and acute lung injury models and suggests that TLT-1 may directly affect neutrophil function. Studies by Derive et. al[45] demonstrate that TLT-1 plays a crucial role in regulating leukocyte activation and modulating sepsis-induced acute inflammatory response. A 17-amino acid sequence (LR17 – 94-LQEEDAGEYGCMVDGAR-110) of the TLT-1 extracellular domain that has great structural similarity but only 35% homology with the TREM family member TREM-1 was identified. These studies demonstrate that LR17 mediates a protective effect in murine sepsis models, reduces organ damage, and mortality in the *treml1*$^{-/-}$ mouse sepsis model. The authors interpret these results as the LR17 competes with the ligand of TREM-1, thereby providing a blockade of TREM-1 as an amplifier of the inflammatory response. A recent study using LR12, a truncated LR17 (94-LQEEDAGEYGCM-105; Figure 1.1c blue ribbon), with a greater structural similarity and 50% homology reproduces the LR 17 results in wild type septic mice[46]. These results argue for the importance of this 12 amino acid region in TLT-1, however caution should be taken in the interpretation of TREM-1 ligand competition. While LR17 and 12 may block TREM-1 function directly, neither platelets nor fibrinogen were carefully evaluated and there is no way to determine if TREM-1 activation was secondary to platelet function in these studies.

The size and distribution differences between aIIbb3 and TLT-1 allow the conjecture that TLT-1 may play a mechanosensory role. In this speculative model, compression of cells, or shear stress increases the probability of fibrinogen clustering.

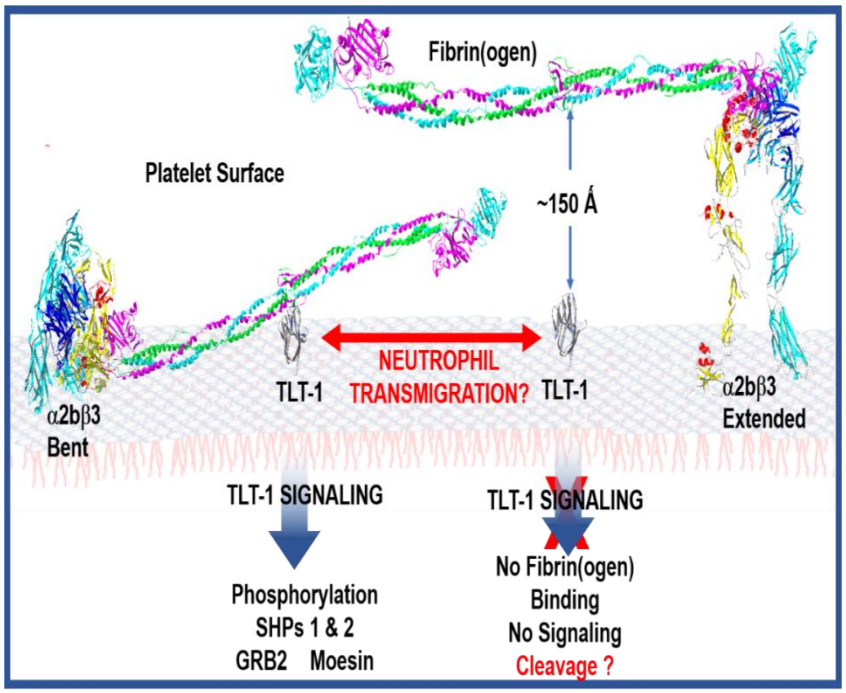


Figure 1.4: Mechanistic Model for TLT-1/Fibrinogen interaction: When in the extended confirmation, the extreme height of a2bb3 may prevent TLT-1 interaction with fibrin(ogen). Transmigration, however, may induce the bent confirmation of a2bb3, allowing fibrin(ogen) to interact with TLT-1 more readily. TLT-1 fibrinogen interaction may induce intercellular signaling that does not happen under conditions of classic hemostasis. TLT-1 fibrinogen would induce TLT-1 clustering, a tighter binding of fibrinogen to the platelet surface, and intercellular signaling. Without the fibrinogen interaction TLT-1 may be cleaved off of the surface more readily.

This hypothesis is supported by the transmigration models in which neutrophils from either wild-type or *treml1*$^{-/-}$ mice (TLT-1$^{-/-}$ null mice) fail to release attached platelets during transmigration over fibrinogen in the presence of *treml1*$^{-\backslash-}$ platelets. Wild-type platelets, with either neutrophils from either wild-type or *treml1*$^{-/-}$ mice transmigrated without attached platelets[19]. Studies using Mn^{++} to activate aIIbb3, suggest that even in the bent form, aIIbb3 is capable of binding fibrin(ogen)[32]. It is possible that transmigration induces the bent form of aIIbb3 and increases fibrinogen interaction with TLT-1, inducing stronger platelet/fibrinogen interactions. The neutrophils transmigrate and the platelets seal the gap[37] Going back to the comparison of Shakira to Michael Jordon, if the competition were singing and not basketball, how the voices worked together would be more important than height.

Cell Signaling & TLT-1

Despite the known importance of TLT-1 in platelet biology and several potentially interesting motifs in its cytoplasmic domain, our knowledge of TLT-1 mechanistic signaling is limited. The cytoplasmic tail contains proline rich domains, two tyrosines (Tyr265 and Tyr281) shown to be phosphorylated[7] and a string of serines demonstrated to be phosphorylated in human platelets[47] (Figure 1.5).

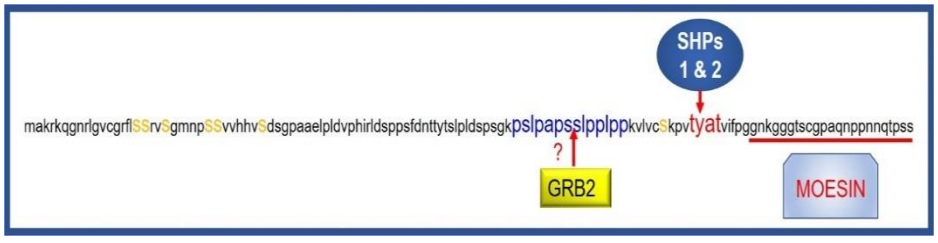


Figure 1.5: The TLT-1 cytoplasmic tail. The serines demonstrated to be phosphorylated are shown in gold, the proline rich region is shown in blue, the ITIM is shown in red. Known cytoplasmic interacting partners are shown in their regions of

suspected interaction. GRB2 is believed to interact with the proline rich domain, SHP 1 & 2 have been demonstrated to bind the ITIM, and Moesin binds the distil region of cytoplasmic tail (last 25 amino acids unpublished observations)

While Y281 is central to a canonical immunoreceptor tyrosine-based activation motif (ITIM) and shown to be important to binding SHP-1 [7], not much is known about the importance of Y265. In the membrane proximal regions, there are a series of serines that may be of great interest. There are models that suggest that the cytoplasmic tails of ITIM containing proteins in the resting state are unstructured but remain buried in the membrane in a way that sequesters access of key residues from interaction partners[48–50]. PECAM-1, also found in platelets, is an example of one of these receptors[51]. It has been demonstrated that upon platelet activation, serines become phosphorylated, unzippering the tail from the membrane granting access to key motifs such as the ITIM. The structure of the TLT-1 cytoplasmic tail containing 7 serines that are phosphorylated and a distal ITIM, is very reminiscent of the PECAM-1 cytoplasmic tail suggesting that TLT-1 may work in a similar fashion to PECAM-1 upon platelet activation.

The first characterized interacting partners of TLT-1 were the SH2 domain-containing protein phosphatases SHP-1 and SHP-2[5,7]. They have been shown to co-immunoprecipitate with TLT-1 following induction of tyrosine phosphorylation. Barrow, *et.al.* demonstrated that phosphorylation of both Y265 and Y281 was regulated by tyrosine phosphatases, however, only phosphorylation of Y281 induced the TLT-1/SHP-2 interaction[7]. Furthermore, phosphorylation of Y281 within the TLT- 1 ITIM was demonstrated to enhance FcεRI mediated calcium mobilization through SHP-2 in rat basophilic leukemia cells which suggests a potential co-stimulatory role in platelets[7].

Interestingly the T280 and the S278 adjacent to the Y281 are also phosphorylated, which may affect the phosphorylation of the Y281 and ultimately affect its binding of SHP-1, and -2[47].

The scaffolding protein moesin was shown to interact with the TLT-1 cytoplasmic tail. Moesin is known to link membrane proteins via its ERM (Ezrin, Radixin, and Moesin) domain to the actin cytoskeleton[18]. This data postulates that the mechanism by which TLT-1 facilitates platelet aggregation involves linking fibrinogen to the platelet cytoskeleton via the ERMs. However, none of the ERM protein deficient mice have a demonstrated bleeding diathesis[52,53] and therefore the importance of these interactions is yet to be determined.

TLT-1 has also been seen to stabilize clot formation by facilitating outside-in signaling of aIIbb3. $\beta3^{Y773}$ phosphorylation ($p\beta3^{Y773}$) in the aIIbb3 tail was examined over time in WT and *treml1*$^{-/-}$ platelets. $p\beta3^{Y773}$ is an indicator of early outside-in signaling[35] Consistent with published data [35] it was found that within 5 minutes of platelet activation, $p\beta3^{Y773}$ increased in *WT* mice. *Treml1*$^{-/-}$ platelets demonstrated ~2X less $p\beta3^{Y773}$ at 5 minutes and a greater deficiency over a 20-minute period. This data suggest that TLT-1 may enhance aIIbb3 stability, although an association with increased phosphorylation is inconsistent with TLT-1 binding to SHP phosphatases.

Schmoker et. al recently published a proteomic analysis to define the TLT-1 protein-protein interactions in both resting and activated platelets[47]. Several novel TLT-1 interacting partners were identified including several RAB proteins and RACK1, the integrin interacting protein. Of great interest, however, are the two proteins validated in platelets, aIIbb3 and GRB2. The amount of aIIbb3 that associates with TLT-1 on

activated platelets is greatly reduced compared to resting platelets. Thus, platelet activation seems to separate aIIbb3 from TLT-1 suggesting that TLT-1 may play a role in maintaining aIIbb3 in its resting or bent form. In line with a mechanosensory role for TLT-1, transmigration may release a subset of aIIbb3 increasing the amount available to bind fibrinogen. It should be mentioned that both aIIbb3 and TLT-1 are found in the a-granules and the reduced interaction seen upon activation may reflect release from the a-granules. At the same time TLT-1 engagement of fibrinogen would lead to the unzippering of the TLT-1 cytoplasmic tail allowing interaction with scaffolding protein moesin thereby further strengthening platelet – platelet interactions and ultimately platelet release from the neutrophil.

GRB2 on the other hand is a linker protein that contains two SH3 and a single SH2 domain, either of which could mediate interactions with TLT-1. Early studies with the SH2 domain of GRB2 did not demonstrate significant interaction (unpublished data) and suggest that TLT-1/GRB2 interaction may be mediated by the proline rich domain. GRB2 has been shown to play an important role in T-cell activation through interactions with SLP-76. It would be of interest to see if GRB2 mediates similar interaction in platelets, since SLP-76 has been implicated in immune-derived bleeding[54].

Conclusions

Current data suggest that the TLT-1/sTLT-1 axis is important for immune hemostasis. To understand how, or even if, fibrinogen directs platelets toward immune function will take a greater understanding of the TLT-1 structure function relationship. Given the size difference and disparity in copy number between TLT-1 and aIIbb3, a better appreciation of the landscape of the platelet surface may also become an important

metric to better understand this association. TLT-1 peptide studies suggest that the b-sheet leading to the CDR3 loop of TLT-1 may be the key to understanding the mechanism behind TLT-1 function[46,55] Crystallographic studies suggest that this region may have flexibility for allosteric change allowing for a peptide to affect TLT-1 function[56] Even though TLT-1 remains an enigmatic platelet receptor, the foundations have been laid to dissect its unique role in platelet function. Given the abundance of TLT-1 in our system we hope to use our knowledge on the structural aspects of TLT-1 to develop interventions designed reducing cardiovascular risk and/or improving survival for those who suffer from lung injury.

*The above section of this chapter has been published as a review on TLT-1: Branfield, S., & Washington, A. V. (2021). The enigmatic nature of the triggering receptor expressed in myeloid cells -1 (TLT- 1). *Platelets*, *32*(6), 753–760. https://doi.org/10.1080/09537104.2021.1881948

CHAPTER 2

THE TREML1- FIBRINOGEN INTERACTION

Abstract

Platelets and fibrinogen, pivotal players in inflammation and hemostasis, orchestrate complex interactions crucial for immune response regulation[1]. Platelets binding to fibrinogen is facilitated by αIIbβ3, the most abundant platelet receptor, and thus it was surprising to discover that Trem-Like Transcript-1 (TLT-1) was identified as a ligand to fibrinogen[2,3]. It is difficult to understand why there are two receptors with the same ligand on the platelet surface[4]. This study explores the binding affinity and molecular dynamics of Trem-Like Transcript-1 (TLT- 1) with fibrinogen. The kinetics assay revealed a robust TLT-1/fibrinogen interaction, with a dissociation constant (KD) of 3.02 ± 0.20nM. Atomic force microscopy suggests that TLT-1 plays a role in fibrinogen polymerization, demonstrating structural similarity to the thrombin-fibrinogen interaction. LP17 and CDR3 we isolated as the binding sites for fibrinogen on TLT-1, while five peptides (TRGGSTYS, YQISVNKY, SMKIRPFF, SGLYFIKP, HYGGFTVQ) were identified as the binding sites for TLT-1 on fibrinogen by immunoprecipitation. Further validation through ex vivo assays showcased the inhibitory role of fibrinogen peptides in platelet aggregation and cell attachment. The interaction's complexity was underscored by 3D modeling, suggesting a multi-site binding interaction on fibrinogen. (Docking information to be inserted here). Notably, this study proposes an alternative therapeutic approach targeting TLT-1/fibrinogen interaction, potentially reducing thrombotic risk without significant bleeding concerns. However, the intricate

nature of this interaction demands further in vivo studies to assess physiological relevance. This work unveils a promising avenue for understanding and manipulating platelet-fibrinogen interactions, holding implications for thromboinflammation, inflammation-related diseases, and potential therapeutic interventions.

Background

Platelets and fibrinogen play integral roles in the intricate orchestration of the body's immune response, particularly in the regulation of inflammation[1,5–7]. Inflammation is a complex biological process that serves as a crucial defense mechanism against infections, injuries, and other threats to the body's homeostasis. Platelets, small cells originating from megakaryocytes, and fibrinogen, a plasma protein, interact to modulate the inflammatory response.

Platelets, traditionally recognized for their role in hemostasis and blood clotting, have emerged as pivotal players in the realm of inflammation. Upon encountering vascular damage or inflammation triggers, platelets undergo activation, releasing a cascade of bioactive molecules[6,8,9]. These molecules, including cytokines, chemokines, and growth factors, exert diverse effects on immune cells, endothelial cells, and other components that facilitate maintenance of the inflammatory environment. One of the key interactions in inflammation regulation involves platelets and leukocytes. Platelets can directly interact with leukocytes, promoting their adhesion and recruitment to the site of inflammation. Moreover, platelets act as rapid responders, arriving at the site of inflammation to initiate immune cell recruitment and orchestrate a dynamic cellular interplay[1].

Fibrinogen, on the other hand, is a multifunctional protein that serves as a major player in blood coagulation[7]. However, beyond its conventional role in clot formation, fibrinogen also actively participates in the regulation of inflammation. Studies demonstrate that aberrant fibrinogen deposition are a common feature in the inflammatory microenvironment, and that deposits can be found at the site of injury post mechanical, infectious or inflammatory injury. During inflammation, fibrinogen is cleaved into fibrin by the action of thrombin, an enzyme involved in blood clotting. Fibrin, in turn, forms a mesh-like structure that contributes to the stabilization of blood clots. Importantly, this fibrin matrix serves as a scaffold for leukocytes, facilitating their migration to the inflamed site and promoting tissue repair. Moreover, vascular endothelial damage at the site of tissue injury leads to platelet activation through the coagulation cascade initiated by thrombin or collagen and subendothelial platelet activating factors exposure, facilitating platelet – fibrinogen binding, clot formation and vascular repair[5,7].

The synergy between platelets and fibrinogen is evident in their coordinated efforts to modulate inflammation. Platelets release various bioactive molecules that not only attract immune cells but also contribute to the activation of the coagulation cascade, leading to the generation of fibrin[10]. This fibrin network, in conjunction with platelet-derived signals, creates a microenvironment that supports immune cell adhesion, migration, and activation. Furthermore, platelets and fibrinogen collaborate in the resolution phase of inflammation by promoting tissue repair and regeneration. Despite their crucial roles in inflammation, dysregulation of platelet-fibrinogen interactions can contribute to pathological conditions. Excessive platelet activation may lead to

thrombosis, while aberrant fibrin formation can result in chronic inflammation and tissue damage. Understanding the delicate balance between platelets and fibrinogen in inflammation is thus essential for developing therapeutic strategies that target specific components of this intricate interplay, aiming to mitigate inflammation-related diseases.

Upon activation, platelets release TREM-Like Transcript-1 (TLT-1) from their a-granules onto their surface. TLT-1 is a type 1 receptor that, like the integrin αIIbβ3, binds fibrinogen and facilitates platelet aggregation[11–13]. However, although TLT-1 may assist in clot formation and hemostasis to arrest bleeding in a non-inflammatory mediated setting, TLT-1's main association is with regulating inflammatory-derived bleeding as demonstrated by increased hemorrhage after inflammatory treatments such as lipopolysaccharide LPS in the treml1$^{-/-}$ mice as compared to controls[14].TLT-1 has also been shown to regulate leukocyte activation and modulate sepsis induced acute inflammatory responses. During platelet activation a soluble form of TLT-1 is cleaved off the platelet surface (s-TLT-1) and has been shown to have biological importance, with increased levels of s-TLT correlating with inflammatory related pathologies such as acute coronary syndrome, (ACS) acute respiratory distress syndrome (ARDS) and sepsis. TLT-1 binding fibrinogen was a surprising discovery since αIIbβ3, the most abundant platelet receptor, also binds fibrinogen and facilitates platelet aggregation[15–17].

It is difficult to understand why there are two platelet specific receptors that have the same ligand, drawing us to question what the difference in function between the two is? Our studies suggest that although TLT-1 may assist in clot formation and hemostasis to arrest bleeding in a non-inflammatory setting like αIIbβ3, TLT-1's main association is with regulating inflammatory-derived bleeding. Very little is known about the TLT-1-

fibrinogen interaction, further studies would set the stage for a better understanding as to why two fibrinogen ligands exist on platelets and potentially outline a novel platelet therapeutic target during hypercoagulable and/or hyperinflammatory states. We set out to determine the binding affinity and localize the binding sites for the TLT-1 Fibrinogen molecular interaction.

Methods

Bio-Layer Interferometry

To assess the binding kinetics of the TLT-1 and fibrinogen interaction, a Bio-Layer Interferometry (BLI) assay was conducted using the Octet QKe instrument. The instrument's sensitivities included an association constant (ka, M-1s-1), dissociation constant (kd, s-1), and equilibrium dissociation constant (KD, M) ranging from 103 to 107 M-1s-1, 10–6 to 10–1 s-1, and 0.1 mM to 10mM, respectively. A 96-well plate was prepared by loading recombinant TLT-1 with the Fc region of human Immunoglobulin type G (IgG) at a concentration of 1.5μg/mL (35nM) in a baseline buffer. A control antibody 4E10 (0.30 ng/mL) was added, and a negative control reference sensor lacked recombinant TLT-1. The association column contained human fibrinogen at concentrations of 125nM, 62.5nM, 31.3nM, and 15.6nM, while the reference well did not contain fibrinogen. Anti-Human Capture (AHC) sensors were soaked in baseline buffer before the assay. During the loading stage, optical sensors with anti-Human IgG were dipped in the association wells for 600 seconds, immobilizing the control antibody 4E10 and recombinant TLT-1. After loading, biosensors were dipped in baseline wells for 120 seconds to wash away non-specifically interacting ligands. Subsequently, biosensors were dipped in association wells for 900 seconds, allowing fibrinogen to bind to

immobilized TLT-1. The dissociation stage involved dipping sensors in baseline wells for 900 seconds to wash away and dissociate bound fibrinogen from TLT-1. Octet Data Analysis High Throughput (HT) software aligned, subtracted reference wells, and fitted curves globally to a 1:1 model, yielding binding kinetics values (ka, kd, KD). The assay was repeated for mean (μ) and standard deviation (σ) calculations of kinetics values.

Immunoprecipitation (IP)

Human fibrinogen was reconstituted with 0.1 M of NH4HCO3, pH 8.5 at a concentration of 11.1 mg/mL. Mass Spec Grade Trypsin/Lys-C or chymotrypsin at 1 μg/μL concentration was added to the reconstituted fibrinogen following a 1:100 weight/weight (w/w) ratio. The fibrinogen digestion was incubated overnight at 37℃. Digestion was stopped using 5 % Trifluoroacetic Acid (TFA) to acidify the solution to a pH 1 – 4, inhibiting trypsin proteolysis. His-Select Nickel Affinity Gel (Sigma Aldrich) beads were washed with double diluted water (dd H2O) three times and centrifuged for one minute at 5,000 RPM. Three different volumes (2 μL, 10 μL and 20 μL) of His – tagged sTLT-1 at concentration of 4 mg/mL were added to the tubes containing the beads. His – tagged sTLT-1 was not added to control beads. Tubes were left incubating in a rotator for 1 hour at 4℃. After incubation, washing buffer (50 mM NaPO3, 0.3 M NaCl, 10 mM Imidazole, pH 8.0) was added to the tubes containing beads. The washing step was repeated after each incubation. Tubes were centrifuged for 1 minute at 5,000 RPM. Supernatant that consisted of unbound sTLT-1 in solution was removed from the tubes. 20 μL of digested fibrinogen peptides were added to the tubes and left incubating in a rotator for 1 hour at 4℃. Fibrinogen peptides that bound specifically to sTLT-1

stayed in the beads, while those that had no interaction with sTLT-1 remained in the supernatant.

Finally, to isolate the peptides bound to sTLT-1 from the beads, an elution buffer (50 mM NaPO3, 0.3 M NaCl, 250 mM Imidazole, pH 8.0) was added to the tubes and left to incubate for 10 minutes. After incubation, the tubes were centrifuged for 30 seconds at 5,000 RPM. The supernatant was removed and added to different tubes which contained the digested peptides that bound specifically to TLT-1 and peptides that bound non-specifically to the beads. These samples and controls were acidified with 5% TFA to a pH 1 – 4 and ready for Liquid Chromatography – Mass Spectrometry (LC – MS/MS).

Liquid Chromatography – Mass Spectrometry (LC – MS/MS)

Using the Easy-nLC1200 instrument from Thermo Fisher Scientific, peptides were injected into a ReproSil-Pur C18-AQ column. Then, they were separated using a gradient elution 7 by increasing and decreasing flow of 0.1% formic acid in 80% acetonitrile, termed Buffer B, in a determined time frame. The separation was obtained using a total gradient time of 69 minutes: 5 - 40% of Buffer B for 45 minutes, 40 - 95% of Buffer B for 6 minutes, 5 - 95% Buffer B for 12 more minutes and 95 - 5% of Buffer B for 6 minutes. Flow rate was set at 300nL/min, and the injection volume was 2µL per sample. The column was analyzed with Q-Exactive Plus Mass Spectrometer (MS) from Thermo Fisher Scientific. First scan MS measured the range of 400 – 1600 m/z. The MS/MS analysis selected the 10 most intense ions for fragmentation. The generated spectra were analyzed in Proteome Discover 2.1 and sequences from Uniprot were used to match the peptides to fibrinogen regions.

Atomic Force Microscopic (AFM) analysis of TLT-1 Fibrinogen interaction

For our control sample, fibrinogen was used at a concentration of 0.9mg/ml in Tris- HCL Ph8.5 buffer and plated on mica (SPI ChemTM Mica Grade V-1 12mm Pk (100) Discs CAS# 12001-26-2) coated coverslips at 100ul of sample, left to incubate at 37 Degrees Celsius for two hours, rinsed with DDH2O, dried with nitrogen and imaged on the AFM. The same preparation was done for our thrombin- fibrinogen sample and TLT-1 fibrinogen sample, however, after dilution of fibrinogen, either thrombin alone, TLT-1 alone or both (at concentrations 0.2 units/1ml Thrombin or 1200pg/ml TLT-1) were added to 100ul of fibrinogen and left to incubate at 37°C for 2 hours before rinsing with ddH20 and drying with nitrogen. Images were analyzed and quantified on gwyddion to analyze plane leveling and height of samples, followed by prism for statistical analysis.

Competitive Enzyme Linked Immunosorbent Assay (ELISA)

96 - well plates were coated with fibrinogen at a concentration of 300 nM and left incubating overnight at room temperature (RT). One control well had fibrinogen at 300nM while two other control wells only had 1X Phosphate-buffered saline (PBS) at pH 7.4. After incubation, the plate was flicked in a container to remove the solution in wells. The plate was also blotted in a paper towel to ensure complete removal of the ligand. To wash any remaining fibrinogen that did not bind to the plate, 350 μL of washing solution (0.03% Tween 20 in 1X PBS) were added to each well, and the plate was flicked and blotted again. This flicking, blotting, and washing step was repeated three times after each incubation. Wells were blocked by 8 adding 350 μL of 2% Bovine albumin serum (BSA) for 1 hour at RT. All subsequent incubations were done for 1 hour at RT. Following the peptide inhibition design of previous studies CDR1, CDR2, CDR3 and LP17 were added individually to fibrinogen coated wells at eight different

concentrations: 30,000nM, 3,000nM, 1,500nM, 750nM, 300nM, 150nM, 60nM and 30nM. Additionally, a mix of all these peptides termed “Peptide Mix”, was added to fibrinogen coated wells at the previously mentioned concentrations. All other standard wells and controls were incubated with 1X PBS.

TLT-1 Fc standards were diluted in the following concentrations: 30nM, 15nM, 7.5nM, 3.88nM and 1.87nM. No TLT-1 was added to the control well that contained fibrinogen, while TLT-1 at 30nM was added to one control well incubated with only 1X PBS. The remaining control well was again incubated with only 1X PBS. TLT-1 Fc at a concentration of 30nM was added to wells that had previously been incubated with control and sample CDRs, LP17 or the Peptide Mix.

Goat Anti-Human IgG Horse-Radish Peroxidase (HRP) linked antibody was diluted to a 1:7,000 ratio in 0.1% BSA, 1X PBS. This solution was added to all the wells and left to incubate as previously mentioned. After the final wash procedure, 3,3',5,5'-Tetramethylbenzidine (TMB) was added to all the wells in the plate and incubated for 5 – 10 minutes at RT. A 0.5 M hydrochloric acid (HCl) solution was added to stop the enzymatic reaction. Infinite 200 Pro Microplate Reader was configured to read plates at 450 nm and to obtain absorbances from each well. Each control and sample well were done in duplicates and this ELISA procedure was repeated three times. This permitted the calculation of the mean (μ) absorbance and standard deviation (σ) for each control and sample. A standard curve was designed with TLT-1 concentration (nM) vs. absorbance at 450 (nm) for the purpose of measuring the quantity of TLT-1 binding to fibrinogen. For samples treated with CDRs, LP17 and the Peptide Mix, similar graphs of TLT-1 peptide concentration (nM) vs. absorbance at 450 (nm) were designed. A linear or nonlinear

regression line was constructed to observe the concentration dependent blocking effect. To compare the effect of each treatment peptide (CDRs, LP17 or Peptide Mix) at the same concentration, one way ANOVA tests and Tukey's honesty significant difference (HSD) were conducted.

Aggregometry

Blood was drawn by standard brachial vein blood collection into citrated tubes, spun down in a centrifuge at 100G for 20 minutes to separate plasma from platelets. Plasma was extracted from transferred into a 15ml conical tube, prostaglandin E2 and apyrase we added to plasma to prevent platelet activation, spun down for 8 minutes at 900G to obtain a platelet pallet. The pallet was resuspended in 10% ACD/Tyrode's buffer with prostaglandin E2 and apyrase and spun a second time at 900G before being resuspended in Tyrode's buffer. Resuspended platelets were counted using a Sysmex hematology analyzer. Lumi-aggregometry was done using 2.5×10^5 washed platelets in the presence or absence of the fibrinogen peptides to determine whether there was a reduction on platelet aggregation in the presence of these peptides using either collagen (at a concentration of 5ug/ml) or thrombin (0.5u/ml) as agonists.

Platelet Fibrinogen Binding by flowcytometry

Using flowcytometry, we incubated washed human platelets with FITC labeled fibrinogen and activated these platelets in the presence or absence of these selected fibrinogen peptides to determine whether the peptides decrease fibrinogen binding. The peptides were introduced at different concentrations to assess their inhibitory potential.

Platelet spreading

Coverslips were precoated with fibrinogen or peptides at a concentration of 50ug/ml, washed and blocked with 5% BSA blocking solution. Pretreat coverslips for activated platelets with either collagen (at a concentration of 5ug/ml) or thrombin (0.5u/ml). Using the protocol described above to collect blood for aggregometry, washed human platelets were obtained and counted with the Sysmex Hematology analyzer. Gentle pipette 130ul of washed platelets resuspended in Tyrode's at a concentration of 2.5 x 10^5 onto either your control fibrinogen coated cover slips or peptide coated coverslips. Incubate for 30 minutes. Wash three times with PBS, fix and permeabilize cells with BD Cytofix/cytoperm (Cat No. 554714) for 30 minutes and wash with BD Perm/Wash Buffer before incubating platelets with 200ul of 50ug/ml fluorescent phalloidin in1% BSA for 25 minutes at room temperature. Rinse three times with PBS and mount coverslips onto slides. Image slides with a confocal microscope and use Image J to quantify platelets, followed by prism for statistical analysis.

Statistics:

In this study, statistical analyses were conducted using standard methods to assess differences between groups. A one-way analysis of variance (ANOVA) was utilized to evaluate variations among means across three or more groups, specifically in our competitive inhibition assays when comparing efficiency of peptides, enabling comprehensive comparisons and elucidating potential group differences. This statistical test was chosen based on its appropriateness for the study design and the nature of the data collected. Results from these analyses were critical in elucidating the relationships between variables and providing statistical support for the study's conclusions.

Results

Trem-Like Transcript-1 (TLT-1) has a strong binding affinity for fibrinogen

As a starting point, it was necessary to determine what the binding affinity for the TLT-1 fibrinogen interaction was. Using the kinetics assay, Octet Qk e Bio-layer Interferometry (BLI) as our assay of choice, we obtained an equilibrium dissociation constant (KD) of 3.02 ± 0.20nM for the TLT-1/Fibrinogen interaction.

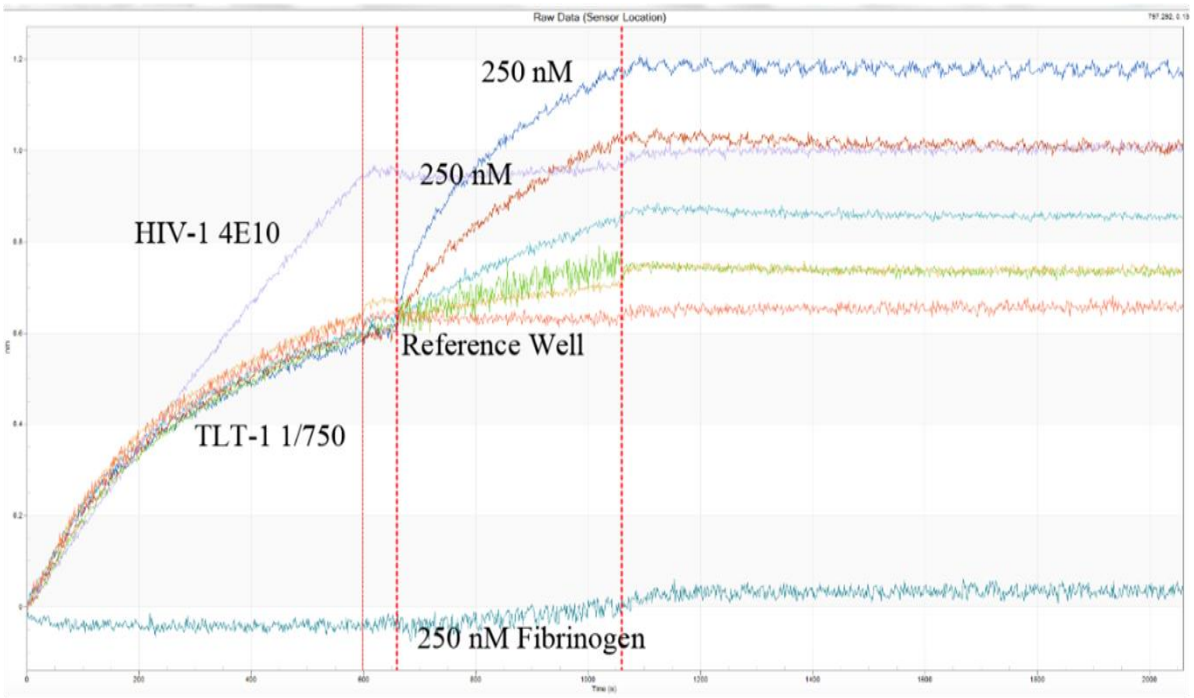


Figure 2.1: Binding of FcTLT-1 to Human Fibrinogen. TLT-1 (Fc Ligand) and Fibrinogen have a concentration dependent bicomplex formation (Green line 31.25nM, Light blue 62.5nM, Red 125nM, dark Blue 250nM) with a concentration of 250nM fibrinogen showing the best bicomplex interaction. HIV-14E10 (Purple line), Sensor (Turquoise line) and Reference (bottom red line) wells show no bicomplex formation.

The curve (Figure 2.1) shows that the TLT-1/Fibrinogen interaction has increasing bimolecular complex formation with increase in concentration of fibrinogen (15.625nM -

250nM), with a concentration of 250nM showing the best bicomplex formation. In the control well with HIV014E10 capture, reference and sensor well, no bicomplex formation is shown, highlighting the specificity of the TLT-1 Fibrinogen interaction. The curve illustrates an association with no dissociation, suggesting a strong interaction between the proteins.

Molecular interaction of the Trem-Like Transcript-1 (TLT-1)- Fibrinogen interaction leads to microclot formation.

Mica plates coated with fibrinogen and viewed under an atomic force microscope (AFM) microscope either alone, in the presence of thrombin, which is known to proteolytically cleave fibrinogen into fibrin, soluble TLT-1 or both, suggests that sTLT-1 mediates fibrinogen polymerization in a similar manner to thrombin. Figure 2 demonstrates that untreated fibrinogen forms a “branch like” structure on mica plates, however in the presence of thrombin, fibrinogen is cleaved and polymerized, forming micro clots. Interestingly, TLT-1 incubation with fibrinogen forms similar micro clots, suggesting that TLT-1 interaction with fibrinogen initiates fibrinogen polymerization. When fibrinogen, thrombin and TLT-1 are incubated together there is not much difference in polymerization, compared to individual incubation. While it was hypothesized that fibrinogen binds TLT-1 to assist in maintaining aggregation and clot integrity, we did not anticipate the interaction to cause a structural change similar to that of thrombin-fibrinogen interaction. These data suggest that the interaction between fibrinogen and TLT-1 is much more complex than we initially assumed, causing structural changes to fibrinogen.

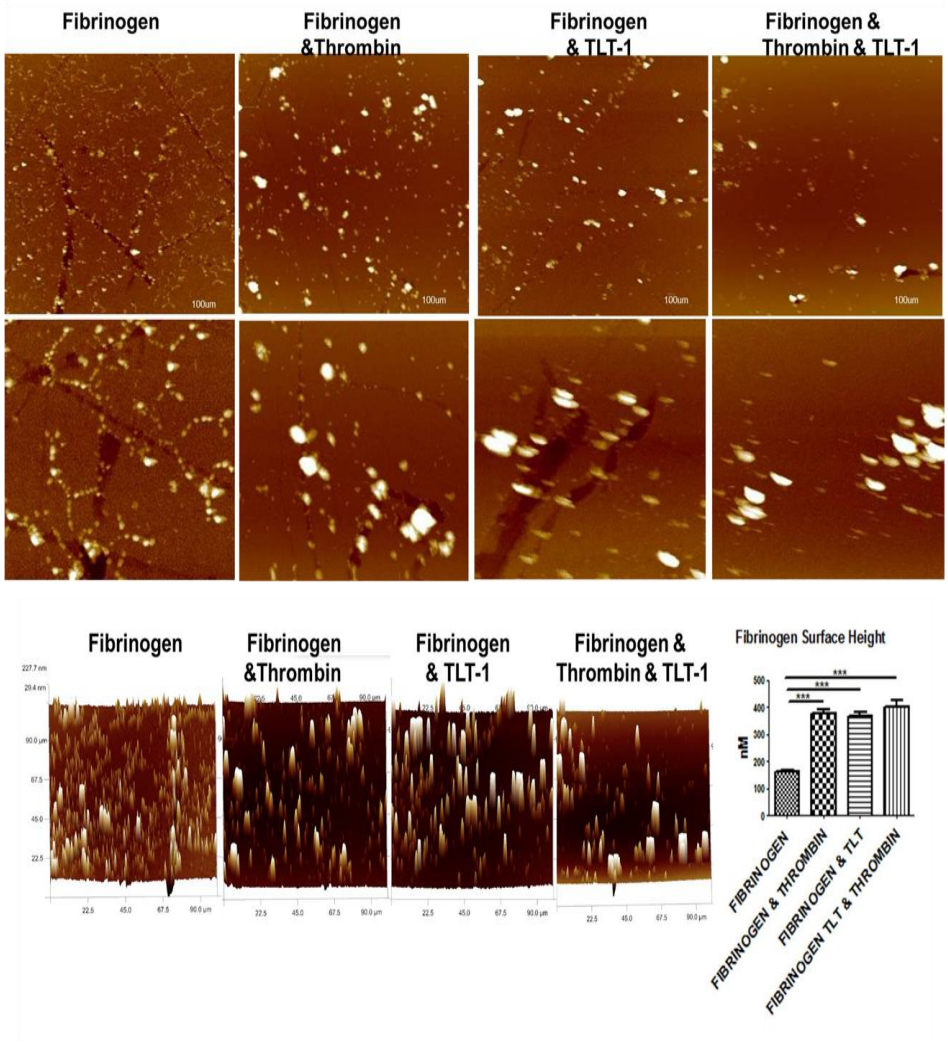


Figure 2.2A: analyzing molecular interaction of trem-like transcript-1 (tlt-1) and fibrinogen by atomic force microscopy (AFM). Comparison of morphological changes between the TLT-1 Fibrinogen interaction and the Thrombin Fibrinogen interaction. AFM Analysis of the TLT-1 Fibrinogen Interaction: The two rows of images are at magnifications of 100um and 24um respectively. Column (A) Fibrinogen only on a Mica surface Column (B) Fibrinogen & Thrombin on Mica Column (C) Fibrinogen & TLT-1 on Mica (D) Fibrinogen, Thrombin & TLT-1 on Mica.3D Model of the Surface

Distribution of Proteins on Mica: Column (A) Fibrinogen only on a Mica surface Column (B) Fibrinogen & Thrombin on Mica Column (C) Fibrinogen & TLT-1 on Mica Column (D) Fibrinogen, Thrombin & TLT-1 on Mica.

Trem-Like Transcript-1 (TLT-1) alters fibrinogen thickness and cross branching

We then wanted to take a closer look at the clots using SEM analysis of clots made with and without sTLT-1. Clots were made with 3.3 ng/mL, 0.56 ng/mL or no sTLT-1, in incubation with thrombin and evaluated by scanning electron microscopy (SEM). In support of the AFM data, there is a distinct difference between fibrin(ogen) clots incubated with or without TLT-1. Fibrin fibers in the presence of sTLT-1 are thinner, more densely packed and have more branching when compared to controls that are in the absence of sTLT-1. Data has demonstrated that clots that have thinner, more branched fibers are more stable and resistant to lysis, suggesting that TLT-1 stabilizes clots, making them more resistant to lysis.

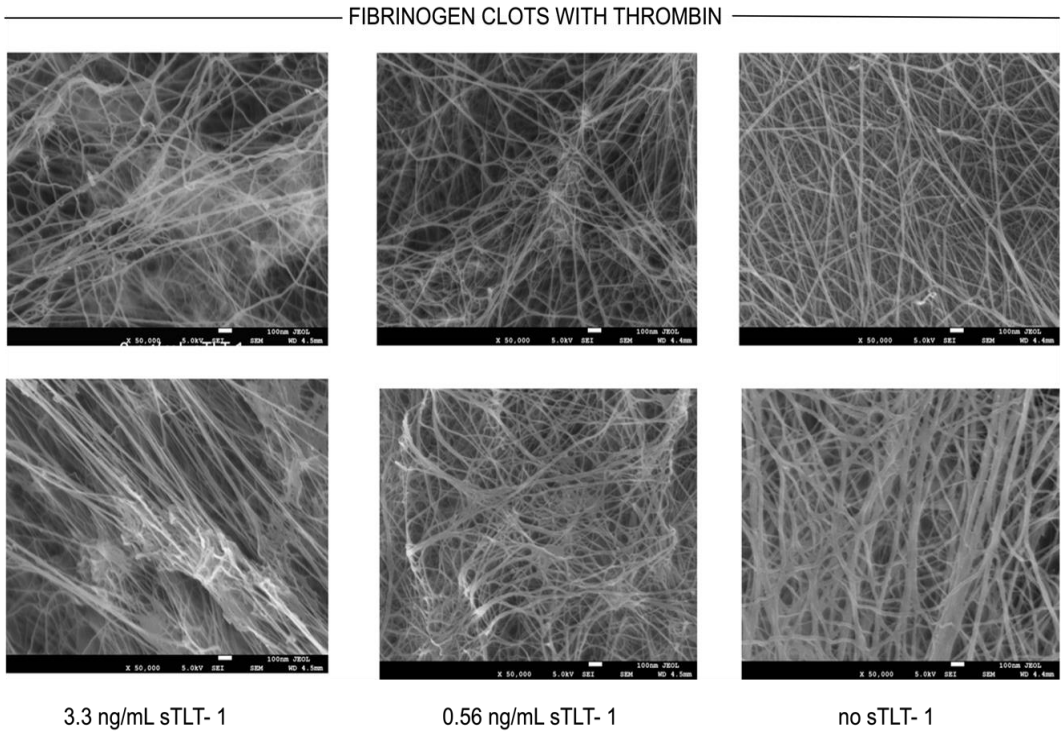


Figure 2b: Comparison of Trem – Like Transcript (TLT-1) Fibrinogen vs thrombin fibrinogen interaction by scanning electron microscope.

LP17 and CDR3 peptides have an inhibitory effect on TLT-1 binding to fibrinogen. The extracellular domain of TLT-1 is comprised of complementarity determining regions (CDRs) formed by beta sheets interactions. Like most Ig domains, TLT-1 has CDR1, CDR2 and CDR3 loops as shown in Figure 2.3C. Therefore, to understand which regions of TLT-1 are most responsible for binding to fibrinogen, purified, individual peptides were added to the wells. In a competitive ELISA where fibrinogen coated plates were incubated with TLT-1 in the - presence of either these Ig domains or LP17, both LP17 and CDR3 decreased the absorbance with increasing concentrations of peptides. This suggests that LP17 and CDR3 inhibit TLT-1 binding and play an important role during this protein-ligand interaction Figure 2.3A. However, and most surprisingly, CDR1 and

CDR2 in Figure 3B had opposite effects, with increasing concentration of these peptides, absorbance increased. The Peptide Mix (PM), which had all four peptides together, also showed a trend for increasing absorbance at higher concentrations

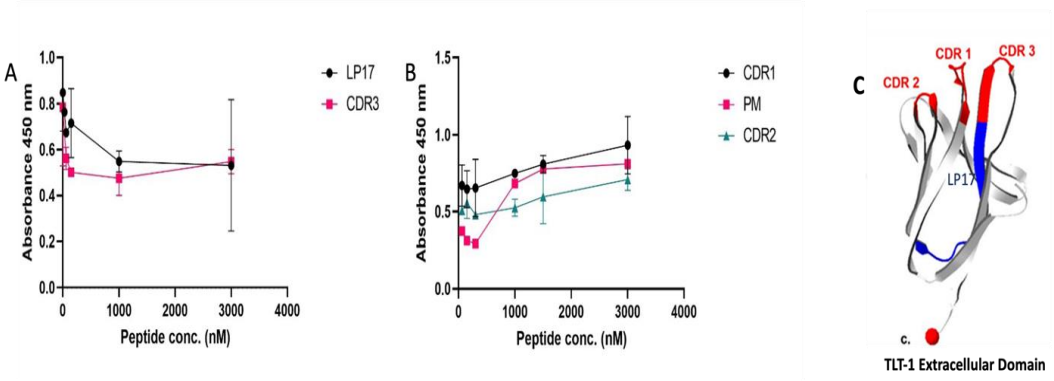
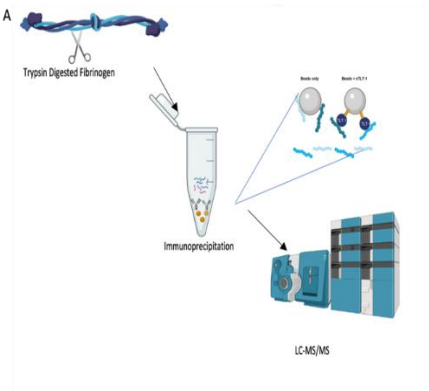


Figure 2.3: LP17 and CDR3 peptides decrease absorbance in competitive ELISA compared to B) peptide mix, CDR1 and CDR2.

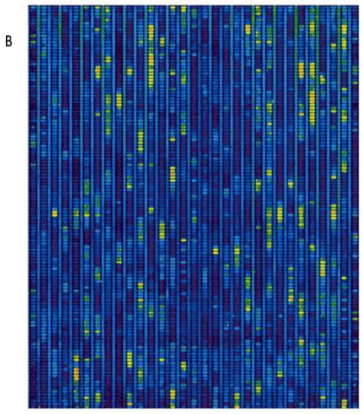
LP17 and CDR3 peptides decrease absorbance in competitive ELISA compared to B) peptide mix, CDR1 and CDR2*CDR and LP17 peptides effect on TLTL-1 binding to fibrinogen.* A) Graph shows that both LP17 (black) and CDR3 (pink) have an inhibitory effect on TLT-1 and fibrinogen binding. With increasing concentration of these peptides, absorbance values decrease. No significance was observed between the effect of both peptides after a One Way – ANOVA ($p > 0.05$). B) Graph shows that CDR1 (black), CDR2 (green) and Peptide Mix (PM) (pink) have an increasing effect of binding of TLT-1 to fibrinogen. Although surprising, a clear trend is observed where increasing concentrations of these peptides increase absorbance values. No significance was observed between the effect of both peptides after a One Way – ANOVA ($p > 0.05$). C) Extracellular TLT-1 domain with highlighted CDR loops (blue) and LP17 (red) taken from Branfield and Washington (2021).

Isolating the Fibrinogen Binding Site for TLT-1

Fibrinogen is a large (340KD) protein consisting of three pairs of polypeptide chains, designated Aα, Bβ and γ, making it difficult to use molecular docking as our initial option to predict possible binding sites for TLT-1 fibrinogen binding (Figure 2.4). Our experimental method of choice to isolate possible fibrinogen peptides that bind TLT-1 was digestion of fibrinogen by chymotrypsin and trypsin followed by immunoprecipitation with soluble TLT-1 and LC-MS/MS. Using this method, we isolated 5 peptides that showed up both trypsin and chymotrypsin digests (Table 1), however because of the differences in proteolytic cleavage sites for trypsin and chymotrypsin there was a need to narrow down the site to smaller regions. As an alternative method, we used a peptide array, which allows high through-put screening of 3000 8-mer peptides from the fibrinogen amino acid sequence attached to a 4K chip and incubated with Alexa Fluor™ 647 labelled TLT-1. We were able to reproduce similar results obtained from the trypsin and chymotrypsin digest, but narrow it down to a more specific, 8 amino acid sequence, as represented in Table 1. These peptides were then synthesized to be used in ex vivo competitive assays to determine whether they have an inhibitory effect on platelet function.



IMMUNOPRECIPITATION TO ISOLATE PEPTIDES OF INTEREST



FIBRINOGEN – TLT-1 PEPTIDE ARRAY

Figure 2.4: Isolating the fibrinogen binding site for TLT-1. Fibrinogen is a large (340KD) protein consisting of three pairs of polypeptide chains, designated Aα, Bβ and γ making it difficult to use molecular docking as our initial option to predict possible binding sites for TLT-1 fibrinogen binding. Trypsin and chymotrypsin digest of fibrinogen for immunoprecipitation with TLT-1 and LC/MS/MS b) Peptide array with fibrinogen peptides 8MERS and CY5 labelled TLT-1.

TRYPSIN	CHYMOTRYPSIN	PEPTIDE ARRAY	LOCATION ON FIBRINOGEN
GGSTSYGTGSETESPR	TSYGTGSETESPR	TRGGSTSY	FIB A 272-280
YQISVNK	TVQNEANKYQISVNKY	YQISVNKY	FIB B 360-374
IRPFFPQQ	SMKIRPFFPQQ	SMKIRPFF	FIB B 481-491
QSGLYFIKPLK	GLYFIKPLK	SGLYFIKP	FIB G 189-199
AHYGGFTVQNEANK	GGFTVQNEANKY	HYGGFTVQ	FIB B 354-367

Table 1.1:Fibrinogen peptide selection. Peptides that appeared in. both immunoprecipitation with trypsin and chymotrypsin, and peptide array were selected as our peptides of interest to perform validation studies.

Molecular docking of peptides of interest.

Interestingly, 3D modeling of our peptide array identified peptides show that the three B chain peptides form a binding "patch" on the B module of the D-domain, with the distance between these peptides being less than 3 angstroms, suggesting that they have a binding interaction between themselves (Figure 2.5a). The peptide from the γ chain was also located on the D-domain, suggesting that instead of having a single binding site, TLT-1 could potentially be "anchored" between the peptides, like stretched out arms

holding fibrinogen on either end. Using alpha fold to run docking predictions on the potential binding of TLT-1 peptides (LP17 and CDR3) to fibrinogen peptides, peptide YQISVNKY on the beta chain residue K344 might interact with Q95 on LP17 peptide on TLT-1 (Figure 2.5b)

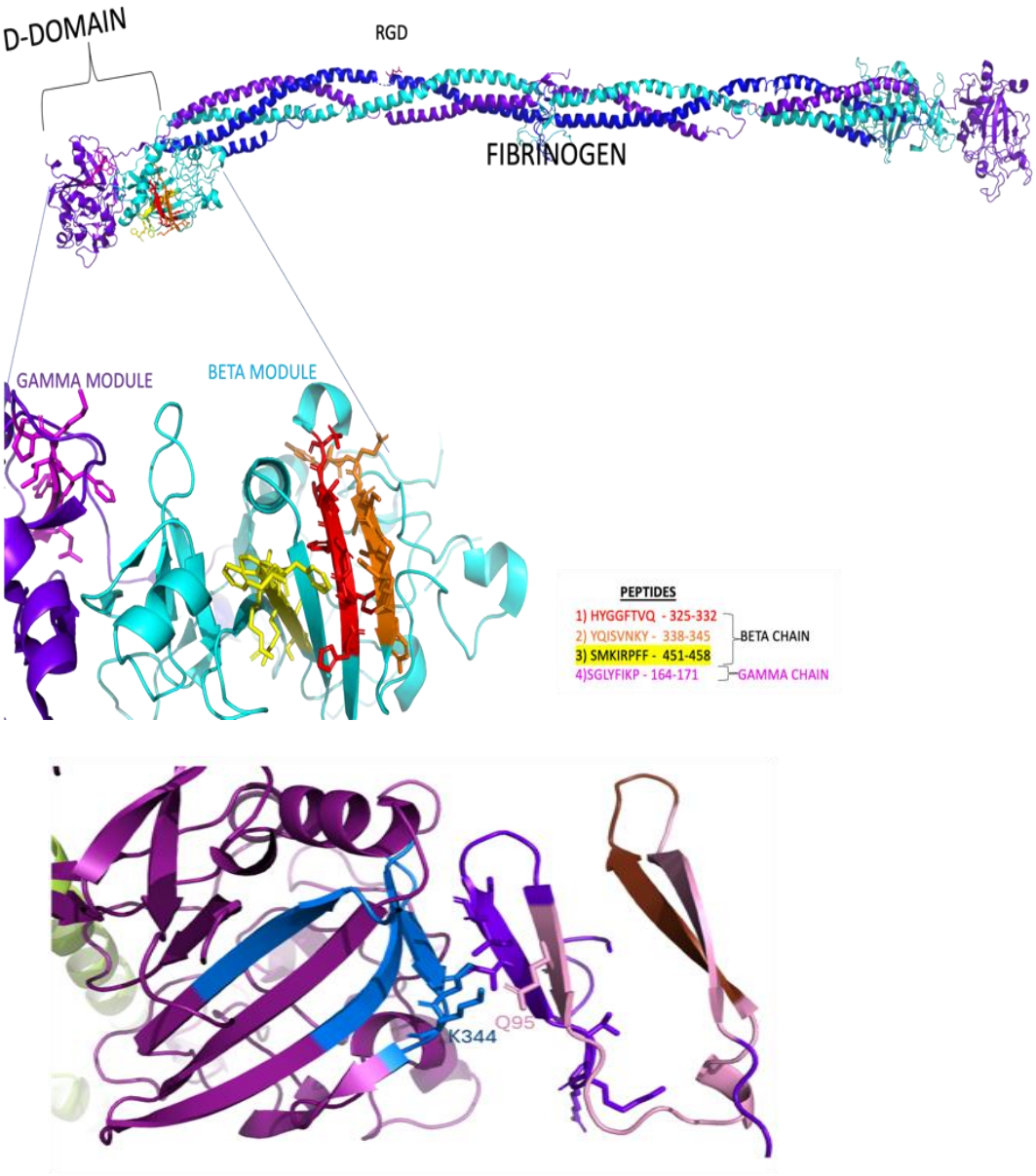


Figure 2.5: a) 3D Model of fibrinogen highlighting peptides of interest for TLT-1 binding, b) Molecular Docking Predictions for TLT-1 Fibrinogen Interaction.

Fibrinogen has a D domain, coiled coils regions and an E domain. The alpha chain is identified in navy blue, beta chain in turquoise and gamma chain in purple. The isolated peptides are identified in the D domains of the fibrinogen molecule, with the three Beta peptides in close proximity, forming a patch. Human Fibrinogen Crystal Structure 3GHG[24] in Protein Data Bank (PDB) format was used and due to crystallization limits of the molecule, one peptide in the alpha chains of fibrinogen that binds to TLT-1 could not be shown in the model.

Validating selected peptides Ex VIVO: Competitive Inhibition Assays

Lumi-aggregometry was done using 2.5 x 10^5 washed platelets in the presence or absence of the fibrinogen peptides to determine whether there was a reduction on platelet aggregation in the presence of these peptides using either collagen or thrombin as agonists. When comparing collagen activated control platelets to platelets incubated with the peptides, all the fibrinogen peptides reduced aggregation, with the highest and most significant inhibition being with SGLYFIKP, SMKIRPFF and a peptide mix with all the peptides, while the scrambled peptides do not have the same effect (Figure 2.6). Similarly, when incubated with thrombin as an activator, there was decreased aggregation, with the most pronounced decrease being with peptides YQISVNKY, SMKIRPFF and the peptide mix with all peptides (data not shown). These results suggest that the isolated fibrinogen peptides work as a complex to facilitate TLT-1 Fibrinogen

binding.

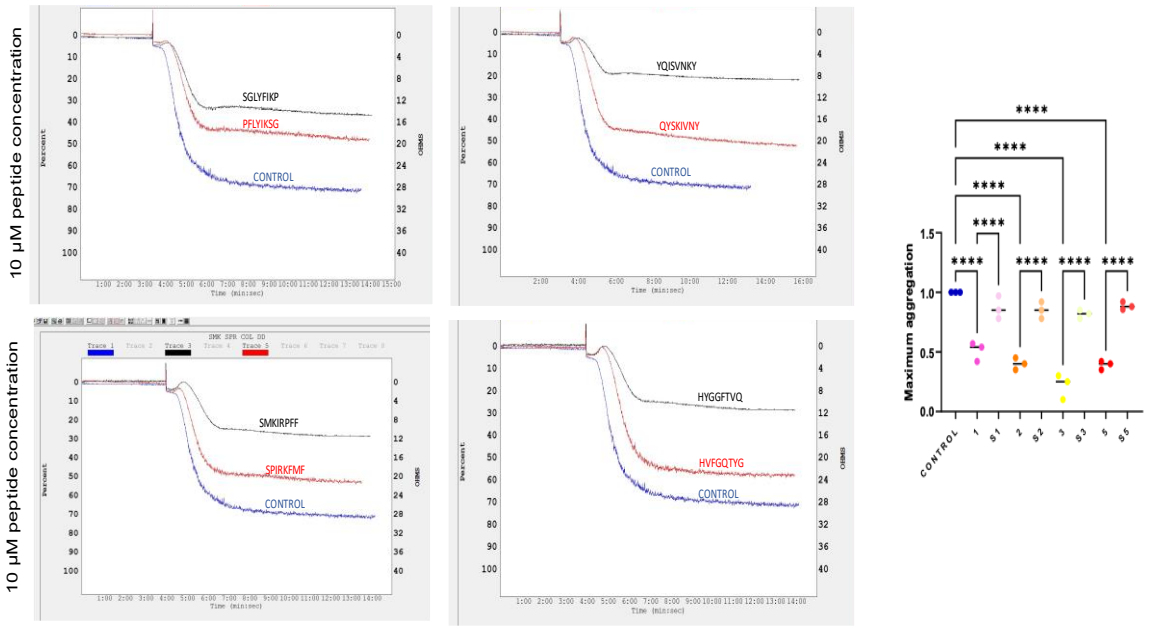


Figure 2.6: Selected fibrinogen peptides competitively inhibit aggregometry with collagen. *Selected fibrinogen peptides competitively inhibit aggregometry with collagen and compare to scrambled peptides.* Aggregometry with washed human platelets demonstrates a decrease in platelet aggregation in samples incubated with the peptides for both collagen and thrombin activation compared to control curves (in blue). The most pronounced decrease in aggregation is seen with the peptide mix, where aggregation decreases to less than 10%.

Competitive inhibition of fibrinogen binding to platelets

In flow cytometry assays evaluating fibrinogen binding to platelets, we incubated activated washed platelet with FITC labelled fibrinogen and activated the platelet in the presence or absence of eptifibatide (an inhibitor of aIIbb3 binding to fibrinogen). We cannot see any differences with just the addition of the peptides, however, in the presence of Eptifibatide, we can demonstrate significant inhibition of fibrinogen binding in the

presence of peptide.

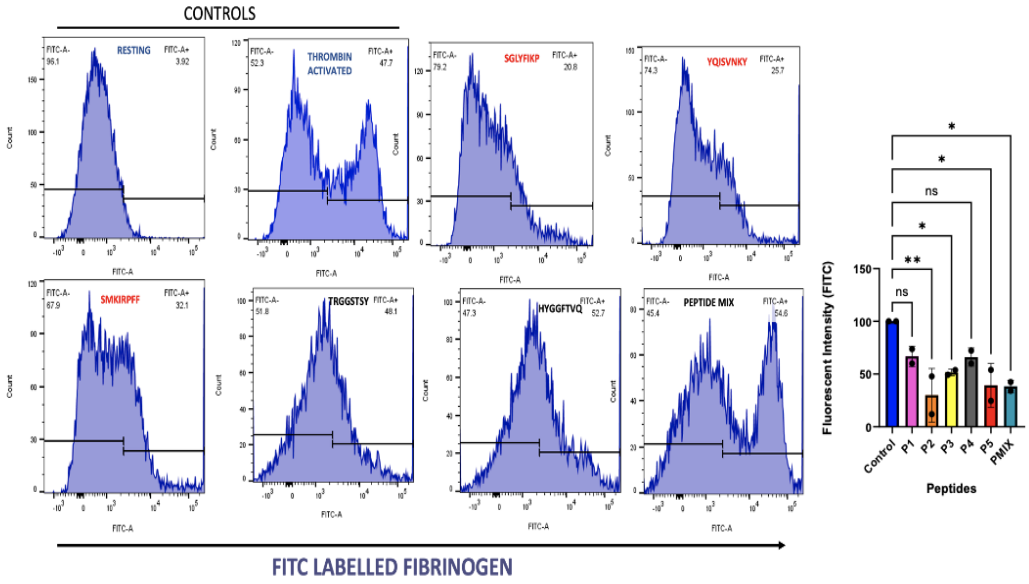


Figure 2.7: Competitive peptide inhibition of fibrinogen binding to platelets using Flow cytometry. Washed platelets were incubated with FITC labeled fibrinogen in the presence and absence of peptides and were evaluated for their ability to reduce fibrinogen binding. We demonstrate varying amounts of inhibition, with the highest inhibition being with peptides 2, 3, 5 and peptide mix.

Washed platelets were incubated with FITC labeled fibrinogen in the presence and absence of peptides and were evaluated for their ability to reduce fibrinogen binding. We demonstrate varying amounts of inhibition, with the highest inhibition being with peptides 2, 3, 5 and peptide mix. These data suggest that these peptides have inhibitory potential and that there may be a synergic effect, in which these peptides work as a binding complex/pocket, as suggested in the 3D model (Figure 2.5a).

Platelet spreading

To determine whether these platelets can directly bind to the peptides of interest, washed platelets (resting or activated) were incubated on either fibrinogen coated coverslips, as a control, or with the peptides of interest. There was a significantly larger number of peptides on the fibrinogen coated coverslip compared to the peptides coated coverslips. This difference could be explained by fibrinogen binding to αIIbβ3 which is expressed on resting platelets and suggests that although αIIbβ3 is also a ligand for fibrinogen, it does not interact with these peptides. When activated with either thrombin or collagen, the difference between the number of platelets binding to the fibrinogen control compared to the peptides decreases, suggesting that platelet activation is necessary to facilitate binding to these peptides, these data support the idea that these peptides are the binding site for TLT-1 since platelet activation is necessary to release TLT-1 from the alpha granules and onto the platelet surface to be exposed to the peptides (Figure 2.8).

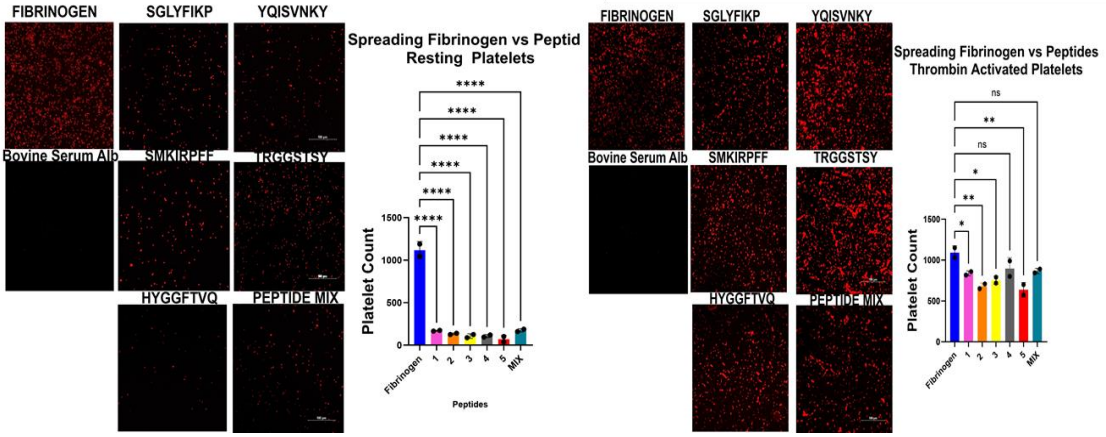


Figure 2.8: Platelet spreading on fibrinogen or peptides. Resting platelets attach well to fibrinogen coated coverslips, compared to peptide coated coverslips, with a significantly higher platelet number on the fibrinogen coated coverslip. Upon platelet activation, TLT-1 is released from the alpha granules onto the platelets and platelets can attach to the peptide coated coverslips, decreasing the significance in platelet quantification on fibrinogen coated cover slips vs peptide coated coverslips.

Discussion

The platelet-fibrinogen interaction has remained a central factor necessary for thrombosis, hemostasis and thromboinflammation. To this end, drugs like eptifibatide and abciximab which binds to the platelet receptor αIIbβ3 to inhibit its interaction with fibrinogen have been used as antiplatelet therapy in patients with high risk of thrombosis[18,19]. While these drugs have proven to be effective antiplatelet drugs there remains a risk of both major and minor bleeding complications in patients taking antiplatelet drugs[20–23]. Knocking out the αIIbβ3 receptor in mutant mice can lead to bleeding complications and internal hemorrhage, demonstrating that it is necessary for

normal physiological function and that the inhibition of this receptor is not optimal for long term usage in high-risk patients, such as those with cardiovascular disease. Our work has been focused on determining why this alternative fibrinogen binding receptor TLT-1, exists on the platelet surface since the interaction between αIIbβ3 and fibrinogen is well defined and facilitates clot formation post vascular injury, and, to take it a step further, introduce potential alternative mechanisms to reduce thrombotic risk while simultaneously minimizing risk of hemorrhage.

To address the "why", several studies have demonstrated that although TLT-1 does facilitate aggregation and clot formation, there seems to be a trend towards an association between plasma soluble TLT-1 (s-TLT-1) levels and thromboinflammation, such as in ARDS, Sepsis and congestive heart failure (CHF). The prevalence of an s-TLT-1 plasma signature in multiple inflammatory pathologies that correlates with disease severity and mortality begs us to question whether TLT-1 extends beyond its role in facilitating fibrinogen binding and clot formation upon platelet activation, to have a functional role in mechanisms driving disease progression. Interestingly, our AFM results point towards a functional role of soluble TLT-1, where incubation of fibrinogen with s-TLT-1 leads to micro clot formation similar to that seen when thrombin is incubated with fibrinogen.

Recent studies on long COVID have demonstrated that amyloid like fibrin micro clots can be found in platelet poor plasma of these patients and increase the risk of autoantibody development and microthrombi in capillaries[24,25]. We question whether the formation of microthrombi could be associated with the TLT-1/fibrinogen interaction as the s-TLT-1 plasma signature remains even months after initial thromboembolic events

(as seen in plasma of patients with CHF, Systemic Lupus Erythematosus, and patients with Long COVID – data not published)?

To introduce an alternative therapeutic intervention, it was necessary to study the fibrinogen TLT-1 molecular interaction. The kinetics studies not only validated the interaction between TLT-1 and fibrinogen, but also demonstrated that this interaction is very strong, with an equilibrium dissociation constant (KD) of 3.02 ± 0.20 nM. Further experiments then led us to isolation of binding sites for this interaction as demonstrated in figures 3 and 4. The results presented in this article pave the way for the development of peptide inhibitors for the TLT-1 fibrinogen interaction, with the potential use of either the TLT-1 or Fibrinogen specific binding sites as targets for inhibition. However, it is important to note that the "multi-site" binding interaction came as an unexpected finding, making this interaction much more complex than we realized and more challenging to inhibit than αIIbβ3 since it would require the use of a peptide mix rather than a singular peptide inhibitor.

Taken together, there may be a use for a therapeutic intervention that inhibits the interaction of TLT-1 and fibrinogen for purposes:

1) Blocking TLT-1 fibrinogen interaction instead of or in combination with αIIbβ3 could allow platelets to still aggregate efficiently but reduce the risk for thromboembolic events and vascular occlusion without having such a pronounced bleeding risk, since knock out mice for the TLT-1 receptor have not been shown to have lethality and high risk for internal hemorrhage, unlike the αIIbβ3 knock out mice.

2) If the soluble form of TLT-1 is function and can remain in the plasma for months after acute phase reactions during immunohemostasis there may be a need to

inhibit its interaction with circulating fibrinogen as it could precipitate micro clot formation and thus increase the risk of capillary occlusion by microthrombi and developing secondary auto-immune diseases.

Conclusions

While the results unravel novel insight on both the structural effects of TLT-1 interaction with fibrinogen and reveal potential binding sites as targets for therapeutic intervention, experiments were done in vitro, and there is need to test these peptides of interest in an in vivo model, such as a FeCl injury model to determine whether vascular occlusion is reduced in the presence of these peptides. However, the major challenge is that only two of the five peptides isolated from human fibrinogen are within 95-100% homology with mouse fibrinogen which was used to conduct these experiments, meaning using the other peptides may not be physiologically relevant.

Overall, this article paves way for the development of an alternative therapeutic target for the platelet fibrinogen interaction.

Bibliography

1. Morrell, C. N., Aggrey, A. A., Chapman, L. M. & Modjeski, K. L. Emerging roles for platelets as immune and inflammatory cells. *Blood* 123, 2759–2767 (2014).

2. Litvinov, R. I., Farrell, D. H., Weisel, J. W. & Bennett, J. S. The Platelet Integrin αIIbβ3 Differentially Interacts with Fibrin Versus Fibrinogen. *J Biol Chem* 291, 7858 (2016).

3. Hantgan, R. R., Stahle, M. C. & Lord, S. T. Dynamic Regulation of Fibrinogen: Integrin αIIbβ3 Binding. *Biochemistry* 49, 9217 (2010).

4. Branfield, S. & Washington, A. V. The enigmatic nature of the triggering receptor expressed in myeloid cells -1 (TLT- 1). *Platelets* 32, 753 (2021).

5. Luyendyk, J. P., Schoenecker, J. G. & Flick, M. J. The multifaceted role of fibrinogen in tissue injury and inflammation. *Blood* 133, 511 (2019).

6. Wagner, D. D. & Burger, P. C. Platelets in Inflammation and Thrombosis. *Arterioscler Thromb Vasc Biol* 23, 2131–2137 (2003).

7. Wolberg, A. S. & Sang, Y. Fibrinogen and Factor XIII in Venous Thrombosis and Thrombus Stability. *Arterioscler Thromb Vasc Biol* 42, 931–941 (2022).

8. Sonmez, O. & Sonmez, M. Role of platelets in immune system and inflammation. *Porto Biomed J* 2, 311 (2017).

9. Foley, J. H. & Conway, E. M. Cross Talk Pathways Between Coagulation and Inflammation. *Circ Res* 118, 1392–1408 (2016).

10. Ferrer-Acosta, Y., González, M., Fernández, M. & Valance, W. A. Emerging Roles for Platelets in Inflammation and Disease. *J Infect Dis Ther* 2, (2014).

11. Valance Washington, A., Quigley, L. & McVicar, D. W. Initial characterization of TREM-like transcript (TLT)-1: A putative inhibitory receptor within the TREM cluster. *Blood* (2002) doi:10.1182/blood-2002-02-0523.

12. Washington, A. V. *et al.* A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 104, 1042–1047 (2004).

13. Washington, A. V. *et al.* A TREM family member, TLT-1, is found exclusively in the α-granules of megakaryocytes and platelets. *Blood* (2004) doi:10.1182/blood-2004-01-0315.

14. Washington, A. V. *et al.* TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. *Journal of Clinical Investigation* (2009) doi:10.1172/JCI36175.

15. Derive, M. *et al.* Soluble Trem-like Transcript-1 Regulates Leukocyte Activation and Controls Microbial Sepsis. *The Journal of Immunology* (2012) doi:10.4049/jimmunol.1102674.

16. Bayrón-Marrero, Z. *et al.* The Characterization and Evaluation of the Soluble Triggering Receptor Expressed on Myeloid Cells-like Transcript-1 in Stable Coronary Artery Disease. *Int J Mol Sci* 24, (2023).

17. Morales-Ortíz, J. *et al.* Platelet-derived TLT-1 is a prognostic indicator in ALI/ARDS and prevents tissue damage in the lungs in a mouse model. *Blood* (2018) doi:10.1182/blood-2018-03-841593.

18. Baker, N. C. & Waksman, R. Eptifibatide. *Antiplatelet Therapy in Cardiovascular Disease* 142–149 (2023) doi:10.1002/9781118493984.ch17.

19. Harvison, P. J. Abciximab. *xPharm: The Comprehensive Pharmacology Reference* 1–5 (2007) doi:10.1016/B978-008055232-3.61147-X.

20. Topol, E. J. *et al.* Comparison of Two Platelet Glycoprotein IIb/IIIa Inhibitors, Tirofiban and Abciximab, for the Prevention of Ischemic Events with Percutaneous Coronary Revascularization. *New England Journal of Medicine* 344, 1888–1894 (2001).

21. Dyke, C. M. Safety of glycoprotein IIb-IIIa inhibitors: A heart surgeon's perspective. *Am Heart J* 138, (1999).

22. Brouse, S. D. & Wiesehan, V. G. Evaluation of bleeding complications associated with glycoprotein IIb/IIIa inhibitors. *Ann Pharmacother* 38, 1783–1788 (2004).

23. Germing, A. *et al.* Bleeding complications associated with glycoprotein IIb/IIIa inhibitors in patients 80 years of age and older undergoing percutaneous coronary intervention. *Exp Clin Cardiol* 15, e57 (2010).

24. Kell, D. B., Laubscher, G. J. & Pretorius, E. A central role for amyloid fibrin microclots in long COVID/PASC: origins and therapeutic implications. *Biochem J* 479, 537–559 (2022).

25. Pretorius, E. *et al.* Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. *Cardiovasc Diabetol* 20, (2021).

CHAPTER 3

IMMUNOLOGICAL AND PLATELET MARKERS FOR MORTALITY RISK IN COVID-19

Abstract

The COVID-19 Pandemic resulted in approximately 6,972,152 reported global deaths. Although the precise disease progression mechanisms remain unclear, multiple studies have indicated that a hyperimmune response, evidenced by heightened plasma cytokine levels, comorbidities, and thromboembolic events, increases the risk of mortality in COVID-19 patients[1–3]. This study compares plasma cytokines and platelet activation markers in two patient cohorts (Puerto Rican and Utah) exhibiting different clinical spectrums (outpatient COVID, COVID-ICU, and long COVID), alongside varying comorbidity levels, to assess correlations with disease severity and mortality risk. Elevated plasma IL6 and D-Dimer levels were notably higher in non-COVID, COVID-ICU, and long COVID patients compared to healthy controls, indicating a pronounced proinflammatory response.

While all COVID-9 patients showed heightened cytokine and platelet activation marker levels, the most significant differences were observed in COVID ICU patients. The levels of plasma cytokines were notably increased in ICU patients with 2, 3, or more comorbidities compared to those with just one comorbidity. TNF-a and IL6 were particularly raised in patients with 2, 3, or more comorbidities, suggesting a[4–8]n

intensified proinflammatory immune response in these cases (Table 5, Figure 1B). Prothrombotic and inflammatory markers (S100A8, IL10, D-Dimers, TPO) were linked to mortality in ICU patients. Using a multiple linear regression we assessed correlation between P-Selectin, a well-known marker of platelet activation and plasma cytokines in ICU COVID-19 patients. P-Selectin levels significantly correlated with proinflammatory markers S1000A8, C5A, IL1-B, GM-CSF, IFNalpha and procoagulant markers such as D-Dimers and TPO (Figure 3). These data indicate that P-Selectin is a highly sensitive biomarker for proinflammatory induced hyper coagulopathy.

Intriguingly, increased Trem-Like Transcript-1 (s-TLT-1), which is also a platelet-specific receptor released upon activation and has been shown to be a highly sensitive marker of platelet activation, correlated with 2, 3, or more comorbidities, S100A8, IL10, and D-Dimers. These findings suggest that a panel consisting of platelet activation markers P-Selectin and s-TLT-1, along with proinflammatory markers S100A8, IL10, and D-Dimers could be developed as a risk assessment tool for predicting mortality and thrombosis in these ICU-COVID patients. Using receiver operator curve (ROC) analysis to establish cutoff points as predictive indicators for mortality in ICU COVID-19 s-TLT-1 is the strongest predictive indicator (with a cutoff of 605pg/ml, a specificity of 0.833 and sensitivity of 0.75), while P-Selectin, IL-10, D-Dimers and S1000A8 have a high sensitivity but not specificity (Figure 5). Looking at fold increase of these predictive parameters, IL10, D-Dimers and s-TLT-1 had the highest fold increase in patients who died compared to patients who survived. Here we introduce the potential for a 6-point mortality risk evaluation scoring system in COVID-19 (MRES-COVID-19), where parameters include hypertension, ischemic events, s-TLT-1 levels above 605pg/ml,

elevated D-Dimers, elevated IL10, and/or concurrence of two or more co-morbidities, where the risk for mortality increases with coexistence of these parameters, and the higher the score the greater the risk of mortality, with those having score ≥5 having the highest risk for mortality

Introduction

The outbreak of COVID-19, instigated by the coronavirus SARS-CoV-2, has caused a global health crisis unparalleled in recent history. This infectious disease exhibits a wide spectrum of clinical manifestations, ranging from mild flu-like symptoms to severe respiratory distress and multi-organ failure[3,9]. Among the diverse aspects of this illness, the immune response, particularly the interaction between platelets and cytokines, has emerged as a crucial area of interest in understanding disease severity, mortality, thrombotic risk, and prognosis, unraveling the underlying molecular processes is imperative for developing effective therapeutic strategies.

The severity of COVID-19 and associated mortality is linked to cytokine storm, an uncontrolled release of proinflammatory cytokines exploiting the immune response. This phenomenon, characterized by elevated levels of cytokines like IL-6, TNF-α, and IL-1β, triggers systemic inflammation, vascular leakage, and coagulation abnormalities. In severe cases, especially those requiring ICU admission, elevated inflammatory markers are consistently observed, indicating a higher risk of adverse outcomes[10–12]. The cytokine storm contributes to mortality through the induction of acute respiratory distress syndrome (ARDS), multiple organ dysfunction syndrome (MODS), and thrombotic complications. The heightened inflammatory state increases the risk of blood clots, leading to complications such as deep vein thrombosis and pulmonary embolism[13].

Efforts to address this involve immunomodulatory agents like tocilizumab and dexamethasone, as well as antiplatelet or anticoagulant medications, aiming to dampen the cytokine storm and reduce platelet activation for improved survival outcomes in COVID-19 patients.

Studies have demonstrated that SARS-CoV-2 virions can be found in platelets and their precursor cells, megakaryocytes. Several studies have demonstrated that platelets in patients with COVID-19 are hyperreactive and that when SARS-CoV-2 interacts with megakaryocytes, it alters the transcriptome[14]. Moreover, COVID-19 has been shown to predispose high risk patients, specifically those in ICU, to both venous and arterial thrombosis[15–19].

P-selectin and Trem-Like Transcript 1 (TLT-1) are alpha granule platelet receptors that are released onto the platelet surface upon activation and are known to be sensitive markers of platelet activation. Several studies have demonstrated a correlation between plasma levels of these proteins and risk for thrombotic complications and disease progression[6,20,2122]. More specific to COVID-19, several studies have demonstrated a correlation between plasma P-Selectin levels (which is expressed on endothelial cells and platelets), critical illness and mortality[23–26]. However, to date there has been no studies to assess correlations between COVID-19 and TLT-1, which has been demonstrated to be a more sensitive marker of platelet activation than P-Selectin[27].

A previous study by Morales et.al demonstrates that in a cohort of patients with Acute Lung Injury (ALI) and acute respiratory distress syndrome (ARDS), high levels of soluble TLT-1 (sTLT-1) in plasma (>1200 pg/mL) were associated with nearly double the mortality risk compared to lower levels (<1200 pg/mL) in these patients with ARDS.

In support of these findings, murine models further revealed that TLT-1 regulates inflammation and hemostasis, increasing fibrinogen deposition, prevalence of platelet-neutrophil conjugates, and tissue damage during ALI/ARDS progression. In vivo administration of sTLT-1 mitigated pulmonary hemorrhage and tissue damage, indicating a potential therapeutic role for TLT-1 in controlling leukocyte activity during ARDS.

Given TLT-1s role in the pathophysiology of ARDS, a sequel of COVID-19, its biological signature as a platelet activation marker as well as its role in coagulation, we designed this study as a comparative analysis of the platelet (P-selectin and Trem-Like Transcript 1 (TLT-1)) and cytokine plasma signature in COVID-19 with the hope of shedding light on the nuanced connections between cytokines, platelet activation, and disease severity, the objective was to determine whether platelet activation markers and cytokines can be used to create a screening panel for disease severity in COVID-19.

Methods

Study Design and Participants

Plasma samples were collected from a cohort of patients (Utah Cohort) who were either donor controls, who do not test positive for COVID-19 but may have other comorbidities, or patients who tested positive for COVID (ICU-COVID n=26, non-ICU COVID, COVID or long COVID). We ordered customized cytokine kits which included inflammatory cytokines and coagulation danger signal molecules such as tissue factor, P-Selectin, Thrombomodulin (LXSAHM16 and LXSAHM-08 from R & D Systems) and an ELISA kit for sTLT-1 quantification to quantify plasma cytokine and coagulation markers in these different cohorts of patients. We also collected patient characteristics for

these ICU COVID patients (including medication use, anticoagulant use co-morbidities and coagulation danger signal molecules such as PT, PTT, D-Dimers) for inpatient UTAH ICU patients, however it was difficult to collect this data for non-CU covid or long covid patients as there was no extensive charting or additional laboratory testing done for these patients who were treated as outpatients. The Puerto Rican cohort of patients were outpatients, who presented to clinics for PCR COVID-19 testing , for these patients we obtained vaccination records, patient characteristics such as sex and age, coagulation danger signal markers such as PT, PTT and platelet count, these patients were categorized into COVID, COVID mycoplasma, mycoplasma only or healthy individuals who tested negative. We did not have access to patient history as these patients had no follow up or additional testing.

Plasma sample collection

Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Plasma samples were stored in the -80 before use for the human Luminex discovery assay and s-TLT-1 ELISA.

Human Luminex Discovery Assay

LXSAHM-16 ANYLATES	LXSAHM-08 ANYLATES
CD31/PECAM-1 (BR45) Coagulation Factor III/Tissue Factor (BR62) Complement Component C5a (BR18) D-Dimer (BR43) GM-CSF (BR46) ICAM-1/CD54 (BR61) IFN-alpha (BR63) IL-1beta/IL-1F2 (BR28) IL-6 (BR13) IL-10 (BR22) S100A8 (BR21) P-Selectin/CD62P (BR48) Thrombomodulin/BDCA-3 (BR47) Thrombopoietin/Tpo (BR55) TNF-alpha (BR12) vWF-A2 (BR15)	Collagen I alpha 1 (BR18) CXCL4/PF4 (BR56) CXCL7/NAP-2 (BR47) MMP-9 (BR14) Properdin (BR67) Serpin C1/Antithrombin-III (BR30) SerpinE1/PAI-1(BR43) u-Plasminogen Activator (uPA)/Urokinase (BR78)

Quantification of plasma cytokines according to manufacturer's protocol [28].

Whole blood collected in citrated tubes was spun down to collect plasma, frozen and kept in the -80°C freezer until s-TLT-1 quantification was done using the Human TREML1/TLT-1 DuoSet ELISA kit (Cat# DY2394; assay range 15.6–1000pg/mL), according to the manufacturers protocol[29].

Statistical analysis

All tests were performed using SPSS version 28.0, R Studio version 4.0.4. and GraphPad Prism version 9.1.0. Statistical significance was set to a $p \leq 0.05$, except for the normality diagnostics. Independent t test for equality of means and Shapiro wilk.
A linear regression was used as a multiple variable comparison to assess the linear relationship between s-TLT-1 and cytokines, comorbidities, or cytokines.

Results

The COVID-19 pandemic has presented an unprecedented challenge to global healthcare systems, necessitating an in-depth exploration of its underlying pathophysiological mechanisms. Herein, we present findings from two separate patient populations, divided into different COVID-19 cohort subgroups, shedding light on the interplay between cytokines, thrombotic events, and disease severity.

Puerto Rican cohort of patients

A total of 72 patients with constitutional symptoms were tested in an outpatient setting for COVID-19, mycoplasma and influenza, blood samples were collected from these patients simultaneously. Of the 72, 28 tested negative and were controls, 16 tested positive for COVID-19, 14 tested positive for both COVID and mycoplasma, while 14 tested positive for mycoplasma alone. The mean age of the patients was 43.66 ± 15.25

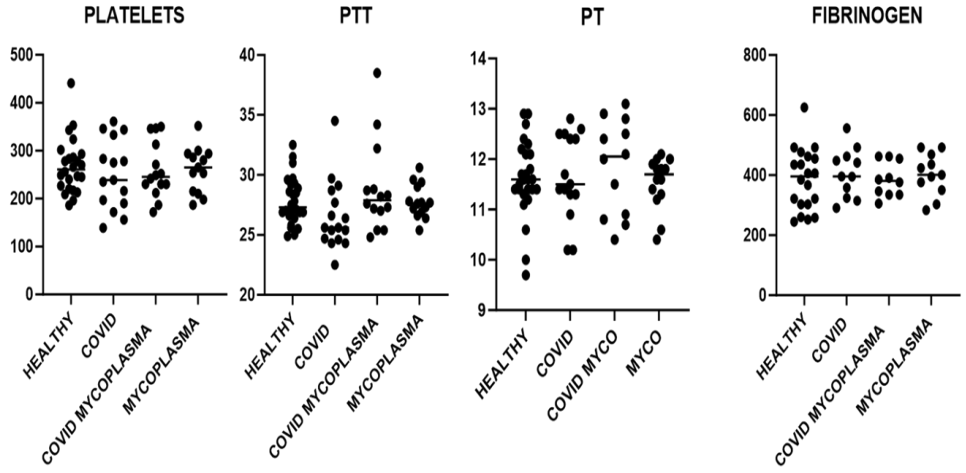
years. From the blood samples, coagulation specific markers PT and PTT were measured before plasma extraction for s-TLT-1 cytokine quantification. Since this cohort was seen in the outpatient department, we did not have access to patient charting/ history or additional blood testing, however, it is observed that even in an outpatient setting D-Dimer and s1000A8 are significantly elevated, making these markers the only two of the measured outputs sensitive to milder COVID-19 infections.

Patient Characteristic		**Mean + SD (0)**	**Mean + SD(1)**	**p Value**
Sex (0=Male n=25, 1=Female n=43)		144.81 ± 180.36	205.29 ± 249.64	0.047
Patients tested positive for COVID-19	29 (39%)	175.81 ± 242.82	183.52 ± 205.55	0.42
Patients tested positive for Mycoplasma	28 (37.3%)	139.15 ± 179.38	238.73 ± 271.17	0.005
Patients tested positive for Mycoplasma and COVID-19	14 (18%)			
Laboratory Parameters				
Platelet Count	257.91 (139-374) ± 63.74			
PT	11.88 (10-22.7) ± 1.55			
PTT	27.74 (25-31) ± 2.57			
Sex (F/M/Unknown)	n=74 34/24/15 (46%/32%/20%)			
Age n=58, 16 unknown	43.87 ± 15.48			
Comorbid diseases (0/1/2/3 or more)	32/15/19/8 (43%/20%/26%/11%)			
Coronary Artery Disease	5 (6.8%)			
Arterial Hypertension	29 (39.2%)			
Diabetes Mellitus 21 (28.4%)	21 (28.4%)			
Peripheral Artery Disease	3 (4.1%)			
Current or Recent Smoker	3 (4.1%)			
Dyslipidemia	3 (4.1%)			
Previous Myocardial Infarction 1	1 (1.4%)			
Previous Percutaneous Coronary intervention	2 (2.7%)			
Previous Coronary Artery Bypass Grafting	2 (2.7%)			
Prior Stroke or Transient Ischemic Attack 1 (1.4%)	1 (1.4%)			
Renal Insufficiency	3 (4.1%)			
On Dialysis 1 (1.4%)	1 (1.4%)			
Other	17.60%			

Table 2.1: Puerto Rican Patient Characteristics. Patient characteristics for Puerto Rican cohort who tested positive for either COVID-19, Mycoplasma, or both.

CYTOKINE	HEALTHY	COVID	COVID & MYCOPLASMA	MYCOPLASMA
ICAM-1/CD54	P=0.3364	P=0.2128	0.0346	P=0.5836
vWF-A2	P=0.1994	P=0.0222	P=0.9495	P=0.4127
S100A8	P=0.1079	P=0.4754	P=0.0490	P=0.0045

Table 2.2: Correlation between s-TLT-1 and Plasma Cytokines. Multiple Linear Regression in Puerto Rico Outpatient Cohort to compare healthy individuals with those who either had COVID-19 or Mycoplasma.



Patient laboratory parameters in Puerto Rican outpatient cohort, using one way ANOVA Utah Cohort of Patients

Baseline characteristics and laboratory parameters of cohort

A total of 70 adult patients (M/F:28/40) (12-Non COVID Donors, 11 Long-COVID, 19 COVID Non-ICU, 26 COVID ICU) were included in the study. The mean age of the patients was 53.5 ± 13.9 years. For statistical analysis patients were divided into four groups) (12-Non COVID Donors, 11 Long-COVID, 19 COVID Non-ICU, 26 COVID ICU), for these groups we were able to use an ANOVA to compare plasma s-TLT-1 levels and plasma cytokines (Figures 3.1a and 3.1b). It is important to note that the s-TLT-1 levels were high in some of the samples we classified as healthy individuals, however, these patients were in a "walk in testing center", no patient history was collected, the only criteria for inclusion within the study was testing negative for COVID-19, yet these patients could have had co-morbidities that potentially could lead to elevated s-TLT-1 levels. Long COVID had the highest s-TLT-1 levels, with significantly higher levels than Non-ICU COVID and ICU-COVID. When comparing cytokines in the four groups, there was no significance between TNF-alpha levels. IL-6 levels were significantly lower in non-COVID Donors than in Non-ICU-COVID and ICU-COVID, VWF-A2 was significantly lower in Non-COVID Donors than in ICU-COVID, D-Dimers were significantly lower in Non COVID Donors than in Non-ICU-COVID, long-COVID and ICU-COVID (Figure 3.1b).

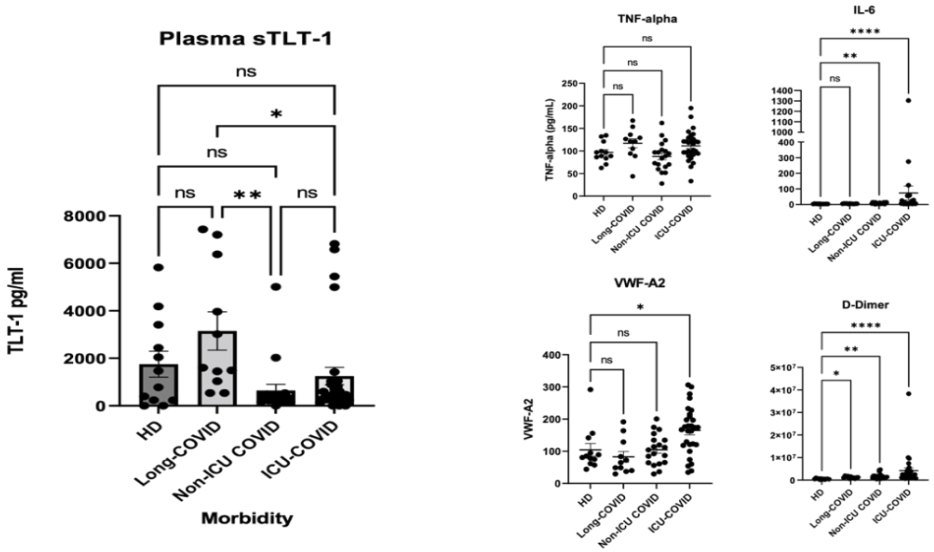


Figure3.1: Plasma cytokine analysis in UTAH COVID cohort of patient's vs healthy donors.

Patient characteristics were difficult to acquire for non-COVID donors, Long-COVID and Non-ICU-COVID since these patients were in the outpatient clinic and either did not have follow ups or did not have further testing. We acquired patient characteristics for the 26 ICU- COVID patients, who had a mean age of 56.22, 25 of these patients had accompanying comorbidities. The most prevalent comorbidities were Diabetes (53.84%), Hypertension (50%) and Asthma (34.6%). Due to high risk of thrombotic complications in COVID-19, all ICU patients were under anti-coagulant treatment even though only 15% were reported to have thrombotic events (deep vein thrombosis or pulmonary embolism.) The most common therapeutic modalities were atorvastatin (42%), lisinopril (23%), metformin (19%), gabapentin (15%) and furosemide (15), which coincided with the frequency of comorbid diabetes and hypertension. Moreover, high blood pressure frequently coincides with hypercholesterolemia and cardiovascular disease, thus validating the high usage of atorvastatin in this cohort of

patients. (Table 2.3)

Sex (F/M) n=26	12/14 (46.1%/53.84%)
Age	56.11
Comorbid diseases (0/1/2/3 or more)	1/6/9/10 (4%/23%/35%/38%)
Diabetes	14 (53.84%)
Asthma	9 (34.6%)
Cardiovascular Disease	4 (15%)
Thrombotic events	4 (15%)
Hypertension	13 (50%)
Pulmonary pathology	4 (15%)
Liver Disease	2 (8%)
Anemia	1 (4%)
Sleep Apnea	3 (11.5%)
Hyperlipidemia	1(4%)
Renal Insufficiency	2 (8%)
Obesity	1 (4%)
Autoimmune Disease	5 (19%)
Thyroid dysfunction	4 (15%)
Treatment /Medications	
Aspirin	15%
Atorvastatin	11(42%)
Gabapentin	15%
Metformin	19%
Dulaglutide	3 (11.5%)
Pioglitazone	2 (8%)
Levothyroxine	4 (15%)
Lisinopril	6 (23%)
Amlodipine	2 (8%)
Metoprolol	1 (4%)
Furosemide	4 (15%)
Omeprazole	2 (8%)
Tamsulosin	1 (4%)
Albuterol	3 (11.5%)
Duloxetine	4 (15%)
Temazepam	1 (4%)
Oxygen Demand	
Oxygen Mask	7 (31%)
Nasal Canula	13 (50%)
CPAP	1 (4%)
Intubation	5 (19%)

Table 2.3: UTAH COVID ICU Patient Characteristics. Patient characteristics for ICU-COVID patients in UTAH, including respiratory vitals and comorbidities.

Oxygen Saturation % (average)	86%
Cell counts: Mean, Range, Standard Deviation	
Platelet Count	271.88 (124-444) ± 108.35
Red Blood cells	5.45 (3.47-13.2) ± 2.79
Lymphocytes	2.05 (0.66-7.43) ± 2.59
PT (*n=6)	12.92 (12.2-13.4) ± 0.385
INR (*n=7)	3.02 (0.9-15.2) ± 4.96
HR	92.7 (66-146) ± 20.85
Temperature	36.95 (35.5-38.4) ± 0.93
D-Dimers (*n=12) (remove outlier?)	74.02 (0.7-820) ± 235.29

Table 2.4:UTAH COVID ICU Patient laboratory parameters. Oxygen saturation. vitals and average cell count in ICU COVID patients.

We selected and designed cytokine panels that could detect and quantify plasma inflammatory and coagulation markers (from bio-techne catalog numbers: LXSAHM-16, LXSAHM-08, as shown in table 8). All ICU patients were administered oxygen supplementation with 31% using oxygen masks, 50% using nasal cannulas, 4% using CPAP and 19% of patients intubated, however, there was no correlation between mortality and ventilation use or between duration of ventilation and mortality (p=0.836). The laboratory parameters : platelets, red blood cells, lymphocytes and PT were all within normal ranges for an adult population. Although the INR ratio was slightly above normal range, all ICU patients were on anticoagulants so this right shift in INR ratios should be expected. Average heart rate was normal and temperatures subfebrile, for this

cohort of patients (Table 2.4). Average D-Dimer levels were elevated in these patients suggesting that they were in a procoagulant state.

Platelet activation marker, s-TLT-1, correlates with cardiovascular disease in ICU-COVID patients.

Although studies have demonstrated an association between restrictive lung disease such as asthma, there was no association between mortality and asthma or between the prevalence of asthma and s-TLT-1 levels in these patients, despite asthma being the most frequently observed pathology in this cohort. In support of previous studies, there was a correlation between cardiovascular disease (CVD) and s-TLT-1 levels, patients with CVD have significantly higher s-TLT-1 levels (3179.64 ± 3237.93) than those without CVD (779.86 ± 1384.00, p=.003). Moreover, patient who presented with risk factors for CVD, hypertension (1814.16 ± 2506.79, p=<.001) and sleep apnea (2514.78 ± 3730.62, p=.024), also had correlation with s-TLT-1 levels.

Use of antihypertensive and statin medications, lisinopril and atorvastatin were also correlated with s-TLT-1 levels, (2173.34 ± 2953.16, p=.007) and 1947.51 ± 2743.67, p=<.001) respectively, this is not surprising since these medications are first line therapies for both hypertension and patients with high cholesterol levels in CVD. Moreover, there was a correlation between number of co-morbidities and s-TLT-1 levels. Patients with 3 or more comorbidities had significantly higher levels of s-TLT-1 compared with those with less than 3, 2140.00 ± 2808.68 and 513.04 ± 399.20, respectively (p=<.001), (Table 3). When comparing association/ relationship between plasma cytokines and s-TLT-1, there was an association between plasma s-TLT-1 and plasma cytokines S1000A8 (p=0.022), IL10 and D-Dimers (p=0.012), (Figure 3.2).

Intriguingly, s-TLT-1 levels in ICU-COVID patients was significantly correlated with mortality, with mortality associated mean s-TLT-1 levels of 2798.18 ± 3046.14,(p=<.001), this supports a study where s-TLT-1 levels above 1200 are shown to be associated with mortality in acute respiratory distress syndrome (ARDS), which, like COVID-19, causes a hyperinflammatory response and pulmonary injury.

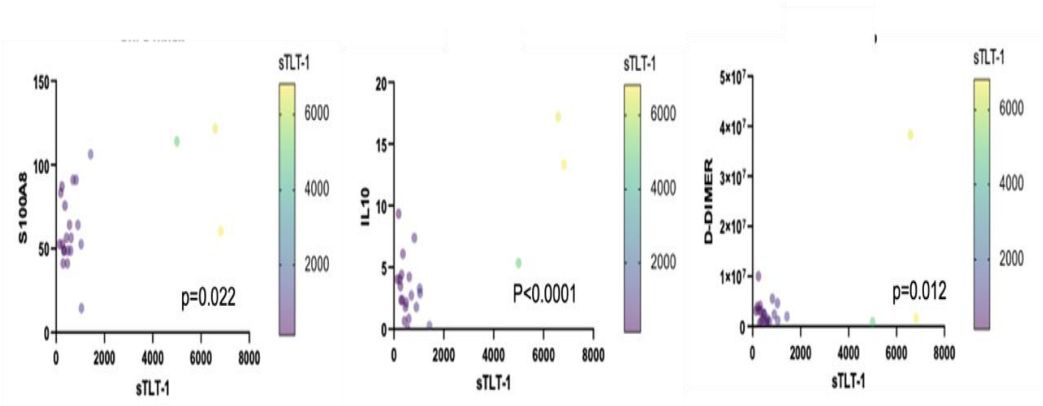


*Figure 3.2: Linear regression- Plasma s-TLT-1 levels compared to plasma cytokines in ICU COVID patients.*s-TLT-1 has a correlation with s100A8, IL-10 and D-Dimers.

Co-morbid Pathologies	**Mean + SD (0)**	**Mean + SD(1)**	**p Value**
Asthma (17=0, 9=1)	936.61 ± 1563.50	1515.015 ± 2462.32	.057
CVD (21=0, 4=1)	779.86 ± 1384.00	3179.64 ± 3237.93	.003
Thrombotic Events (0=20, 1=5)	999.97 ± 1689.93	1819.21 ± 2835.73	.166
Hypertension (0=13, 1=13)	459.48 ± 432.90	1814.16 ± 2506.79	<.001
Pulmonary Pathology (0=22, 1=4)	1085.51±1852.93	1419.03 ± 2388.03	.365
Sleep Apnea (0=22, 1=3)	986.71 ± 1616.39	2514.78 ± 3730.62	.024
Renal Insufficiency (0=24, 1= 2)	1154.44 ±1972.27	925.43 ± 148.67	.289
Auto Immune Disease (0=21, 1=5)	1005.54 ± 1692.46	1688.22 ± 2757.49	.178
Correlated to # of Co-morbidities			
1 Comorbidity (0=20), (1=6)	1300.44 ± 2134.51	591.4 ± 373.78	.075
2 Comorbidities (0=17, 1=9)	1467.57 ± 2277.30	512.08 ± 392.90	.012
3 or more Comorbidities (0=15, 1=10)	513.04 ± 399.20	2140.00 ± 2808.68	<.001
Mortality (0=20, 1=6)	**638.42 ± 1067.75**	**2798.18 ± 3046.14**	<.001
Medication usage			
Aspirin (0=21, 1=5)	1306.93 ± 2072.41	422.34 ± 363.09	.119
Gabapentin (0=22, 1=4)	1013.3 ± 1648.53	1816.02 ± 3179.85	.064
Metformin (0-21, 1=5)	1305.99 ± 2075.92	426.31 ± 260.41	.097
Lisinopril (0=18, 1=6)	874.99 ± 1478.33	2173.34 ± 2953.16	.007
Atorvastatin (0=13, 1=11)	569.57 ± 569.57	1947.51 ± 2743.67	<.001
Amlodipine (0=24, 1=2)	1209.69 ± 1955.23	262.33 ± 36.68	.263
Furosemide (0=22, 1=4)	1025.04 ± 1642.09	1751.63 ± 3223.48	.058
Rivaroxaban (0=24, 1=2)	888.21 ± 1557.37	4120.14 ± 3810.13	.059
Duloxetine (0=22, 1=4)	992.50 ± 1614.74	1930.59 ± 3258.42	.050
Albuterol (0=22, 1=4)	1301.70 ± 2018.18	229.96 ± 233.58	.137
Losartan – Hydroclorothiazide (0=24, 1=2)	1187.53 ± 1962.83	528.34 ± 412.88	.377

Where 0= absent/no , 1=present/yes

Table 2.5: sTLT-1 correlation with comorbidities and medications in UTAH COVID ICU cohort. Levels of s-TLT-1 were correlated with number of comorbidities, mortality and medication usage.

p-Selectin, a platelet activation marker is a highly sensitive cytokine storm biomarker in ICU C0VID-19.

p-Selectin is a well-known marker of platelet activation and has been proposed as a biomarker for several diseases. While s-TLT-1 levels were correlated with CVD and its risk factors, hypertension and sleep apnea, we did not find a similar correlation between

p-Selectin and these pathologies, or any other specific commodities within the study cohort. However, like s-TLT-1, p-Selectin levels were significantly elevated in patients with three or more co-morbidities (12704.98 ± 9828.76, p=.012), supporting the notion that these patients had hyperreactive platelets, increasing the risk for thrombosis (Table 5). On the contrary, p-Selectin seems to be more sensitive to changes in cytokine levels, as demonstrated by a greater number of correlations with plasma cytokines in ICU-COVID patients. Like s-TLT-1 it has a correlation with S100A8, IL-10 and D-Dimers, however it also correlates with C5A, IL-1B, GM-CSF, TPO, IFN-alpha, making it a better biomarker for cytokine over production than mortality.

Elevation of cytokine levels correlates with disease severity and number of comorbidities.

In the Utah cohort of patients, a comprehensive comparison of cytokines in all four subgroups demonstrates that proinflammatory marker IL-6 is elevated in all subgroups of patients with COVID-19, as compared to non-COVID-19 donors. Although multiple studies have demonstrated changes in TNF-alpha, these data did not show any significant changes in TNF-alpha when comparing the different subgroups (Figure 1b). Comparison of all other cytokines between subgroups did not show any significant differences (supplemental data). When comparing correlation of cytokine levels to co-morbidities in ICU-COVID patients, these data support a dramatic change in cytokine profiles, specifically with increasing number of comorbidities, supporting previous studies that suggest there is increased risk of mortality in COVID-19 patients with comorbidities, a cytokine storm being a key hallmark of pathophysiological progression. These data demonstrate a significant correlation between one comorbidity (n=6) with cytokines F3 (p=.042) and MMP9 (p=<.001). While patients who had two comorbidities

(n=9), had a significant correlation with cytokines TNF-Alpha (p=.013), IL-6 (p=.004), IL-1B (p=.021), collagen 1A1 (p=.013), CXCL7NAP-2 (p=.002), Properdin (p=.034) and uPA (p=.022), (Table 4).

One Comorbidity			
F3 (0=20, 1=6)	28.06 ± 10.04	19.02 ± 4.82	.042
MMP9 (0=20, 1=5)	242.35 ± 321.53	893.15 ± 1716.78	<.001
Two Comorbidities			
(0=16, 1=9)			
TNF-Alpha	2.41 ± 3.08	5.99 ± 10.30	.013
IL-6	19.52 ± 19.57	55.16 ± 91.37	.004
IL-1B	144.70 ± 49.67	129.05 ± 23.68	.021
COLLAGEN 1A1	2.41 ± 3.08	5.99 ± 10.30	.013
CXCL7NAP-2	45.09 ± 22.05	125.89 ± 156.98	.002
Properdin	33083.83 ± 9207.56	40230.78 ±15828.59	.034
uPA	22.15 ± 5.65	26.43 ± 12.64	.022
Three Comorbidities			
(0=15, 1=10)			
TNF-alpha	5.22 ± 8.60	1.89 ± 1.82	.031
IL10	2.39 ± 1.63	7.07 ± 4.86	.003
IL-1B	134.37 ± 33.29	151.50 ± 53.40	.020
D-DIMER	2444726.85 ± 2214834.90	7269846.18 ± 11234999.53	.028
P-Selectin	8960.16 ± 3193.46	12704.98 ± 9828.76	.012
MMP9	551.00 ± 1045.36	145.74 ± 159.13	.051
Collagen 1A1	5.22 ± 8.60	1.89 ± 1.82	.031

*Table 2,6: Correlation of # of co-morbidities with cytokines in UTAH COVID ICU cohort.*The number of comorbidities was compared to cytokine levels to determine whether there is a correlation, those will statistical significance are included in this table.

Interestingly, patients with 3 or more co-morbidities also had significant correlation with TNF-alpha (p=.031), Collagen 1a1 (.031), MMP9 (p=.051), however, when compared to those with either one or two comorbidities, the levels were lower. IL-1B (p=.020) showed a dramatic increase in patients with 3 or more comorbidities compared to those with two (7269846.18 ± 11234999.53 vs 129.05 ± 23.68, respectively). Patients with three or more comorbidities also had significant correlations with IL10 levels (p=.003), (Table 2.7), all other cytokines had no significant correlations with number of comorbidities in COVID-19.

1.5 Cytokines as predictive markers of mortality on COVID-ICU patients.

Patients that succumbed (n=6) to COVID-19 had significant correlation with plasma cytokines S1000A8 (p=.036) and IL10 (p=<.001), both inflammatory cytokines were elevated compared to patients who survived. Prothrombotic markers, D-Dimers (.001) and TPO (p=018) were also elevated in patients that succumbed to COVID-19 compared to controls (Table 2.7).

N=26 , Alive =20, Dead=6	**Mean + SD (Alive)**	**Mean + SD(Dead)**	**p Value**
S100A8	66.026 ± 20.29	67.96 ± 39.42	.036
IL10	3.29 ± 2.30	7.05 ± 6.65	<.001
D-Dimers	3163822.5 ± 2668914.57	8097438.66 ± 14833232.57	.001
TPO	1089.88 ± 233.72	1109.88 ± 421.64	.018

Table 2.7: Mortality correlation with cytokines in UTAH COVID ICU cohort. List of cytokines that are significantly correlate with mortality

These data point towards the potential of developing a screening panel for risk assessment of mortality in ICU-COVID patients, receiver operating curves to develop cut of points with highest sensitivity and specificity are discussed below.

The comparison of these different patient populations and severity categorization (inpatient -ICU COVID vs outpatient) outlines the similarities and differences in plasma inflammatory and thrombosis markers shedding light on some of the fine differences and complexities of immune responses in disease progression of different severities, that could elucidate effective treatment strategies categorized according to severities.

Discussion

This study delves into four distinct patient groups - non-COVID donors, Long-COVID, COVID Non-ICU, and COVID ICU. Notably, Long-COVID patients exhibited elevated s-TLT-1 levels, implicating a potential link between s-TLT-1 and disease chronicity. Moreover, cytokine disparities were observed among these groups, revealing distinctive patterns between ICU and non-ICU patients. Despite challenges in obtaining comprehensive patient data, a thorough analysis was conducted among the ICU cohort, delineating prevalent comorbidities and associated therapeutics.

Inflammatory and prothrombotic state is intensified in ICU-COVID compared to all other categories of COVID-19 infections

Although inflammation and immune response to infection is the bodies protective mechanism to fight infection, it is well known that cytokine storm and hyperreactivity of the coagulation system is associated with poor outcomes in patients. IL-6 is significantly increased in all COVID-19 patient subgroups (Figure 3.1b) compared to non-COVID donor controls, these data suggest that there is stimulation of an acute phase reaction, hematopoiesis and increased transcription of inflammatory gene products in COVID-19 patients compared to control group

Platelet activation markers as a predictive indicator of procoagulant state in COVID-19.

Before drawing any conclusions, it is important to note that despite all ICU-COVID patients being on anticoagulants, there was still a significant difference in platelet activation markers. In the pathophysiological mechanisms of thrombus formation, it is well known that a central dogma for clot formation is platelet activation, upon activation, p-Selectin and TLT-1 are released from the alpha granules onto the

platelet surface. Studies have demonstrated that these receptors are highly sensitive as markers of platelet activation[27]. At baseline, in healthy individuals, soluble forms of these receptors should be at very low, if not undetectable levels, on the contrary, in inflammatory states that trigger platelet activation, soluble forms of these receptors are cleaved off the platelet surface and are a platelet activation "signature/footprint" in the plasma.

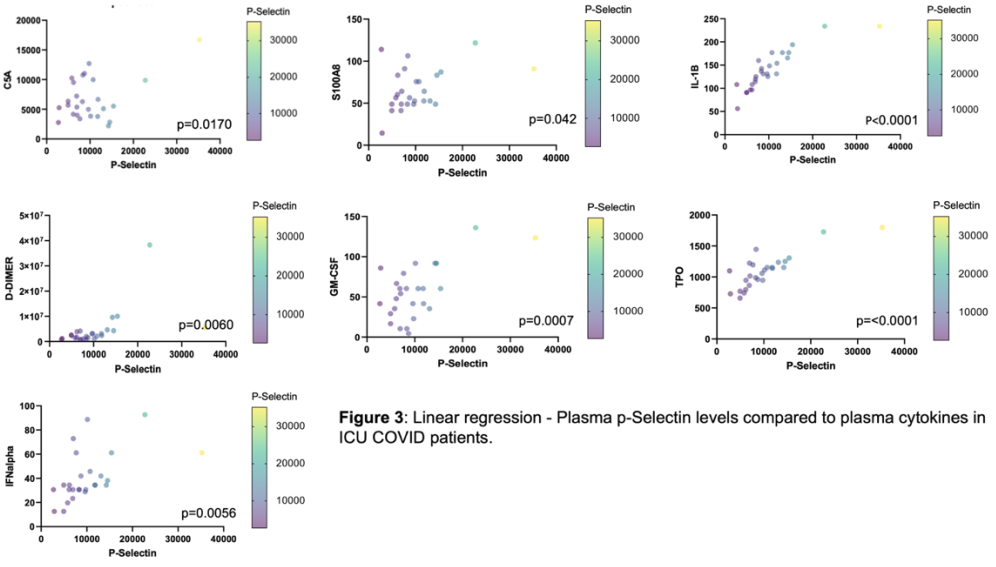


Figure 3: Linear regression - Plasma p-Selectin levels compared to plasma cytokines in ICU COVID patients.

Figure 3.3: Linear regression for plasma p-Selectin levels compared to plasma cytokines in ICU COVID patients. Linear regression highlights cytokines that p-Selectin correlates with.

In this study, s-TLT-1 levels significantly correlated with CVD, these data are consistent with previous studies where both acute coronary syndrome in two separate emergency departments and stable CVD demonstrate a correlation between s-TLT-1 levels and CVD prevalence. In stable CVD, a peak frequency of 544pg/ml was determined as a cutoff point, a significant association with congestive heart failure was identified. Interestingly, in this study, we demonstrate that even though all ICU patients are on anticoagulants, the

average s-TLT-1 levels are approximately six-fold (3179.64 ± 3237.93, p=.003)[30] higher when compared to those an average of 544pg/ml in stable CVD. These data suggest that COVID-19, specifically in patients that are in critical condition (ICU patients), exacerbates/precipitates a hyper coagulopathy, increasing the risk of thrombosis (deep vein thrombosis or pulmonary embolism). Moreover, a previous study has demonstrated that patients with ARDS have elevated plasma s-TLT-1 levels and that higher levels of s-TLT-1 correlated with mortality [31]. These studies support the data in this study and the potential use of s-TLT-1 as a prognostic indicator not just of thrombosis, but also mortality in COVID-19.

Studies have demonstrated that hypertension leads to endothelial dysfunction, Renin Angiotensin hyperactivation, decreased platelet nitrous oxide and shear stress leading to platelet degranulation, moreover, hypertension is a leading risk factor for the development of CVD, all four patients who had CVD concurrently had hypertension, thus it is not surprising that this study demonstrates that ICU COVID patients who have hypertension have significantly higher s-TLT-1 levels than those who did not 1814.16 ± 2506.79 and 459.48 ± 432.90, respectively, p=<.001.

To strengthen and support these data, sleep apnea significantly correlated with elevated s-TLT-1 levels in these COVID-ICU patients 2514.78 ± 3730.62 compared to those without sleep apnea, 986.71 ± 1616.39, p=.024.Studies have illustrated that sleep apnea increases the risk of both high blood pressure and cardiovascular disease, in this study, all three patients with hypertension had sleep apnea. Taken together, when CVD and its risk factors (hypertension and sleep apnea) are comorbid diseases of COVID-19,

there is increased risk of platelet activation and hypercoagulable state. In support of this, s-TLT-1 levels correlate with D-Dimer levels (p=0.012, Figure 3.2).

Plasma cytokines markers as a predictive indicator of disease severity in COVID-19.

The cytokine profile in COVID-19 seems to progress with an intricate interplay between pro and anti-inflammatory cytokines. We see a notable difference between outpatient covid patients (both in the Puerto Rican and UTAH cohorts) and ICU-COVID, with ICU-COVID patients showing significantly elevated cytokines compared to other groups (Figures 1 and 4). Several studies support our data, demonstrating that with increased severity of COVID-19 there is increased IL6 and S100A8, which contribute towards initiating an acute phase response and modulating the cytokine storm, respectively. While simultaneously the human host defense attempts to minimize cytokine storm damage by compensation with elevated levels of IL10, an anti-inflammatory cytokine.

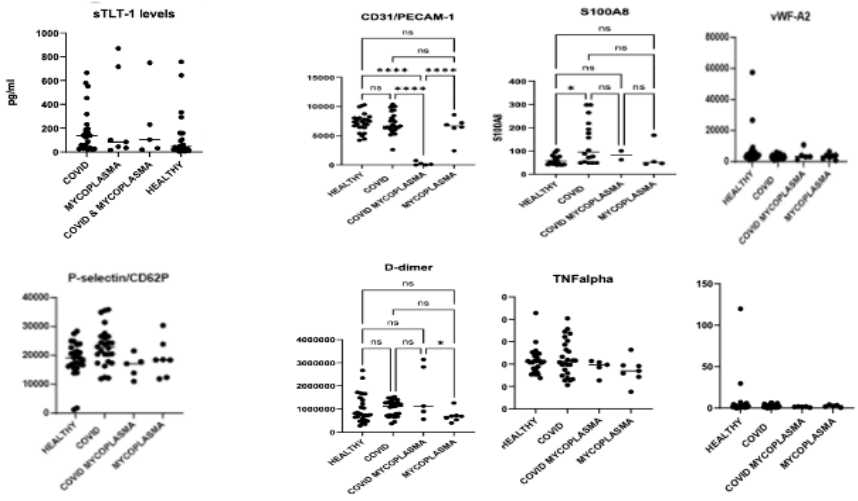


*Figure 3.4: Quantification of cytokines and s-TLT-1 in Puerto Rican outpatient cohort, using one way ANOVA.*Patients with both COVID and mycoplasma have significantly higher D-Dimer levels.

Comparison of cohorts according to severity of disease

We compared a general outpatient cohort (Puerto Rican cohort) with an ICU patient cohort (UTAH) and screened for the cytokines commonly associated with a viral inflammatory response, as well as platelet specific markers, and compared these variables to mortality. Our data suggests that there are no key biomarkers of disease in an outpatient setting and that the immune response is not as exaggerated as in ICU patients, on the contrary, there is potential for the use of a plasma cytokines and platelet marker panel for early screening of risk for mortality in ICU patients.

Developing a mortality risk evaluation scoring system in COVID-19 (MRES-COVID-19).

Our results support previous studies illustrating that D-Dimers, P-selectin, IL10 and S100A8 levels are elevated in COVID-19 ICU patients and that they correlate with mortality. Moreover, our studies suggest that s-TLT-1, a platelet specific activation marker is correlated with mortality. These data suggest the potential for the development of a predictive model for the risk of mortality in ICU-COVID-19 patients, we thus decided to use receiver operating curves (ROC) to determine whether cutoff values could be determined for a mortality risk evaluation prognostic panel in ICU-COVID patients: "Mortality Risk Evaluation Score in COVID-19" (MREs-COVID-19).

Our results demonstrate that although all these variables have a high sensitivity with the selected cutoff values, it is only s-TLT-1 that has a high specificity, making s-TLT-1 a potential biomarker for risk of mortality in these patients (Figure 3.5). We then looked at all the patients who had s-TLT-1 levels over the ROC determined 605pg/ml cutoff point and found that 5 of the 6 patients (83.3%) who died had s-TLT-1 levels

above 605pg/ml, moreover, when the average levels of those who died was compared to all patients who survived but had levels higher than the cutoff, those who died had 1.6 times higher the levels of s-TLT-1, than those who survived. In support of our initial linear regression findings, D-Dimer levels in those who died were 2.9 times higher than those who survived with levels of s-TLT-1 over 605pg/ml and IL-10 levels were 1.9 times higher in those who died compared to those who survived with elevated s-TLT-1, surprisingly, we did not see any significant differences with S1000A8 and P-Selectin.

On the contrary, P-Selectin levels seem to be almost double in the patients who lived compared to those who die. It was also noted that over 80% of the patients who had TLT-1 levels above the cutoff point presented to the hospital with two or more comorbidities, with four of the six patients who died having had an ischemic/thrombotic event (PE, Stroke or MI) and/or hypertension. Interestingly, none of the patients who survived had a history of ischemic events (Table 6). Although the linear regression comparing thrombotic events and mortality did not show any significance, there was a correlation with CVD (which is a high-risk factor for an ischemic event) and hypertension.

Taken together, these results suggest that clinicians could develop a 6-point scoring system “Mortality Risk Evaluation Score in COVID-19” (MRES-COVID-19), where the presence of any of the risk factors (hypertension, ischemic events, s-TLT-1 levels above 605pg/ml, D-Dimers above 1,000,000 and IL10 levels above 2 ,concurrence of two or more co-morbidities) would increase the risk for mortality by 1 point, with each parameter in the model representing one point, and the higher the score, the greater the risk of mortality.

Conclusion

Several studies have demonstrated that comorbid disease, thrombosis, and cytokine storm, in COVID-19 increase the risk for mortality. Here we looked at patients ranging from outpatient to ICU hospitalized with COVID-19 with the objective of determining which platelet activation markers and plasma cytokines had a correlative pattern with either/both disease severity and mortality. While the outpatient Puerto Rican cohort had elevated plasma levels of both platelet and cytokine markers, the levels and correlation with mortality paled in comparison to ICU patients in a UTAH cohort, emphasizing the role of cytokines and activated platelets in the pathophysiological progression of COVID-19.

Using an ROC comparative model, we determined the specificity and sensitivity of parameters that correlated with mortality in the UTAH ICU cohort and found TLT-1 to be the most specific and sensitive predictive indicator of risk for mortality. There is potential for the development of a 6 point “Mortality Risk Evaluation Score in COVID-19” (MRES-COVID-19), using hypertension, ischemic events, s-TLT-1 levels above 605pg/ml, elevated D-Dimers and IL10 levels , and concurrence of two or more co- were more likely to die (66%), however , this model would have to be tested on a larger scale, since we only had six patients who died of COVID-19 (Table 2.8).

Table 2.8: Comparing parameters for potential mortality risk predictive panel in patients who succumb vs those who survive. This table describes patient characteristics and average s-TLT-1 levels to determine whether they could be used as a predictive panel.

PATIENT	OUTCOME	COMORBIDITIES	COMORBIDITIES	D-DIMER	IL10	S100A8	sTLT-1	P-Selectin	Age
1	DEAD	≥ 3	HTN, PE, Dementia, AAA, Alcohol abuse	4,130,698.97	4.40	48.79	305.56	6946.98	66.00
2	DEAD	≥ 3	Stroke, Cardiac Arrhythmia, DM, MDD, HTN, Hyperlipidemia, Atrial fibrillation, OSA	1,653,731.13	13.33	60.27	6814.31	6132.90	71.00
3	DEAD	≥ 2	Ischemic Heart Disease, MI, HTN	1,417,411.55	4.21	56.44	616.47	5787.14	62.00
4	DEAD	≥ 3	SLE, Sjogren's disease, MG, GI disease, Asthma, HTN	38,296,523.27	17.21	121.62	6583.63	22733.56	49.00
5	DEAD	≥ 2	Stage 4 lung Adenocarcinoma, PE	1,956,804.99	0.25	106.27	1425.97	8337.32	49.00
6	DEAD	≥ 1	Rheumatoid Arthritis	1,129,462.08	2.90	14.42	1043.15	2856.21	70.00
			AVERAGE LEVELS	**8,097,438.66**	**7.05**	**67.97**	**2798.18**	**8799.02**	**61.17**
7	ALIVE	≥ 3	Asthma, Atrial Flutter, Cholecystitis, COPD, HTN	823,593.74	5.34	113.95	4999.52	2689.22	65.00
8	ALIVE	≥ 2	HTN, OSA	784,330.93	0.82	48.79	595.40	8247.58	61.00
9	ALIVE	≥ 3	Diabetes, HTN, CKD, Hyperlipidemia	5,530,000.00	7.39	90.93	820.30	35235.66	49.00
10	ALIVE	≥ 1	Diabetic	2,383,663.77	1.77	64.10	898.72	11834.23	59.00
11	ALIVE	≥ 3	CKD, HTN, DM	4,630,688.78	3.28	52.62	1030.57	13083.07	59.00
			AVERAGE LEVELS	**2,830,455.44**	**3.72**	**74.07**	**1668.90**	**14217.95**	**58.60**
			PROPORTION/ FOLD INCREASE COMPARISON (AVERAGE DEAD/AVERAGE ALIVE)	2.9	1.9	0.92	1.68	0.61	1.04

HTN = Hypertension, PE = Pulmonary Embolism, AAA = Abdominal Aortic Aneurism, DM = Diabetes Mellitus, MDD = Major Depressive Disorder, OSA = Obstructive Sleep

Apnea, MG = Myasthenia Gravis, SLE – Systemic Lupus Erythematosus, CKD = chronic kidney disease. * Where YES= 1 point, No = 0

PATIENT ID	1	2	3	4	5	6	7	8	9	10	11
Hypertension	1	1	1	1	0	0	1	1	1	0	1
Thrombotic events	1	1	1	0	1	0	0	0	0	0	0
s-TLT-1 ≥ 605pg/ml	0	1	1	1	1	1	1	0	1	1	1
D-Dimers ≥ 1,000,000 pg/ml	1	1	1	1	1	1	0	0	1	1	1
IL10 ≥ 2 pg/ml ≥ 2	1	1	1	1	0	1	1	0	1	1	1
Comorbidities	1	1	1	1	1	0	1	1	1	0	1
TOTAL POINTS	5	6	6	5	4	3	4	2	5	3	5
OUTCOME	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	ALIVE	ALIVE	ALIVE	ALIVE	ALIVE

*Table 2.9: Mortality Risk Evaluation Score in COVID-19).*Using suggested parameters does not seem to be a good predictive indicator of mortality since two patience that are alive have a score of 5.

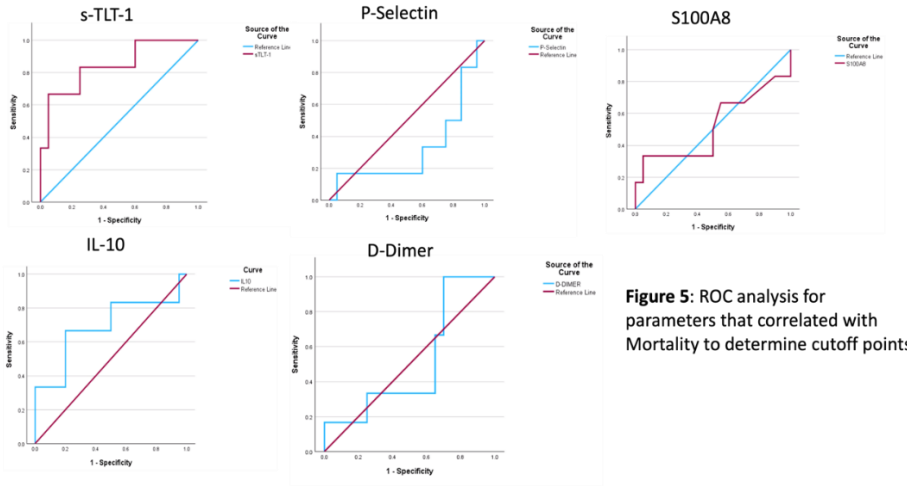


Figure 5: ROC analysis for parameters that correlated with Mortality to determine cutoff points

Figure 3.5: ROC analysis for parameters that correlated with mortality to determine cutoff points. s-TLT-1 and IL-10 have the highest sensitivity and specificity as mortality predictive indicators.

Bibliography

1. Djaharuddin, I. *et al.* Comorbidities and mortality in COVID-19 patients. *Gac Sanit* 35, S530–S532 (2021).

2. Comorbidities and mortality in COVID-19 patients - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8677356/.

3. van den Berg, J. *et al.* Von Willebrand factor and the thrombophilia of severe COVID-19: in situ evidence from autopsies. *Res Pract Thromb Haemost* 7, 100182 (2023).

4. Carlini, V. *et al.* The multifaceted nature of IL-10: regulation, role in immunological homeostasis and its relevance to cancer, COVID-19 and post-COVID conditions. *Front Immunol* 14, (2023).

5. Ragab, D., Salah Eldin, H., Taeimah, M., Khattab, R. & Salem, R. The COVID-19 Cytokine Storm; What We Know So Far. *Front Immunol* 11, (2020).

6. Fenyves, B. G. *et al.* Plasma P-selectin is an early marker of thromboembolism in COVID-19. *Am J Hematol* 96, E468–E471 (2021).

7. Islam, H., Chamberlain, T. C., Mui, A. L. & Little, J. P. Elevated Interleukin-10 Levels in COVID-19: Potentiation of Pro-Inflammatory Responses or Impaired Anti-Inflammatory Action? *Front Immunol* 12, 677008 (2021).

8. Gupta, A. *et al.* Lipocalin-2, S100A8/A9, and cystatin C: Potential predictive biomarkers of cardiovascular complications in COVID-19. *Exp Biol Med* 247, 1205–1213 (2022).

9. Tang, Y. *et al.* Cytokine Storm in COVID-19: The Current Evidence and Treatment Strategies. *Front Immunol* 11, (2020).

10. Grasselli, G. *et al.* Baseline Characteristics and Outcomes of 1591 Patients Infected with SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. *JAMA - Journal of the American Medical Association* 323, 1574–1581 (2020).

11. Grasselli, G. *et al.* Risk Factors Associated with Mortality among Patients with COVID-19 in Intensive Care Units in Lombardy, Italy. *JAMA Intern Med* 180, 1345–1355 (2020).

12. Abdi, S. *et al.* Trends of in-hospital and ICU mortality in COVID-19 patients over the fourth and fifth COVID-19 surges in Iran: A retrospective cohort study from Iran. *International Journal for Quality in Health Care* 34, (2022).

13. Mokhtari, T. *et al.* COVID-19 and multiorgan failure: A narrative review on potential mechanisms. *J Mol Histol* 51, 613 (2020).

14. Zaid, Y. & Guessous, F. The ongoing enigma of SARS-CoV-2 and platelet interaction. *Res Pract Thromb Haemost* 6, (2022).

15. Landi, A. & De Servi, S. The burden of thrombotic complications in critically ill patients with COVID-19: charting the uncharted. *Intern Emerg Med* 15, 893–895 (2020).

16. Thomas, W. *et al.* Thrombotic complications of patients admitted to intensive care with COVID-19 at a teaching hospital in the United Kingdom. *Thromb Res* 191, 76–77 (2020).

17. Rosen, R. J. Thrombotic complications in critically ill patients with COVID 19. *Thromb Res* 191, 56 (2020).

18. van Nieuwkoop, C. COVID-19 associated pulmonary thrombosis. *Thromb Res* 191, 151 (2020).

19. Martinelli, I. *et al.* Pulmonary embolism in a young pregnant woman with COVID-19. *Thromb Res* 191, 36–37 (2020).

20. Branfield, S. & Washington, A. V. The enigmatic nature of the triggering receptor expressed in myeloid cells -1 (TLT- 1). *Platelets* 32, 753 (2021).

21. Kappelmayer, J., Nagy, B., Miszti-Blasius, K., Hevessy, Z. & Setiadi, H. The emerging value of P-selection as a disease marker. *Clin Chem Lab Med* 42, 475–486 (2004).

22. Washington, A. V. *et al.* TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. *J Clin Invest* 119, 1489–1501 (2009).

23. Barrett, T. J. *et al.* Platelet and Vascular Biomarkers Associate with Thrombosis and Death in Coronavirus Disease. *Circ Res* 127, 945 (2020).

24. Vassiliou, A. G. *et al.* ICU Admission Levels of Endothelial Biomarkers as Predictors of Mortality in Critically Ill COVID-19 Patients. *Cells* 10, 1–13 (2021).

25. Hottz, E. D. *et al.* Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. *Blood* 136, 1330 (2020).

26. Goshua, G. *et al.* Endotheliopathy in COVID-19-associated coagulopathy: evidence from a single-centre, cross-sectional study. *Lancet Haematol* 7, e575–e582 (2020).

27. Smith, C. W. *et al.* TREM-like transcript 1: a more sensitive marker of platelet activation than P-selectin in humans and mice. *Blood Adv* 2, 2072 (2018).

28. Human Luminex Discovery Assay (LXSAHM) | Bio-Techne. https://www.bio-techne.com/p/luminex-assays/human-luminex-discovery-assay_lxsahm.

29. Human TREML1/TLT-1 DuoSet ELISA DY2394: R&D Systems. https://www.rndsystems.com/products/human-treml1-tlt-1-duoset-elisa_dy2394.

30. Bayrón-Marrero, Z. *et al.* The Characterization and Evaluation of the Soluble Triggering Receptor Expressed on Myeloid Cells-like Transcript-1 in Stable Coronary Artery Disease. *Int J Mol Sci* 24, (2023).

31. Morales-Ortíz, J. *et al.* Platelet-derived TLT-1 is a prognostic indicator in ALI/ARDS and prevents tissue damage in the lungs in a mouse model. *Blood* (2018) doi:10.1182/blood-2018-03-841593.

Abstract

Nephrotic Syndrome (NS), characterized by glomerular dysfunction and proteinuria, poses challenges in predicting thromboembolism (TE), a severe complication associated with increased morbidity. We investigated the suitability of platelet alpha granule proteins, P-Selectin and Trem-Like Transcript 1 (TLT-1), as biomarkers for NS severity and TE risk. Patients without reported thrombotic events exhibited minimal baseline platelet activation, challenging conventional understanding. Elevated s-TLT-1 levels with NS progression raised questions about its detectability in non-thrombotic individuals. Despite minimal baseline procoagulant activity, our data indicated low-level platelet activation, not correlating significantly with disease progression based on proteinuria. Ex vivo studies supported minimal baseline activation but revealed significant differences upon agonist stimulation, emphasizing the need for anticoagulant therapy in NS. While P-Selectin and TLT-1 may not serve as optimal biomarkers, our findings underscore the importance of understanding platelet dynamics in NS for early therapeutic interventions, potentially revolutionizing TE prevention and reducing associated morbidity and mortality.

Introduction

Nephrotic syndrome (NS) is a chronic kidney disease in children, with a prevalence of 2-7 per 100,000 globally.[1–3] NS is characterized by glomerular filtration barrier changes leading to hypoalbuminemia, plasma accumulation of larger proteins (>80kDa), urinary loss of low molecular weight proteins (<80kDa in size), hyperlipidemia and edema. Although the clinical outcomes are usually resolution with corticosteroid or alternate immunosuppressive treatments, these symptoms can be superimposed by complications such as thromboembolism, acute renal failure, hypovolemia, cardiovascular disease, infection and hypertension. Thromboembolic events (TE) remain the most threatening complication of NS in children, with increased risk of mortality.

The exact pathophysiological mechanism leading to the onset of TE complications in NS is difficult to characterize as it has been reported that there is imbalance of pro vs anticoagulant and pro vs antifibrinolytic factors, on a patient-to-patient basis. Studies have proposed that this discrepancy in coagulation profile is associated with increased urinary loss of proteins that regulate platelet production and function with the more severe cases of NS allowing more, and larger protein permeability into the urine during glomerular filtration. Increased urinary loss of pituitary adenylate cyclase-activating polypeptide (PACAP), an inhibitor of megakaryopoiesis, in children with nephrotic syndrome, was shown to correlate with thrombocytosis and hyperaggregability[4–7]. It has also been reported that there is increased release of active

substances and elevated surface expression of activation dependent platelet surface markers (P-Selectin)[8].

While the mechanisms underlying platelet hyper functionality in NS are multifactorial, related to alterations in coagulation and fibrinolytic proteins, to date, there is no clear outline as to what the "tell-tale" indicator of the progression from hypercoagulability to TE is, making it difficult to predict the risk of TE in patients with NS. Antiplatelet therapy is not included on routine therapeutic guidelines for pediatric NS and can lead to bleeding complications, this introduces the conundrum of not knowing which NS patients should be administered prophylactic antiplatelet medication.

The current risk predictors of TE are coagulation markers, fibrinolysis and platelet levels, which may serve as an indication for the early detection and possible prevention of TE, however, contradictory results have been reported, with some studies reporting increased platelet counts, whilst others report that counts remain unchanged. Platelet aggregation is increased in some studies with activators ADP, collagen and arachidonic acid, whilst some studies report that it remains unchanged with collagen and arachidonic acid. The platelet surface expression marker P-selectin has been reported to be increased in two separate studies, yet, to date, there is no clear biomarker of platelet hyper functionality in NS[8–10].

Early detection of the onset of TE would decrease the risk of mortality and embolic complications due to coagulopathy in children with NS. Several studies have proposed the use of soluble platelet receptor markers (such as soluble P-Selectin and Trem Like Transcript-1) as biomarkers for the early detection and prediction of mortality in diseases associated with platelet dysfunction[11–15]. We hypothesized that an activation

dependent surface receptor for platelets could be used to detect platelet hyperactivity and predict the risk of developing TE in NS. To achieve this, we carried out the below experiments.

Methods

Plasma quantification of platelet markers, s-TLT-1 and s-P-Selectin by ELISA from the Columbus (N=19) and nephrotic syndrome study network (neptune; n=35) patient cohorts [16,17] , s-TLT-1, human TREML1/TLT-2 Duoset ELISA catalog # DY2394 and s-P-Selectin, Invitrogen CAT#BMS219-4) by ELISA, using the kit protocol.

Nephrotic (podocin-DTR mouse) Mice experiments

Using a podocin-Cre, inducible-DTR mouse model (a transgenic mouse that carries transgenes for both podocin-promoter driven Cre recombinase expression and loxP inducible). In this model, administration diphtheria toxin damages only the podocytes to induce nephrotic syndrome[18].

To compare platelet counts in mice with NS vs control counterparts, whole blood was extracted by heart puncture and quantified using a SYSMEX CELL counter.

Platelet Aggregation

To assess whether surface expression of these receptors is associated with changes in hyperreactivity of platelets, we carried out ex vivo platelet aggregation assays using washed platelets at concentration of 2.5×10^6 in NS mice compared to control counterparts[19] using platelet activators collagen and thrombin.

Flowcytometry

Add 5ul of whole blood to your flowcytometry panel (4ul GPRP, 5ul CD41 antibody (PE) , 5ul CD62P (FITC) BD Catalog No:553744, 46ul 1x Tyrode's with

calcium) and incubate in the dark for 30 minutes. whole blood, add 500ul 1x FACS lysing buffer (BD sciences) and incubate for 30 minutes, add 500ul of 1 x Tyrode's without calcium and incubate for 30 minutes, read on flow cytometer.

ELISA

Plasma quantification of platelet markers, s-TLT-1 and s-P-Selectin s-TLT-1 and s-P-Selectin, Invitrogen Cat# EMSELP, in podocin-DTR mice (n=44) by ELISA using the kit protocols.

Statistics

Graph Prism was used to analyze all data using a t-test where test subjects were categorized according to age or nephrotic syndrome quartile, or linear regression for multiple variable comparison of either s-TLT-1 or s-P-Selectin with patient characteristics, protein: creatinine ratios or thrombin generation.

Results

Soluble plasma levels of platelet receptors suggest baseline platelet activation in nephrotic syndrome (NS).

P-selectin and Trem-Like Transcript 1 (TLT-1) are alpha granule platelet receptors that are released onto the platelet surface upon activation. Several studies have demonstrated a correlation between plasma levels of these proteins and the risk of thrombosis. In this study, we quantified plasma levels of these receptors in NS to assess their utility as biomarkers for early detection of platelet activation and risk for thrombosis. There was no significant difference in soluble P-selectin (s-P-Selectin) or soluble TLT-1 (s-TLT-1) plasma levels among healthy, pediatric, and adult individuals in the NCH NS cohort. Furthermore, comparisons of s-P-Selectin or s-TLT-1 plasma levels

with proteinuria, protein-to-creatinine ratio, or thrombin generation showed no significant correlations (Figure 3.6).

When we stratified s-TLT-1 levels based on the peak frequency distribution to be <544 pg/mL, there was a trend toward higher peak thrombin levels in patients with s-TLT-1 > 544 pg/mL, but this trend was not statistically significant (Figure 3.7). The cutoff of 544 was selected from a previous study that identified 544 pg/mL as a sensitive marker for congestive heart failure progression. However, due to the sample size of this cohort of patients, we were unable to perform a ROC analysis specific to s-TLT-1 in NS.

Similarly, there was no statistical significance between plasma s-P-Selectin or s-TLT-1 levels in NEPTUNE NS patients stratified according to spot urine protein (spotupr) into four quartiles. However, s-TLT-1 weakly correlated with spotupr, suggesting that s-TLT-1 could be a sensitive marker of NS severity (Figure 3.8). s-TLT-1 levels were higher in the first two quartiles compared to Q3 and Q4, supporting higher proteinuria in these quartiles. Taken together, these data suggest that s-TLT-1 and P-Selectin would not be optimal markers for NS severity or increased thrombin activity.

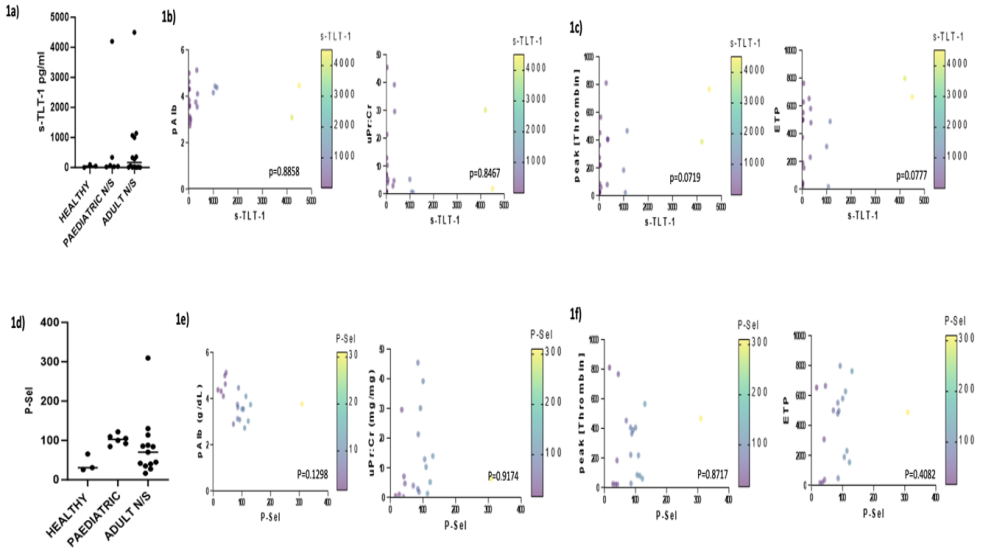


Figure 3.6: Quantification of plasma proteins in Columbus cohort of Nephrotic patients

a) plasma s-TLT-1 and correlation of s-TLT-1 with b) plasma albumin and protein: creatinine ratio or c) peak thrombin and endogenous thrombin potential d) plasma s-P-Selectin and correlation with e) plasma albumin and protein: creatinine ratio, or f) peak thrombin and endogenous thrombin potential in pediatric and adult nephrotic syndrome patients from the Columbus Cohort.

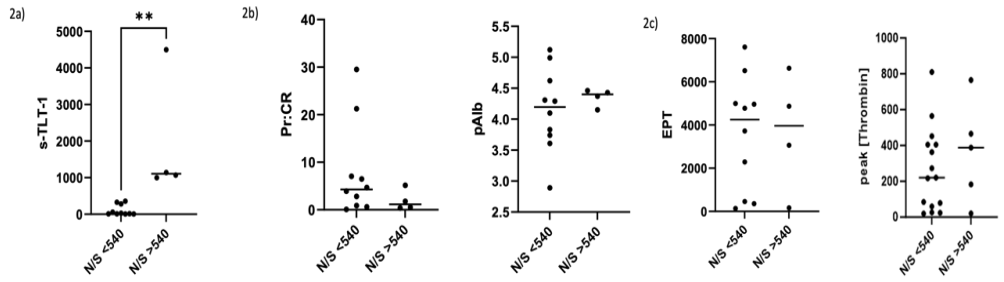


Figure 3.7: Adult patients from the Columbus Cohort stratified based on s-TLT-1 peak frequency distribution (544pg/mL) to compare a) s-TLT-1 levels, b) protein: creatinine ratio and plasma albumin or c) endogenous thrombin potential and peak thrombin.

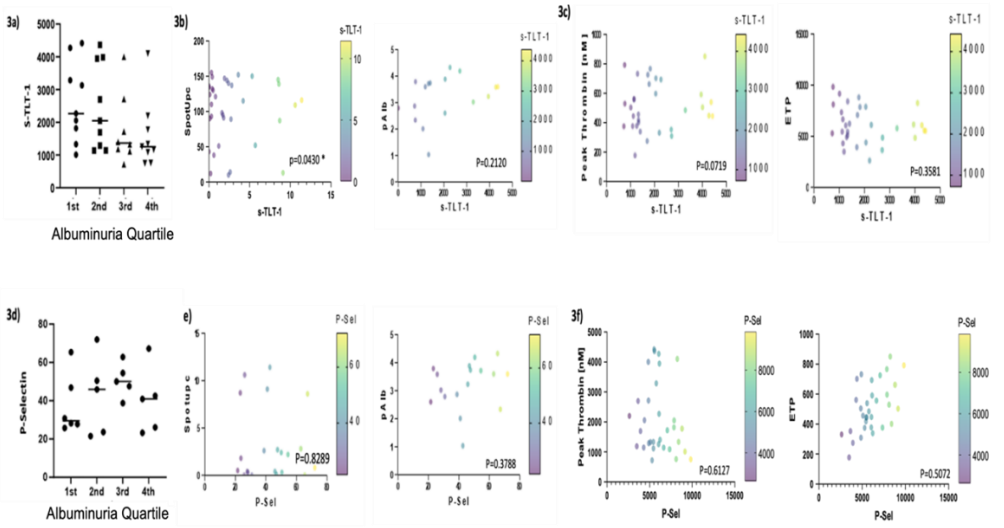


Figure 3.8: quantification of plasma proteins in Neptune *cohort of nephrotic patients* a) plasma s-TLT-1 and correlation of s-TLT-1 with b) protein: creatinine ratio and plasma albumin or c) peak thrombin and endogenous thrombin potential d) plasma s-P-Selectin and correlation with e) protein: creatinine ratio and plasma albumin, or f) peak thrombin and endogenous thrombin potential in nephrotic syndrome patients from the NEPTUNE Cohort.

In the mouse model, plasma quantification of soluble Trem-Like Transcript 1 (s-TLT-1) and soluble P-Selectin (s-P-Selectin) was a more sensitive marker of disease severity in nephrotic syndrome (NS) than what we observed in human plasma samples (Figure 3.9). However, the results were inverse, with s-TLT-1 levels higher with disease severity. This result is intriguing since s-TLT-1 is a small protein (17 kDa), leading us to question whether there is dimerization of s-TLT-1. Using a linear regression, there was no correlation between TLT-1 levels and albumin-to-creatinine ratio in NS mice, with increased s-TLT-1 negatively trending with a decrease in albumin-to-creatinine ratio. In contrast, P-Selectin exhibited an inverse trend in mice, with disease progression leading to a decrease in plasma levels of P-Selectin (Figure 3.9).

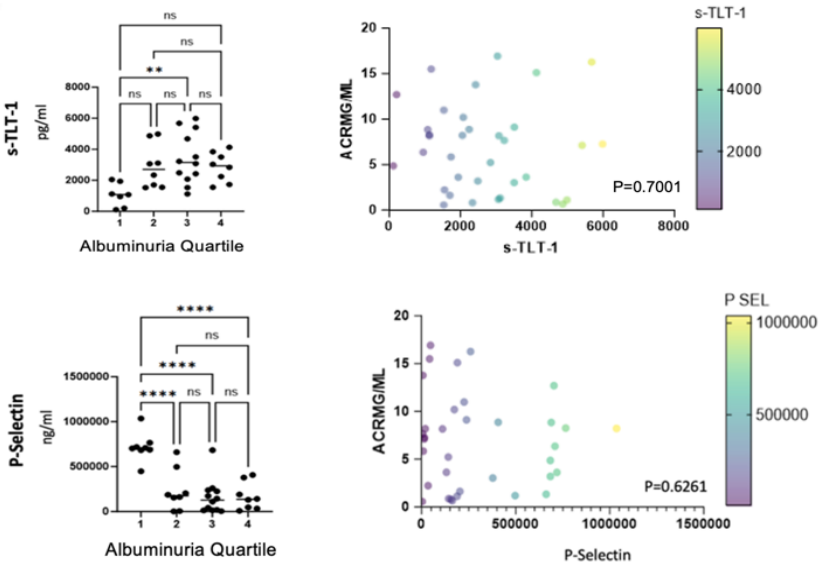


Figure 3.9: Plasma quantification of s-TLT-1 and s-P-Selectin and correlation of Albumin : Creatinine ration to s-TLT-1 and s-P-Selectin in nephrotic mice.

Platelets in nephrotic mice are procoagulant when challenged with a platelet agonist.

While studies have suggested platelet dysfunction during NS, the exact mechanisms, and the correlation between platelet alterations in NS and the onset of thromboembolic phenomena remain unclear. To better understand the pathophysiological mechanisms driving platelet dysfunction in NS, there is a need to characterize these changes in platelets. We thus studied ex vivo platelets collected from the mouse NS model.

These data demonstrate that, when activated, platelets from nephrotic mice are procoagulant compared to wild-type controls with different agonists (Q2 of graphs in Figure 3.10). In support of these data, nephrotic mice exhibit higher platelet aggregation than wild-type controls (Figure 3.11).

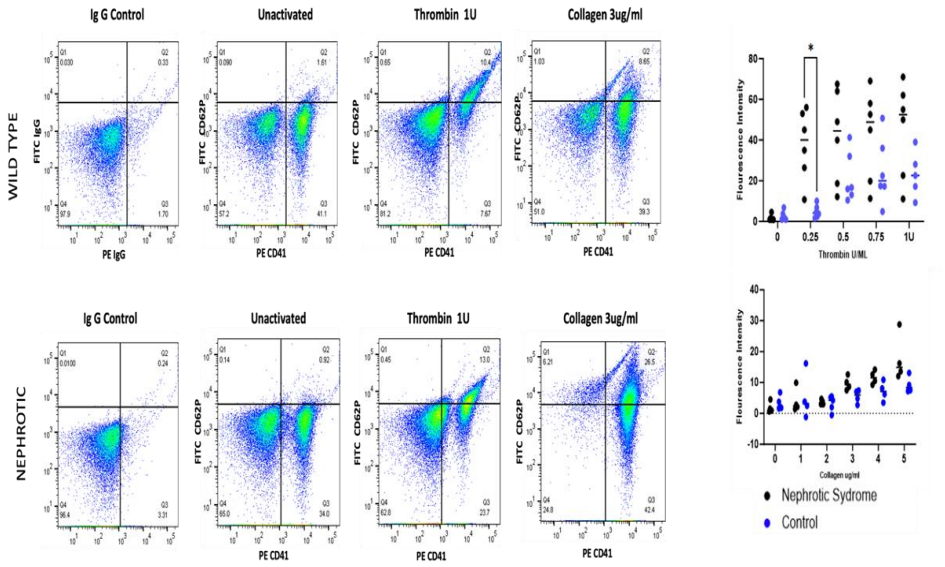


Figure 3.10: Comparison of platelet activation from whole blood in Nephrotic and Wild Type mice. Nephrotic platelets have higher platelet activation than wild type mice.

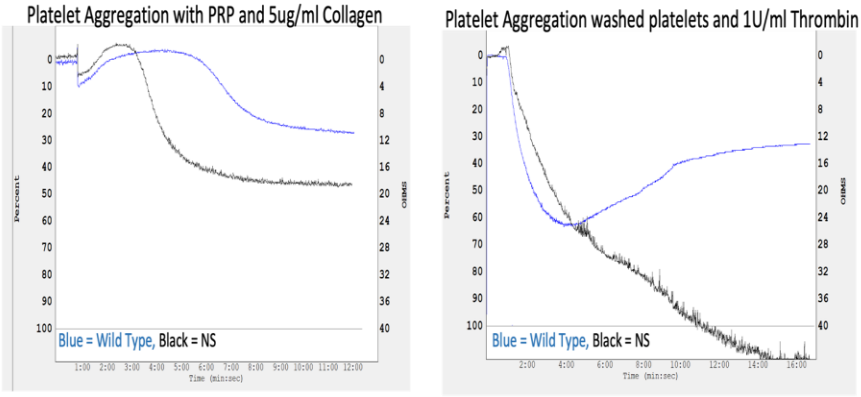


Figure 3.11: Platelet aggregation using platelet rich plasma from Nephrotic and Wild Type mice. Nephrotic mice have higher platelet aggregation with both collagen and thrombin.

Discussion

Platelet alpha granule proteins, namely P-Selectin and TLT-1, undergo translocation onto the platelet surface upon activation, promoting thrombus formation through interaction with ligands. P-Selectin has been proposed as a biomarker for infection and survival in acute respiratory distress syndrome and vaso-occlusive crisis in sickle cell disease. Notably, increased plasma levels of soluble Trem Like Transcript-1 (s-TLT-1) have been associated with poor outcomes and increased mortality in acute respiratory distress syndrome. Elevated soluble TLT-1 levels have also been linked to Acute Coronary Syndrome, Colorectal cancer, and hepatocellular carcinoma, all characterized by platelet dysfunction. These studies suggest the potential use of activation-dependent surface markers for diseases complicated by platelet dysfunction. Despite the conventional use of P-Selectin as a marker for platelet activation, a recent

study reports Trem-Like transcript 1 as a more sensitive marker of platelet activation in both humans and mice.

All patients selected for the Neptune and Columbus Nephrotic Syndrome cohorts had no reported thrombotic events in their patient history, suggesting minimal baseline platelet activation. In non-thrombotic patients, this is considered as none or barely detectable in plasma. However, our data indicate higher s-TLT-1 levels with NS progression, raising questions about the detectable levels in patients with no known thrombotic events. Notably, ETP and peak thrombin did not significantly correlate with s-P-Selectin or s-TLT-1, suggesting minimal procoagulant activity at baseline but an idling, low-level platelet activation. This activation does not significantly correlate with disease progression when stratifying patients according to proteinuria, indicating unpredictability in progression to thromboembolism (TE) as a complication of NS.

Ex vivo studies support minimal platelet activation at baseline in NS. However, stimulation by an agonist reveals significant differences between controls and nephrotic mice, with higher aggregation and platelet activation (with collagen or thrombin) in nephrotic mice. This emphasizes the importance of considering anticoagulant therapy in NS patients, especially those with preexisting coagulopathies. Interestingly, collagen activation leads to a shift in a second population of cells to Q2, suggesting potential "platelet-white blood cell" conjugate formations, possibly neutrophils or monocytes. Although unstained for other cells, this change in platelet phenotype may be related to an inflammatory pathophysiological mechanism, requiring further studies for clarification.

Conclusion

Nephrotic Syndrome is a chronic kidney disease initiated by podocyte injury or dysfunction, characterized by a dysfunctional glomerular filtration barrier. This dysfunction results in increased permeability to low molecular weight proteins, leading to their loss into urine and a decrease in plasma protein levels, particularly albumin. Clinically, the optimal approach is to initiate early therapeutic management to prevent the development and progression of TE in NS. The diverse patient profile, coupled with hemostatic, coagulation, and fibrinolytic anomalies, alongside increased TE risk from therapeutic agents, diuretic-induced blood viscosity, creates a challenging cycle. Predicting and treating TE before onset is difficult, increasing the risk of TE-associated acute kidney injury (AKI) and mortality. Although various risk predictors for NS have been proposed, a standardized predictor for TE onset in NS is yet unknown. The identification of a predictive indicator or biomarker could enable preventative treatment or early TE detection.

Our data suggest that while elevated platelet-specific markers indicate platelet activation in NS, P-Selectin and TLT-1 may not be suitable biomarkers for disease progression or a prothrombotic state in NS. However, ex vivo studies support a procoagulant phenotype in Nephrotic Syndrome, specifically altered platelet activation and aggregation. Future directions should focus on further characterizing platelet morphological changes and signaling in NS. Understanding the triggers precipitating this procoagulant phenotype could introduce novel therapeutic approaches to TE in NS.

Bibliography

1. Downie, M. L., Gallibois, C., Parekh, R. S. & Noone, D. G. Nephrotic syndrome in infants and children: Pathophysiology and management. *Paediatr Int Child Health* 37, 248–258 (2017).

2. Welegerima, Y., Feyissa, M. & Nedi, T. Treatment Outcomes of Pediatric Nephrotic Syndrome Patients Treated in Ayder Comprehensive Specialized and Mekelle General Hospitals, Ethiopia. (2021) doi:10.2147/IJNRD.S310567.

3. Jin Park, S. & il Shin, J. Complications of nephrotic syndrome. *Korean J Pediatr* 54, 322 (2011).

4. Eneman, B. *et al.* Pituitary adenylate cyclase-activating polypeptide (PACAP) in zebrafish models of nephrotic syndrome. (2017) doi:10.1371/journal.pone.0182100.

5. Eneman, B., Levtchenko, E., Van Den Heuvel, B., Geet, C. Van & Freson, K. Platelet abnormalities in nephrotic syndrome. doi:10.1007/s00467-015-3173-8.

6. Walter, E., Deppermann, D., Andrassy, K. & Koderisch, J. Platelet hyperaggregability as a consequence of the nephrotic syndrome. *Thromb Res* 23, 473–479 (1981).

7. Machleidt, C. *et al.* Multifactorial genesis of enhanced platelet aggregability in patients with nephrotic syndrome. *Kidney Int* 36, 1119–1124 (1989).

8. Tkaczyk, M. & Baj, Z. Surface markers of platelet function in idiopathic nephrotic syndrome in children. *Pediatric Nephrology* 17, 673–677 (2002).

9. Sirolli, V. *et al.* Platelet activation markers in patients with nephrotic syndrome: A comparative study of different platelet function tests. *Nephron* 91, 424–430 (2002).

10. (PDF) Platelet Activation Markers in Patients with Nephrotic Syndrome. https://www.researchgate.net/publication/246511212_Platelet_Activation_Markers_in_Patients_with_Nephrotic_Syndrome.

11. Morales-Ortíz, J. *et al.* Platelet-derived TLT-1 is a prognostic indicator in ALI/ARDS and prevents tissue damage in the lungs in a mouse model. *Blood* (2018) doi:10.1182/blood-2018-03-841593.

12. Esponda, O. L., Hunter, R., Río, J. R. R. Del & Washington, A. V. Levels of soluble TREM-Like transcript 1 in patients presenting to the emergency department with chest pain. *Clinical and Applied Thrombosis/Hemostasis* 21, 30–34 (2015).

13. Vázquez-Otero, I. *et al.* Association of soluble TREM-like transcript-1 with clinical features and patient reported outcomes in systemic lupus erythematosus. *Eur J Rheumatol* 5, 244–248 (2018).

14. Derive, M. *et al.* Soluble Trem-like Transcript-1 Regulates Leukocyte Activation and Controls Microbial Sepsis. *The Journal of Immunology* 188, 5585–5592 (2012).

15. Shen, L. *et al.* P-selectin (CD62P) and soluble TREM-like transcript-1 (sTLT-1) are associated with coronary artery disease: A case control study. *BMC Cardiovasc Disord* 20, 1–7 (2020).

16. Abdelghani, E. *et al.* Exploring the Role of Antithrombin in Nephrotic Syndrome-Associated Hypercoagulopathy: A Multi-Cohort Study and Meta-Analysis. *Clin J Am Soc Nephrol* 18, 234–244 (2023).

17. Waller, A. P. *et al.* Nephrotic syndrome disease activity is proportional to its associated hypercoagulopathy. *Thromb Res* 201, 50–59 (2021).

18. Guo, J. K. *et al.* Increased tubular proliferation as an adaptive response to glomerular albuminuria. *J Am Soc Nephrol* 23, 429–437 (2011).

19. Kerlin, B. A. *et al.* Disease severity correlates with thrombotic capacity in experimental nephrotic syndrome. *Journal of the American Society of Nephrology* 26, 3009–3019 (2015).

TLT-1 AS A REGULATOR OF ATHEROSCLEROSIS

Abstract

The Triggering Receptor Expressed on Myeloid Cells-like transcript (TLT-1) has emerged as a significant player in cardiovascular disease (CVD) pathology, particularly in atherosclerosis. This study aimed to elucidate the role of TLT-1 in atherosclerosis progression using a combination of human coronary artery analysis and an apoe$^{-/-}$/treml1$^{-/-}$ mouse model. Initial investigation into TLT-1 expression in human atherosclerotic lesions revealed abundant TLT-1 staining in diseased vessels, persisting throughout lesion progression from early to advanced stages. Subsequent analysis using the apoe$^{-/-}$/treml1$^{-/-}$ mouse model demonstrated reduced atherosclerotic lesion size in the aortic sinus compared to apoe-/- controls, independent of serum cholesterol levels. Plasma cytokine analysis revealed elevated levels of TNF-α and IL-10 in apoe$^{-/-}$/treml1$^{-/-}$ mice, accompanied by increased weight gain and liver damage compared to heterogenous littermates. While no significant difference in macrophage content within the lesions was observed, a trend towards higher macrophage positivity was noted in apoe$^{-/-}$/treml1$^{-/-}$ mice at later time points.

Further examination indicated that TLT-1 regulates fibrinogen deposition within atherosclerotic lesions, implicating its involvement in lesion development These findings underscore the critical role of TLT-1- Fibrinogen dependent mechanism underlying atherosclerotic lesion progression and highlight its potential as a therapeutic target in

CVD. Further investigation into the molecular pathways underlying TLT-1-mediated effects on lesion development is warranted to advance our understanding of CVD pathogenesis.

Introduction

Atherosclerosis is a leading cause of vascular occlusive disease, and commonly precedes pathophysiological occlusion of vessels, leading to ischemic damage to tissues supplied by the blood vessel, such as in cardiovascular disease, the leading cause of mortality globally[1,2]. Atherosclerosis is a complex process that involves the accumulation of plaque within the walls of arteries often preceded by damage to the inner walls of arteries. It can affect multiple arterial beds throughout the body, including the coronary arteries supplying the heart, the carotid arteries supplying the brain, and the peripheral arteries supplying the limbs[3]. The risk factors for the development of atherosclerosis are high blood pressure, smoking, diabetes, and high cholesterol levels, which lead to of the endothelial cell damage [4–7]. Once damaged, they become permeable to lipids and allow them to infiltrate the arterial wall. Low-density lipoproteins (LDL) penetrate the damaged endothelium and accumulate within the arterial wall. These lipids become oxidized and trigger an inflammatory response. Oxidized LDL particles within the arterial wall attract immune cells, particularly monocytes, to the site of injury[8]. Monocytes differentiate into macrophages, which engulf the oxidized LDL particles, forming foam cells. Foam cells are characteristic of early atherosclerotic lesions, known as fatty streaks[9–11].

The accumulation of foam cells, along with smooth muscle cells and extracellular matrix proteins, forms a fibrous plaque within the arterial wall. This plaque can protrude into the lumen of the artery, narrowing its diameter and impeding blood flow.

Additionally, the plaque can become calcified over time, making it harder and more stable[3,12]. Advanced plaques are prone to rupture, exposing their lipid-rich core to the bloodstream, and trigger the formation of blood clots (thrombosis) at the site of rupture. If a clot becomes large enough to block blood flow completely, it can lead to tissue ischemia and result in a heart attack or stroke[13–15].

As cells that play a central role in immunohemostasis, platelets are involved in the initiation, progression, and complications of atherosclerosis through their ability to adhere to damaged endothelium, promote inflammation, interact with lipids, stimulate smooth muscle cell proliferation, and contribute to thrombus formation. When the inner lining of an artery (endothelium) becomes damaged or inflamed due to factors like high blood pressure, smoking, or high cholesterol levels, platelets are among the first responders to the injury. They adhere to the damaged endothelium and become activated[15,16].

Activated platelets release various substances, including thromboxane A2 and platelet-derived growth factor (PDGF), which promote further platelet activation and recruitment. This leads to the formation of platelet aggregates, which can contribute to the formation of a thrombus (blood clot) at the site of the damaged endothelium. Platelets can also interact with low-density lipoprotein (LDL) cholesterol, particularly oxidized LDL. This interaction can further activate platelets and promote their aggregation, exacerbating the formation of atherosclerotic plaques[9,14].

Platelets release inflammatory mediators, such as cytokines and chemokines, which contribute to the recruitment of immune cells (such as monocytes and macrophages) to the site of arterial injury. This sets the stage for the development of

inflammatory plaques characteristic of atherosclerosis. Moreover, platelet-derived growth factors, such as PDGF, stimulate the proliferation of smooth muscle cells within the arterial wall. These smooth muscle cells contribute to the formation of the fibrous cap overlying the atherosclerotic plaque. In advanced stages of atherosclerosis, unstable plaques can rupture, exposing the underlying thrombogenic material to the bloodstream. Activated platelets bind to their ligand, fibrinogen, and play a key role in the formation of thrombi at the site of plaque rupture, which can lead to partial or complete blockage of the affected artery, resulting in myocardial infarction or stroke[14,15,17].

Along with platelets, fibrin(ogen), a plasma protein that binds platelets through the platelet receptors αIIbβ3 and The Triggering Receptor Expressed on Myeloid Cells-like transcript (TLT-1) to form a stable clot, is often elevated in individuals with atherosclerosis risk factors, it potentiates platelet aggregation and thrombus formation, heightening the risk of acute cardiovascular events[17,18]. Beyond its hemostatic functions, fibrinogen serves as a molecular scaffold within the atherosclerotic plaque, modulating leukocyte recruitment, endothelial permeability, and smooth muscle cell migration. Additionally, fibrinogen deposition within the arterial wall promotes the activation of inflammatory pathways and the production of pro-inflammatory cytokines, perpetuating vascular inflammation and plaque vulnerability.

The Triggering Receptor Expressed on Myeloid Cells-like transcript (TLT-1) is a receptor found in platelet α-granules, emerging upon platelet activation. It exists in both membrane-bound and soluble forms (sTLT-1), with the latter enhancing platelet activation and interactions with endothelial cells[19]. Elevated levels of sTLT-1 in plasma signify platelet activation and are correlated with severity and progression of multiple

pathologies such as acute respiratory distress syndrome (ARDS), sepsis and acute coronary syndrome[20–22]. Several studies revealed a link between high sTLT-1 levels and coronary artery disease (CAD). However, its function in chronic inflammatory diseases, like atherosclerosis, remain unclear.

This study identified positive staining for TLT-1 staining in atherosclerotic lesions of patients, compared to controls which were negative for TLT-1, prompting investigation into TLT-1's role in chronic inflammatory diseases. Using a TLT-1/apoe null mouse model, a connection between TLT-1, cardiovascular disease (CVD), obesity, and lipid regulation was established. Despite higher cholesterol levels and inflammatory cytokines, these mice exhibited smaller atherosclerotic lesions in the aorta compared to controls, suggesting TLT-1 as a potential therapeutic target in CVD, validated using a single-chain antibody against human TLT-1

Methods

Mice: Animal care and procedures were performed in accordance with the procedures outlined in Guide for the Care and Use of Laboratory Animals IACUC 1150-1128 at Oakland University, Michigan.

Inducing atherosclerosis

TLT-1 (*treml1*$^{-/-}$) mice were crossed with Apolipoprotein E (*apoe*$^{-/-}$) mice which display reduced platelet aggregation and are more prone to hypercholesterolemia, to create a double null mice (AT-DKO) TLT-1 (*treml1*$^{-/-}$) and Apolipoprotein E (*apoe*$^{-/-}$) double null (AT-DKO) mice. The mice we selected (*treml1*$^{-/-}$ mice, (wt) mice, *apoe*$^{-/-}$ and *apoe*$^{-/-}$/*treml1*$^{-/-}$ (AT-*DKO*) were placed on western diet for 4, 12 and 20 Weeks.

Histology and cytokine analysis

Mice were weighed once a week and food intake monitored weekly. At 20 weeks adipose tissue, livers and hearts were harvested for OsO4, H & E , Macrophage and fibrinogen staining for microscopic analysis. Plasma cytokines were measured by flow cytometry using a multiplex kit (Adiponectin leptin, resistin, TnF-a, IL6, IL8, IL10, angiopoietin-2,C Reactive Protein,CD36,CD2). Plasma samples were also used for a chemistry panel, evaluating ALT, AST, triglycerides, cholesterol, glucose. After overnight fast, blood was collected through the retro- orbital venous plexus and serum was analyzed for total cholesterol (Vitros 250 Chemistry Analyzer, Rochester, NY).

Quantification of aortic sinus lesions

Sections were collected in Poly-L-Lysine slides for Oil Red O and Hematoxilyn staining (Sigma)

Antibody administration

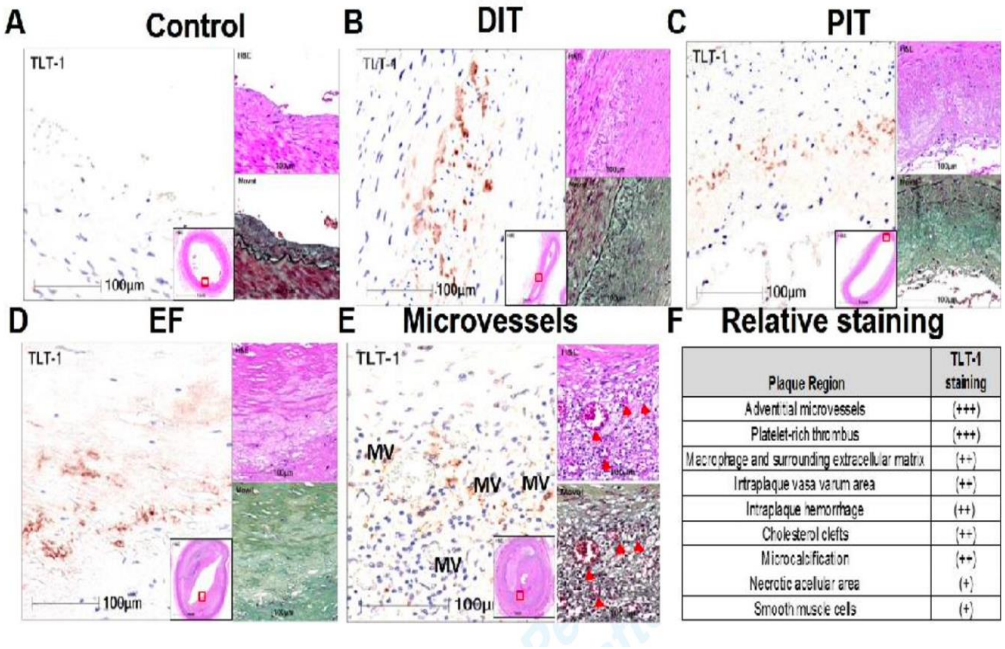
As a comparative analysis to determine whether anti-TLT-1 can potentially be used as a therapeutic intervention for atherosclerosis, (apoe-/-/treml1-/+) mice were fed a western diet for eight weeks while being administered IV anti TLT-1 antibody (C-10)

Results

TLT-1 associates with cardiovascular disease pathology in humans

We investigated TLT-1 expression in human atherosclerotic lesions in diseased and non-diseased arteries using an antibody that detects the extracellular domain of TLT-1. TLT-1 staining was absent in non-diseased coronary arteries (Figure 3.12). However, there was abundant TLT-1 staining seen in diseased vessels. TLT-1 staining is detected as early as the diffused and pathological intima thickening stages ([DIT]-Figure 1B, ;PIT]-1C) and shows even higher staining intensity in the advanced early fibroatheroma (EF)

stage (Figure 1D). Interestingly, relative TLT-1 staining scores revealed that the areas of highest staining are thrombi and around the nascent microvessels within the plaque (Figure 1E, F). The persistence of TLT-1 throughout lesion progression suggest that TLT-1 plays a role from the earliest stages of plaque progression, beginning with the DIT and warranted the investigation to determine if TLT-1 affects lesion progression.



Plaque Region	TLT-1 staining
Adventitial microvessels	(+++)
Platelet-rich thrombus	(+++)
Macrophage and surrounding extracellular matrix	(++)
Intraplaque vasa vasum area	(++)
Intraplaque hemorrhage	(++)
Cholesterol clefts	(++)
Microcalcification	(++)
Necrotic acellular area	(+)
Smooth muscle cells	(+)

Figure 3.12: TLT-1 associates with cardiovascular disease pathology in human. Immunohistochemistry of human coronary arteries. Representative images of TLT-1 staining in defined regions of atherosclerotic plaque, n=3/stage. TLT-1 in (A) control non-diseased artery, (B) diffused intimal thickening (DIT) stage, (C) Pathological Intimal Thickening (PIT). (D) Early fibroatheroma (EF) stage; and staining showing TLT-1 association with microvessels in Late Fibroatheroma. (E) (F) Scoring of relative TLT-1 staining in defined regions of atherosclerotic plaque. H&E and Movat Pentachrome

staining accompany each panel. Original magnification 20x. Red arrows denote microvessels.

Decreased atherosclerosis in the aortic sinus of DKO mice.

To establish a role for TLT-1 in CVD and gain mechanistic insights into TLT-1 function, we crossed our treml1-/- mouse onto the apoe-/- background. Ten-week-old apoe-/- mice have elevated plasma sTLT-1 levels compared to wild-type controls, (Figure 2A; $p \leq 0.01$). To evaluate lesion progression, apoe-/- and apoe-/-/treml1-/- (DKO) mice were fed a western diet (WD) and monitored over a period of 20 weeks. Histological analysis of aortic sinus lesions revealed markedly reduced lesion area in the DKO mice (Figure 3.13 B, C). At four weeks of WD, DKO mice showed a 2.3-fold reduction in lesion size ($p \leq 0.05$) and a 1.8-fold reduction after 12 weeks compared to apoe-/- ($p < 0.01$). After 20 weeks, lesions were only slightly smaller in the DKO mice. These data are consistent with finding TLT-1 staining in human coronary DIT lesions.

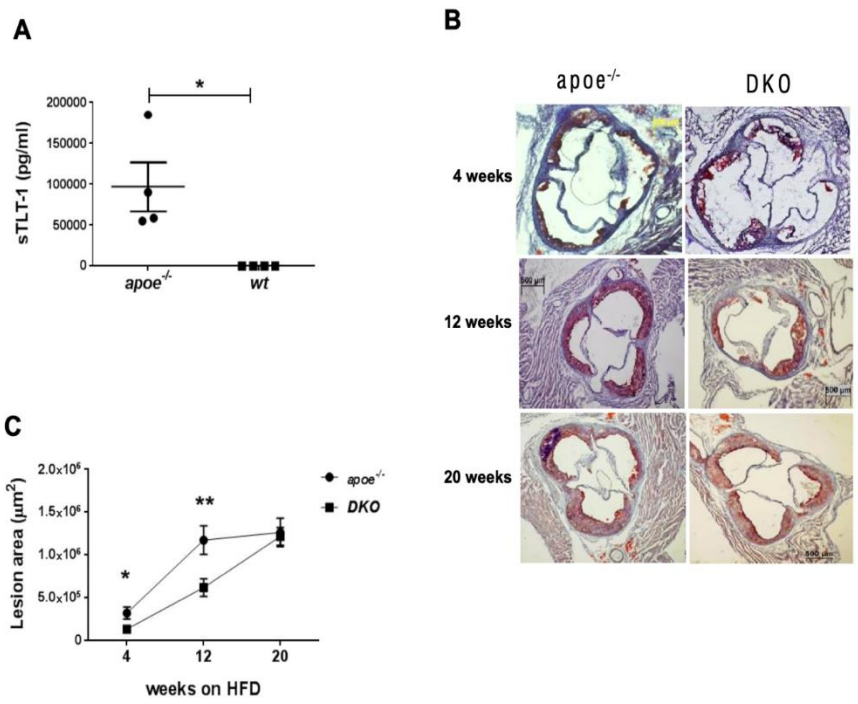


Figure 3.13: TLT-1 deficiency significantly delays atherosclerosis progression in the aortic sinus of apoe-/- mice. (A) Plasma concentration of sTLT-1 was measured by ELISA in 10 weeks old apoe mice and control mice (n=4 per genotype, ***p=0.0001), concentrations represented in pg/ml. (B) Representative pictures of aortic sinus cryosections of mice after WD for the indicated time points (original magnification: 100X). (C) Mean aortic sinus lesion area at indicated time points, n=10 and n=11 for 4 weeks, n=9 and n=10 for 12 weeks, n=11, and n=11 for 20 weeks in apoe$^{-/-}$ and apoe$^{-/-}$ /treml1$^{-/-}$ respectively. Presented are mean ± SEM; **p< 0.01, *p<0.05, student-t test. (D) Serum cholesterol levels from double knockouts and (E) single knockout mice on WD after the indicated time points. Points represent individual cholesterol levels, error bars= means ± SEM **p<0.01. (F) Serum total triglyceride levels after 8 weeks WD, p<0.01.

Plasma cytokine levels are elevated in DKO mice when compared to heterogeneous mice (apoe-/-/treml1-/+)

We questioned why the AT-DKO gave such a pronounced phenotypical difference as compared our controls? To address this question, we compared our AT-DKO to AT hets (heterozygous for *treml1* (apoe$^{-/-}$/*treml1*$^{+/-}$)). Mice were fed high fat diet for 20 weeks. Overall AT-DKO mice gained more weight compared to AT-hets (12.94±1.90 vs 8.51±1.70 grams p=0.02). Plasma analysis demonstrates that the AT-DKO have significantly higher levels of TNF-a (0.54±0.60 vs 0.118±0.17 pg/ml p=0.03), and IL-10 (2.50±1.40 vs 1.50±2.10 pg/ml *p*=0.004) compared to littermate controls. Histological analysis of livers illustrates increased lipid vacuoles and inflammatory foci in the AT-DKO mice as compared to controls, while preliminary data is not significant for these differences, liver damage in the AT-DKO was significantly greater as demonstrated by increased AST levels (166.21±91.00 vs 102±68.10 U/L p=0.02).

Moreover, the AT-DKO mice had higher levels of ALT, direct bilirubin, cholesterol, PAI-1, triglycerides and lower IL-6 and adiponectin however these differences were not significant (Table 1). While the comparison of the AT-DKO to AT hets demonstrated the same trends as seen in the comparisons with apoe-/-, the differences were not as pronounced in the AT-het suggesting a possible gene dose affect. serum triglycerides in both DKO and treml1-/- mice compared to their respective controls ($p<0.01$,data not shown), no significant difference was found in HDL-c or LDL-c (data not shown). It is clear that decreased lesion progression in the DKO mice is not derived from the differences in serum cholesterol.

TLT-1 does not mediate changes in macrophage content within the lesion.

CHARACTERISTIC	AT-DKO MICE	AT-HET MICE	P VALUES
NUMBER	22 (9M, 13F)	15 (7M, 8F)	
AGE	20 WEEKS	20 WEEKS	
AVERAGE WEIGHT GAIN	12.94 +/- 1.90 grams	8.51+/-1.70 grams	0.02**
CYTOKINE ANALYSIS	**pg/ml**	**pg/ml**	
IL-10	2.50+/-1.40	1.50+/-2.10	0.004***
TNF-a	0.54+/-0.60	0.118+/-0.17	0.03**
IL-6	7.80+/-10.70	12.40+/-13.40	0.4936
CHEMISTRY PANEL	**pg/ml**	**pg/ml**	
CHOLESTEROL	789.70+/-412.90	728.00+/-237.40	0.588
TRIAGLYCERIDES	183.10+/-70.80	153.00+/-69.20	0.473
AST	166.21+/-91.00	102.00+/-68.10	0.02**
ALT	51.11+/-49.85	33.00+/-23.50	0.209

Table 3.1:*Plasma cytokine and chemical panel of Obese Mice*. Levels of IL-10 and AST are significantly higher in the double knock out mice.

Lesion progression in the DKO mice is independent of cholesterol levels.

At 20 weeks of western diet, the difference in serum cholesterol levels slightly higher in DKO mice but was not significant (Table 3.1). Previous parallel studies in the laboratory using the treml1-/- and WT mice further implicated that these cholesterol differences were a direct consequence of the treml1 mutation. We found that treml1-/- mice had significantly increased serum cholesterol levels after four weeks of WD compared to WT (mean ± SEM=125.0 ± 3.2 mg/dl, n=11 vs 102.0 ± 9.9 mg/dl, n= 12; $p \leq 0.05$, data not shown). These differences remained after 12 weeks of WD, although not statistically

significant. After 20 weeks of WD, blood cholesterol levels were similar in both treml1$^{-/-}$ and WT mice.

To dissect the mechanism by which TLT-1 accelerates atheroma formation, we investigated macrophage content in the aortic sinus by immunofluorescence microscopy. There was no significant difference between groups, although there was a clear trend toward higher macrophage positive area at the 20week time point in the DKO mice (Figure 3.14A and 3.14B) This data suggests that TLT-1 does not modulate macrophage content within the lesion.

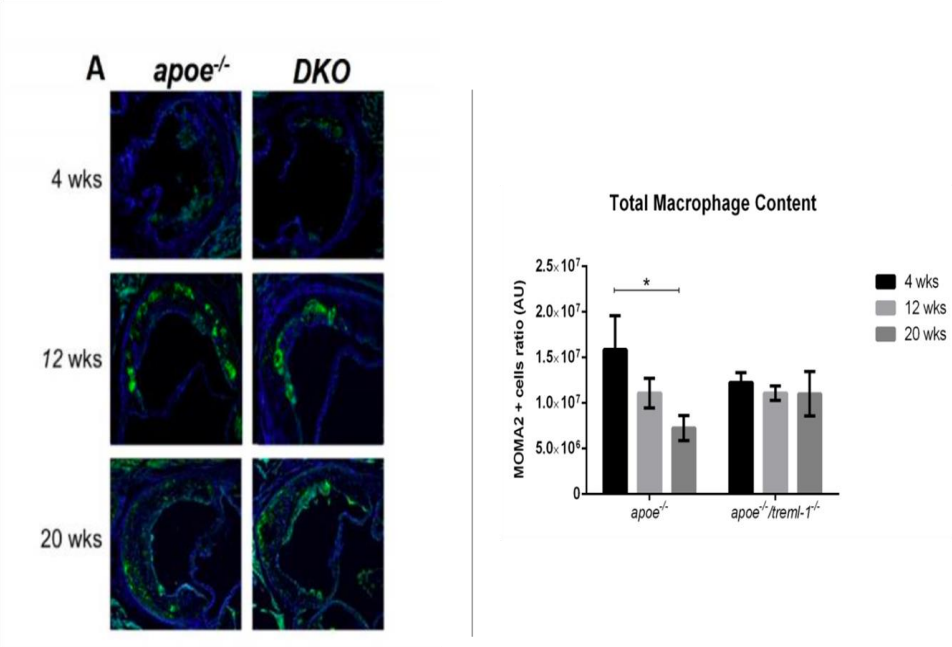


Figure 3.14: Macrophage infiltration during atherosclerosis in the thoracic aorta. (A) Representative images of macrophage content in the aortic sinus (original magnification: 10x)

TLT-1 regulates fibrinogen deposition within the lesion

To determine whether there was a difference in fibrinogen deposition within the atherosclerotic lesions we stained lesions for fibrinogen after 12 weeks of western diet. The apoe$^{-/-}$ mice had significantly higher levels of fibrinogen deposition than the DKO mice, suggesting that TLT-1 mediates fibrinogen deposition within the atherosclerotic lesion.

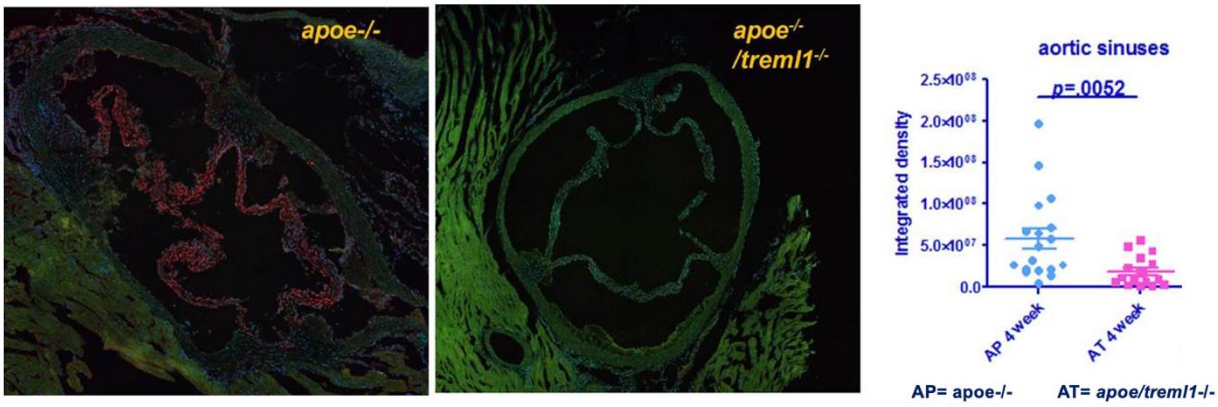


Figure 3.15: Fibrinogen staining in atherosclerotic lesions in apoe-/- mice (AP) compared to apoe-/-/treml1-/-. Apoe-/- mice have significantly higher (p=0.0052) levels of fibrinogen deposition than apoe-/-/treml1-/- mice.

Discussion

Cardiovascular disease (CVD) is the leading cause of mortality globally with one of the main precipitating conditions being atherosclerotic occlusion of the coronary vessels. Atherosclerosis can lead to acute coronary syndrome, and ischemic damage to the heart. Currently, in a clinical setting statin (to reduce cholesterol), antiplatelet medications (to reduce platelet activation) and fibrinolytic therapies (to break up clot post onset of symptoms of acute coronary syndrome) are used as treatment of choice for

acute coronary syndrome. However, antiplatelet drugs are taken as a preventative measure for an indefinite or prolonged period, they mainly target αIIbβ3 and may lead to bleeding complications, which can be life threatening. While fibrinolytic agents are affective for reperfusion of a vessel, however, this is within a short window post onset of symptoms, preferably within 3-6 hours , and anyone presenting to the hospital after that time frame may not have benefits from this therapy. With advancements in biomedical sciences there is still a need to find alternative therapeutic options that will improve quality of life for patients with CVD.

In this study we introduce the potential for targeting an alternative platelet receptor, TLT-1, which like αIIbβ3 binding fibrinogen and facilitates platelet aggregation, however, the genetically modified knock out mouse model does not have a severe bleeding phenotype like αIIbβ3 knock out mouse, suggesting it would be a good alternative therapeutic target as an antiplatelet therapy.

The findings of this study shed light on the role of Triggering Receptor Expressed on Myeloid Cells-like transcript (TLT-1) in CVD pathology, particularly in atherosclerosis. The investigation began with an examination of TLT-1 expression in human atherosclerotic lesions, revealing abundant TLT-1 staining in diseased vessels, starting from the early stages of plaque development. Notably, TLT-1 staining was particularly prominent in areas associated with thrombi and growing micro vessels within the plaque, indicating its involvement throughout lesion progression.

To better understand TLT-1's impact on CVD, a TLT-1/apoe null mouse model was developed. These mice exhibited reduced atherosclerotic lesion size in the aortic sinus compared to apoe-/- controls, despite elevated plasma sTLT-1 levels. This reduction in lesion size was observed early during lesion progression. Interestingly, this decrease in lesion progression in the TLT-1/apoe null mice was not attributed to differences in serum cholesterol levels, as both DKO and treml1-/- mice had similar cholesterol levels compared to their respective controls.

We then compared plasma cytokines in a DKO compared to heterozygous mice, analysis demonstrated elevated levels of TNF-α and IL-10 in DKO mice compared to heterozygous controls, accompanied by increased weight gain and liver damage. However, the differences in cytokine levels between DKO and heterozygous mice were less pronounced, suggesting a possible gene dosage effect.

Interestingly, while there was no significant difference in macrophage content within the lesions between the groups, although a trend towards higher macrophage positivity was observed in DKO mice at later time points. This suggests that the mechanism underlying the TLT-1 regulation of atherosclerotic progression is not macrophage dependent. However, interestingly, in support of previous studies, TLT-1 was found to regulate fibrinogen deposition within atherosclerotic lesions, as evidenced by significantly lower levels of fibrinogen deposition in DKO mice compared to apoe-/- controls.

Overall, these findings highlight the critical role of TLT-1 in atherosclerosis progression and suggest its potential as a therapeutic target in CVD. The study provides valuable insights into the mechanisms underlying TLT-1-mediated effects on lesion development. The mechanism seems to be independent of macrophages or changes in cholesterol levels but rather, due to the direct interaction of TLT-1 with fibrinogen, where the absence of TLT-1 leads to a reduction in fibrinogen deposition within the atherosclerotic lesion. These results underscore the importance of further elucidating the molecular pathways involved in CVD pathogenesis. We are currently testing an anti TLT-1 antibody as a potential therapeutic target to decrease atherosclerotic lesion progression and will report our results in future work.

Bibliography

1. Herrington, W., Lacey, B., Sherliker, P., Armitage, J. & Lewington, S. Epidemiology of Atherosclerosis and the Potential to Reduce the Global Burden of Atherothrombotic Disease. *Circ Res* 118, 535–546 (2016).

2. Moran, A. E. *et al.* The global burden of ischemic heart disease in 1990 and 2010: The global burden of disease 2010 study. *Circulation* 129, 1493–1501 (2014).

3. Jebari-Benslaiman, S. *et al.* Pathophysiology of Atherosclerosis. *Int J Mol Sci* 23, (2022).

4. Third Report of the National Cholesterol Education Program (NCEP) Expert ... - National Cholesterol Education Program (U.S.). Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults - Google Books. https://books.google.com/books?hl=en&lr=&id=VKpLAQAAIAAJ&oi=fnd&pg=PA1&dq=+The+Expert+Panel.+Third+Report+of+the+National+Cholesterol+Education+Program+(NCEP)+Expert+Panel+on+Detection,+Evaluation,+and+Treatment+of+High+Blood+Cholesterol+in+Adults+(Adult+Treatment+Panel+III).+Final+report.+Circulation.+2002%3B106:+3143%E2%80%933421.&ots=jXjp-pJFiu&sig=GpWVG_FfffCJ7bMjpRMYEysRTBs#v=onepage&q&f=false.

5. Van Der Meer, I. M. *et al.* Inflammatory mediators and cell adhesion molecules as indicators of severity of atherosclerosis: The Rotterdam Study. *Arterioscler Thromb Vasc Biol* 22, 838–842 (2002).

6. Fruchart, J. C., Nierman, M. C., Stroes, E. S. G., Kastelein, J. J. P. & Duriez, P. New Risk Factors for Atherosclerosis and Patient Risk Assessment. *Circulation* 109, (2004).

7. Korkmaz, Ş. A. & Kızgın, S. Neutrophil/high-density lipoprotein cholesterol (HDL), monocyte/HDL and platelet/HDL ratios are increased in acute mania as markers of inflammation, even after controlling for confounding factors. *Curr Med Res Opin* 39, 1383–1390 (2023).

8. Berliner, J. A. & Watson, A. D. A role for oxidized phospholipids in atherosclerosis. *N. Engl. J. Med.* 353, 9–11 (2005).

9. Shashkin, P., Dragulev, B. & Ley, K. Macrophage Differentiation to Foam Cells. *Curr Pharm Des* 11, 3061–3072 (2005).

10. Bouhlel, M. A. *et al.* PPARg activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab.* 6, 137–143 (2007).

11. Libby, P. Molecular and cellular mechanisms of the thrombotic complication of atherosclerosis. *J. Lipid Res.* 50, S352-s357 (2009).

12. Libby, P., Ridker, P. M. & Hansson, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature 2011 473:7347* 473, 317–325 (2011).

13. Bentzon, J. F., Otsuka, F., Virmani, R. & Falk, E. Mechanisms of plaque formation and rupture. *Circ Res* 114, 1852–1866 (2014).

14. Gawaz, M. Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. *Cardiovasc Res* 61, 498–511 (2004).

15. Lindemann, S., Krämer, B., Seizer, P. & Gawaz, M. Platelets, inflammation and atherosclerosis. *Journal of Thrombosis and Haemostasis* 5, 203–211 (2007).

16. Gawaz, M., Langer, H. & May, A. E. Platelets in inflammation and atherogenesis. *Journal of Clinical Investigation* 115, 3378–3384 (2005).

17. Cerit, L. Fibrinogen and Atherosclerosis. *Arq Bras Cardiol* 108, 189 (2017).

18. Jiang, P. *et al.* Relationship between fibrinogen levels and cardiovascular events in patients receiving percutaneous coronary intervention: A large single-center study. *Chin Med J (Engl)* 132, 914–921 (2019).

19. Washington, A. V. *et al.* A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 104, 1042–1047 (2004).

20. Morales-Ortíz, J. *et al.* Platelet-derived TLT-1 is a prognostic indicator in ALI/ARDS and prevents tissue damage in the lungs in a mouse model. *Blood* (2018) doi:10.1182/blood-2018-03-841593.

21. Gibot, S. *et al.* A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *Journal of Experimental Medicine* (2004) doi:10.1084/jem.20040708.

22. Esponda, O. L., Hunter, R., Río, J. R. R. Del & Washington, A. V. Levels of soluble TREM-Like transcript 1 in patients presenting to the emergency department with chest pain. *Clinical and Applied Thrombosis/Hemostasis* 21, 30–34 (2015).

CHAPTER 4

CHARACTERIZING PLATELET FUNCTION IN PATHOPHYSIOLOGICAL MODELS

Platelet receptors as biomarkers for disease

P-selectin and Trem-like transcript 1 (TLT-1) are two important markers of platelet activation that have garnered significant attention in the field of biomarker research. These molecules are involved in various aspects of platelet activation and play crucial roles in thrombosis, inflammation, and vascular pathology. Their detection in circulation serves as indicators of platelet activation and may have diagnostic and prognostic implications in various clinical settings.

P-selectin is a cell adhesion molecule stored in alpha granules of resting platelets and endothelial cells. Upon platelet activation, P-selectin is rapidly translocated to the cell surface, where it mediates platelet-leukocyte interactions and facilitates platelet aggregation. Additionally, P-selectin plays a role in the recruitment of leukocytes to sites of inflammation and thrombosis, further amplifying the inflammatory response.

The measurement of soluble P-selectin levels in plasma or serum serves as a valuable biomarker of platelet activation. Elevated levels of soluble P-selectin have been associated with various cardiovascular diseases, including acute coronary syndromes, stroke, and peripheral artery disease. Monitoring P-selectin levels may aid in risk

stratification, prognosis assessment, and monitoring therapeutic efficacy in these conditions.

TLT-1 is a transmembrane glycoprotein expressed on platelets, megakaryocytes, and endothelial cells. Recent studies have identified TLT-1 as a novel platelet receptor involved in fibrinogen binding and platelet activation. TLT-1 interacts with the γ-chain of fibrinogen, facilitating platelet aggregation and thrombus formation.

Similar to P-selectin, TLT-1 has emerged as a promising biomarker of platelet activation and thrombotic risk. Elevated TLT-1 levels have been observed in patients with acute coronary syndromes, ischemic stroke, and venous thromboembolism, suggesting its potential utility as a diagnostic and prognostic marker in these conditions.

Both P-selectin and TLT-1 hold promise as biomarkers of platelet activation in various clinical scenarios. Their measurement in blood samples provides valuable insights into the degree of platelet activation and may help guide therapeutic decision-making in patients with cardiovascular diseases, thrombotic disorders, and inflammatory conditions.

Furthermore, the combination of P-selectin and TLT-1 with other biomarkers, imaging modalities, or clinical risk factors may enhance their diagnostic and prognostic value, enabling more accurate risk stratification and personalized management strategies.

P-selectin and TLT-1 represent important markers of platelet activation with potential clinical utility as biomarkers in various disease states. Further research is warranted to validate their diagnostic and prognostic value and elucidate their role in the pathophysiology of thrombosis, inflammation, and vascular disease.

Abstract

Leukemia, contributing to 3% of all cancer cases in the United States, presents a significant challenge in treatment due to its diverse subtypes, each displaying unique genetic and molecular abnormalities. The aggressive growth of abnormal blood cells compromises immune function and poses challenges in developing effective therapeutic approaches. Unlike solid tumors, surgical resection is not an option, limiting treatment strategies to chemotherapy, immunotherapy, and radiation therapy, which may be nonspecific and lead to organ damage and toxicity.

Anthracyclines, widely used in hematological tumors, face limitations due to severe cardiotoxicity, causing progressive cardiomyopathy and, in severe cases, congestive heart failure. Heart failure incidence is significantly higher in cancer patients treated with anthracyclines for more than 5 years. Early detection of left ventricular dysfunction and cardiotoxicity is crucial to mitigate progression to congestive heart failure (CHF) in this population.

There are currently no standardized methods for early detection of CHF in anthracycline induced cardiotoxicity. Using echocardiography, Troponin-I (Trop-I), and NT-proBNP for early congestive heart failure (CHF) diagnosis presents challenges due to limited specificity and high costs.

Recent research by Bayrón-Marrero et al. reveals that lower levels of plasma soluble TREM-Like Transcript-1 (s-TLT-1), a platelet-specific receptor, correlate with left ventricular dysfunction in cardiovascular disease patients. Those with s-TLT-1 levels exceeding 544 pg/mL have a significantly higher risk of congestive heart failure. These findings suggest the potential use of s-TLT-1 as a diagnostic screening tool for early

detection and treatment of anthracycline-induced CHF in leukemia patients. This review introduces the potential for a combined diagnostic strategy, using markers of myocardial damage (Troponin-1), s-TLT-1 and echocardiography (where available/affordable to patient), depending in geographic location and access to healthcare.
MeSH/Keywords: keyword triggering receptor expressed in myeloid cells-like transcript (TLT-1); blood platelets; cardiovascular diseases; left ventricular ejection fraction (LVEF); biomarker; anthracyclines, anthracycline induced cardiotoxicity, leukemia, Troponin I, cardiotoxicity, congestive heart failure (CHF), cardiomyopathies, Echocardiography, Heart Failure, Biomarkers, Coronary artery disease, Cardiovascular disease, Platelet receptors.

Challenges posed by a hematological cancer

Leukemia stands as a prevalent hematologic malignancy, casting a shadow on the lives of thousands globally. The National Cancer Institute predicts that there will be an estimated 59610 new cases of leukemia and 23710 estimated deaths in 2023[1]. Leukemia poses a formidable challenge for treatment due to its complex nature and the heterogeneity of the disease. Its various subtypes, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL), exhibit diverse genetic and molecular abnormalities. This diversity makes it challenging to develop a one-size-fits-all therapeutic approach. The aggressive growth of abnormal blood cells interferes with normal blood cell production, leading to compromised immune function and anemia.

Furthermore, leukemia cells can infiltrate other organs, complicating treatment strategies, and unlike solid tumor cancers, surgical resection of the tumor is not an option,

limiting strategies to chemotherapy, immunotherapy, and radiation therapy, which are sometimes nonspecific, leading to organ damage and toxicity. The etiology of leukemia is multifaceted, often involving genetic mutations, exposure to certain environmental factors, and a combination of genetic predisposition.

At its core, leukemia disrupts the hematopoietic processes. The pathophysiology of this malignancy unfolds within the bone marrow, where abnormal hematopoietic stem cells undergo unregulated differentiation, leading to an overproduction of immature and dysfunctional white blood cells[1–4]. This aberrant cellular proliferation infiltrates the bloodstream, compromising the immune system function and giving rise to a cascade of detrimental consequences [2,3]. The disease manifests in various forms, with acute and chronic subtypes, and can affect the myeloid and lymphoid cell linages, each presenting its own set of challenges in diagnosis and treatment.

Despite advances in medical research and therapeutic interventions, the blood born nature of leukemia remains a challenge, necessitating continuous efforts to unravel its intricacies and develop more effective treatment strategies.

On a molecular level, leukemia often involves mutations in key genes responsible for regulating cell cycle progression, apoptosis, and DNA repair[4–7]. These genetic aberrations disrupt the balance that ensures the controlled division and differentiation of blood cells of the myeloid or lymphoid stem line. The resultant accumulation of malignant cells not only impairs the production of healthy blood cells but also leads to the infiltration of leukemic cells into vital organs, further exacerbating the disease's impact on overall health.

From a clinical standpoint, leukemia manifests as a relentless battle for those affected. The compromised immune system leaves patients vulnerable to infections, anemia, and bleeding disorders[8–14]. Despite advancements in therapeutic approaches, the intricate interplay between genetic mutations and the dysregulation of hematopoietic processes continues to pose challenges in selecting an optimum treatment strategy and achieving long-term remission.

Leukemia treatment strategies and their complications

The goal of leukemia treatment is remission and ultimately cure. Complete remission is defined as the patient's bone marrow being free of malignant cells, which can be confirmed microscopically, post biopsy. In the management of leukemia, therapeutic interventions encompass a spectrum of modalities, including chemotherapy, targeted therapy, immunotherapy, radiation, and stem cell transplantation, each, with distinct advantages and limitations.

Chemotherapy, the primary strategy in leukemia treatment, exerts its efficacy through a systemic approach, facilitating its widespread impact on leukemia cells within diverse anatomical compartments.

Often used as a combination of multiple drugs with different mechanisms of actions, its cytotoxic mechanism disrupts the cell cycle, impeding the rapid division of malignant cells. While chemotherapy's versatility renders it applicable across various leukemia subtypes, its non-selectivity induces collateral damage to normal, rapidly dividing cells, culminating in adverse effects ranging from mild, such as nausea and fatigue, to severe such as tumor lysis syndrome, cardiotoxicity, suppressed fertility, and myelosuppression (suppression of bone marrow leading to anemia, thrombocytopenia and

neutropenia, and susceptibility to hemorrhage and infection), with the major causes of treatment related mortality being hemorrhage and infection[15–20].

Chemotherapy can either be used as a stand-alone therapy or in combination with other treatments such as targeted and radiation therapy, or as an adjuvant therapy, to shrink a tumor before surgical intervention.

In contrast, targeted therapies offer a precision-oriented paradigm (monoclonal antibodies to receptors on cancerous cells that either mark cancer cells for immune recognition, carry toxins to target cells or have cytostatic effects), selectively addressing leukemia cells harboring specific molecular or genetic aberrations, thereby mitigating off-target effects. Nonetheless, limitations include the potential development of resistance and applicability restricted to certain genetic subtypes[21–24].

Immunotherapy, an evolving frontier, capitalizes on the immune system's intrinsic capacity to recognize and eliminate leukemia cells. While immunotherapy produces sustained responses, in some cases, its variable efficacy and potential for immune-related adverse events necessitate careful consideration. An example of immunotherapy is Chimeric Antigen Receptor CAR-T cell therapy. These cells are taken from the patient or a donor and genetically modified to recognize cancer cell antigens[25]. Currently, CAR-T cell therapy has FDA approval for B-cell acute lymphoblastic leukemia, in both pediatrics and adults. However, despite the specificity, CAR-T cells have been shown to cause multiple adverse effects, including cytokine release syndrome, allergic reactions and dyspnea[26–33]. Moreover, recent data has shown that CAR-T cell therapy has been linked to secondary malignancy development, emphasizing the need for both regulation

and further investigation before determining that the benefits outweigh the risks in this cohort of patients.

Radiation therapy is used in combined treatment strategies, usually alongside chemotherapy or before a bone marrow transplantation, to destroy leukemia cells, alleviate discomfort caused by enlarged liver, spleen or lymph nodes or treat pain from bone damage. Total body irradiation (TBI) is usually the choice prechemotherapy or stem cell transplantation (suppresses the immune system to reduce probability of donor stem cell rejection). External bean radiation therapy (EBRT) is often used to reduce swelling in the liver, spleen or lymph nodes, while total bone marrow irradiation (TMI) is used for precise delivery of radiation to the bone marrow, commonly used for patients undergoing stem cell transplantation, however, radiation has not been shown to be effective as an individual treatment modality. Some of the side effects of radiation therapy include nausea, vomiting, diarrhea, and lowered blood cell counts[34].

Stem cell transplantation remains the optimum treatment for leukemias, as replenishment of bone marrow stem cells with healthy cells would result in remission or cure. However, it is a challenge to find a donor and even with a donor, allogeneic transplantation can lead to graft-versus-host disease (GVHD), which can range from mild to life threatening[35]. In delineating the treatment landscape, the distinct attributes of each modality prompt an individualized approach, often involving combination therapies to optimize therapeutic outcomes while mitigating associated drawbacks. The treatment of choice is determined by overall health of the patient, possible metastasis of the cancer, age, and subtype of leukemia. Due to the frequency of complications with current treatment strategies and the immunosuppressive effects of therapeutic options, it is

important for ongoing research to seek to refine existing strategies and develop diagnostic approaches for early detection of treatment complications to prevent mortality due to secondary causes.

Leukemia and Cardiovascular health

Leukemia can specifically affect cardiovascular health in several ways. Firstly, leukemia can lead to anemia due to the overcrowding of the bone marrow by abnormal white blood cells, which can result in fewer healthy red blood cells. Anemia reduces the blood's oxygen-carrying capacity, leading to symptoms such as fatigue, weakness, and shortness of breath, ultimately affecting the cardiovascular system's efficiency[36,37]. Secondly, leukemia can disrupt the normal function of platelets, which are essential for blood clotting. This disruption can increase the risk of bleeding complications such as hemorrhage, as well as thrombotic events like stroke or heart attack[38,39]. Additionally, the aggressive treatments used to combat leukemia, such as chemotherapy and radiation therapy, can have cardiotoxic effects, potentially leading to cardiomyopathy, arrhythmias, or heart failure.

Screening for cardiovascular health in leukemia patients holds unique benefits compared to the general population due to their increased susceptibility to cardiovascular complications. Regular monitoring of cardiovascular health parameters, such as blood pressure, lipid levels, and cardiac function, allows for the early detection and management of potential cardiovascular issues in leukemia patients. Furthermore, screening can help identify underlying risk factors exacerbated by leukemia or its treatments, enabling healthcare providers to implement preventive strategies tailored to the individual patient's needs. Given the heightened cardiovascular risks associated with

leukemia and its treatments, proactive screening not only improves overall health outcomes but also enhances the safety and efficacy of leukemia treatment regimens. Therefore, personalized cardiovascular screening plays a crucial role in optimizing the care and well-being of leukemia patients, addressing their specific cardiovascular health challenges more effectively than general population screening approaches.

Anthracycline - induced cardiotoxicity

Anthracyclines, also known as ANTs, and including doxorubicin and daunorubicin are a class of drugs that are developed from *Streptomyces species* to treat many types of cancers, including leukemias. The structure of anthracyclines contains a planar anthracyclinone core with substituents of quinones, phenolic groups, and other groups. Of the four rings in this structure, one is saturated. While these molecules are water-insoluble, water solubility can be provided by mono- or multisaccharide substitution in the saturated ring. This substitution creates not only water solubility, but also stability for future DNA binding[40].

These chemotherapy drugs have become widely used since their discovery fifty years ago and have drastically reduced the mortality rate of cancer for patients, becoming one of the leading treatment options for several different cancers. This class of drugs work in several different ways including their interaction with topoisomerase-II, inhibition of DNA and RNA synthesis, the formation of free radicals, and the formation of adducts with DNA to stop the replication[41–44] .

Topoisomerase-II is an enzyme that is critical to the maintenance of DNA. This enzyme uses the hydrolysis of ATP to cut both strands of the DNA helix to wind and unwind DNA during replication and transcription. ANTs interact with Topoisomerase-II

to form a complex that prevents the re-ligation of the cuts in the double stranded DNA. In this way, ANTs inhibit cell growth and promote cell death. Another way that ANTs inhibit DNA and RNA synthesis is by intercalating DNA, thereby inhibiting DNA and RNA synthesis. Finally, ANTs have been found to combine with DNA to form adducts, which induce cell death by blocking transcription factors (Figure 4.1). In addition to these functions that prohibit DNA and RNA synthesis, ANTs are also involved in the formation of free radicals and Reactive Oxygen Species (ROS), which can damage lipids, proteins, and nucleic acids. With this damage, cell death is induced[41,42,45,46].

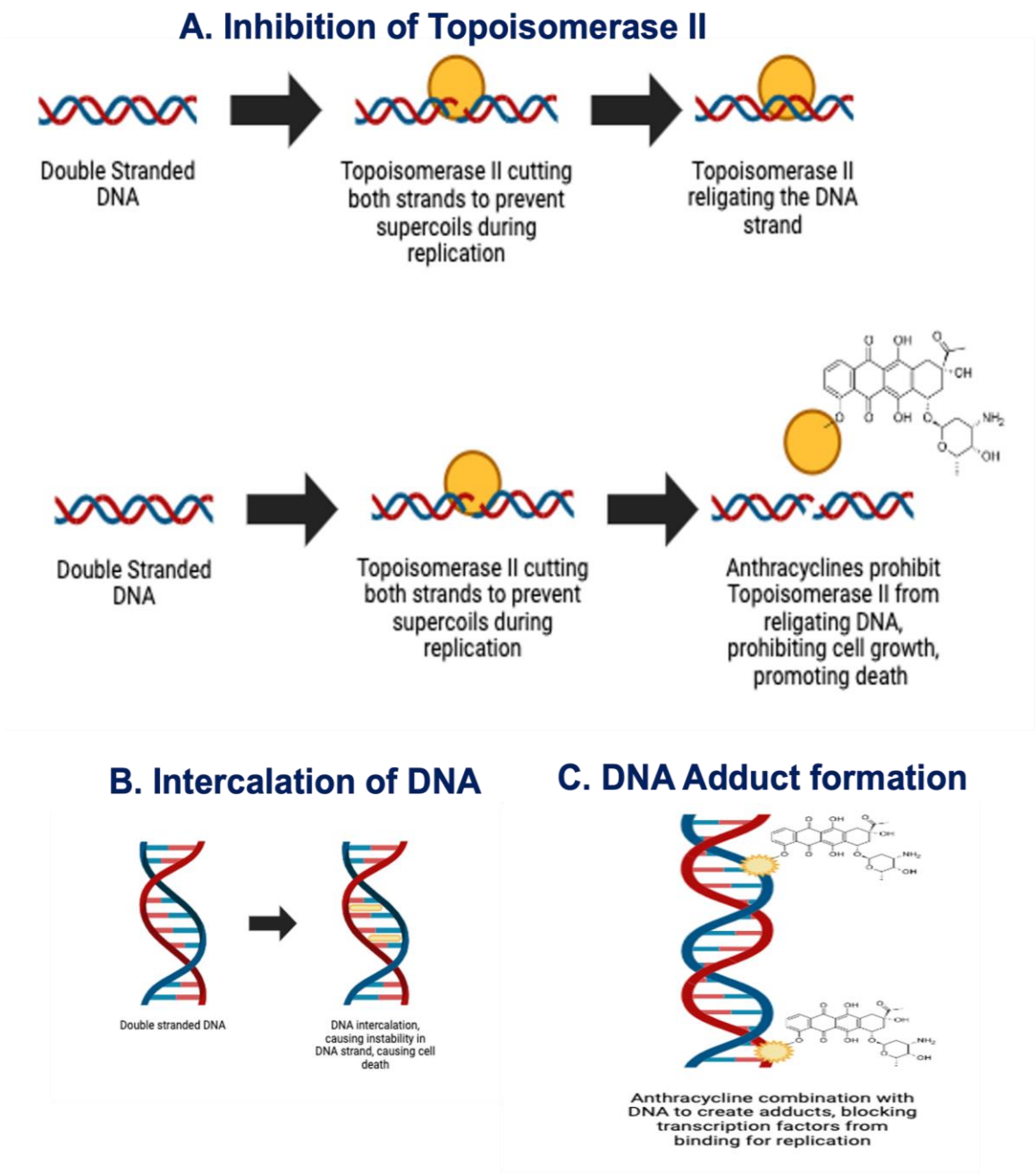


Figure 4.1: Anthracycline induced cardiotoxicity: mechanism of action. “Created with “Biorender”

This multifaceted cytotoxicity targets rapidly dividing cancer cells, including those in the bone marrow, making anthracyclines particularly effective against the proliferative nature of leukemia. Their role extends beyond induction therapy, as anthracyclines are integral components of consolidation and maintenance regimens, contributing to enhanced outcomes. Despite their efficacy, anthracycline use is tempered by the risk of cardiotoxicity, emphasizing the necessity for vigilant monitoring and judicious dosing. Ongoing research seeks to refine treatment protocols, minimize adverse effects, and uncover strategies to optimize the therapeutic benefit of anthracyclines in leukemia management.

Chemical agents that induce cardiotoxicity are classified as either Type-I or Type-II agents. Type-I agents are those that cause irreversible cardiac damage and are dose dependent while Type-II agents are those that cause reversible cardiac damage and are not dose dependent. Patients may develop a variety of cardiac conditions such as arrhythmia, ischemia, pericarditis, systolic dysfunction, diastolic dysfunction, and congestive heart failure. Anthracyclines are considered a Type-I agent, thus limiting their usage as a treatment for cancer due to their high correlation with these adverse cardiac effects. Currently, there are thought to be 2 million individuals in the United States that are at risk for delayed anthracycline toxicity. With this in mind, it is imperative to understand the mechanism by which ANT's act to reduce the cardiotoxicity risk for patients[47]

Anthracycline-induced cardiotoxicity can be defined as acute (after one dose or a single course presenting within 14 days), early onset chronic (presenting within a year), or late onset chronic (more than a year) with the latter two being irreversible[48]. The

irreversibility of cardiomyocyte damage is a recognized concern in the realm of cancer treatment, particularly following the administration of anthracycline chemotherapy for leukemia and other malignancies. This cardiotoxicity stems from a complex interplay of mechanisms, including the generation of free radicals, mitochondrial dysfunction, cardiomyocyte apoptosis, inflammatory responses, disruption of calcium homeostasis, and the promotion of fibrosis and scarring. Anthracyclines' capacity to induce oxidative stress and trigger apoptotic pathways in cardiomyocytes contributes to structural and functional changes in the heart. Furthermore, these drugs may impact blood vessels, causing endothelial dysfunction and compromising overall cardiac function. Anthracycline toxicity may also present up to 20 years after exposure to anthracyclines, complicating its prevention and diagnosis[40].

CHF as a manifestation of anthracycline induced cardiotoxicity

Congestive heart failure, also referred to as CHF, is a cardiac syndrome in which the affected individual has insufficient blood supply to the body because of inefficient myocardial performance. This syndrome can be a result of a variety of conditions but is characterized by decreased ventricular filling or ventricular ejection to circulation. CHF significantly decreases the quality of life of those that are affected by it and increases the risk of cardiovascular associated mortality[49].

The incidence of anthracycline induced cardiotoxicity is dose dependent, ranging between 3-5% with 400mg/m^2, but can be as high as 18-48% patients who receive high dose regimens of anthracyclines (700 mg/m^2)[50,51]. Studies suggest that the potency of these drugs leads to cardiac injury at first drug administration, as supported by increased troponin release from myocardiocytes. While not yet fully understood, the

pathophysiological progression of anthracycline toxicity is preceded by cardiomyocyte injury that eventually manifests as decrease in left ventricular ejection fraction (LVEF), and if not diagnosed and treated on time can eventually lead to congestive heart failure (Figure 4.2).

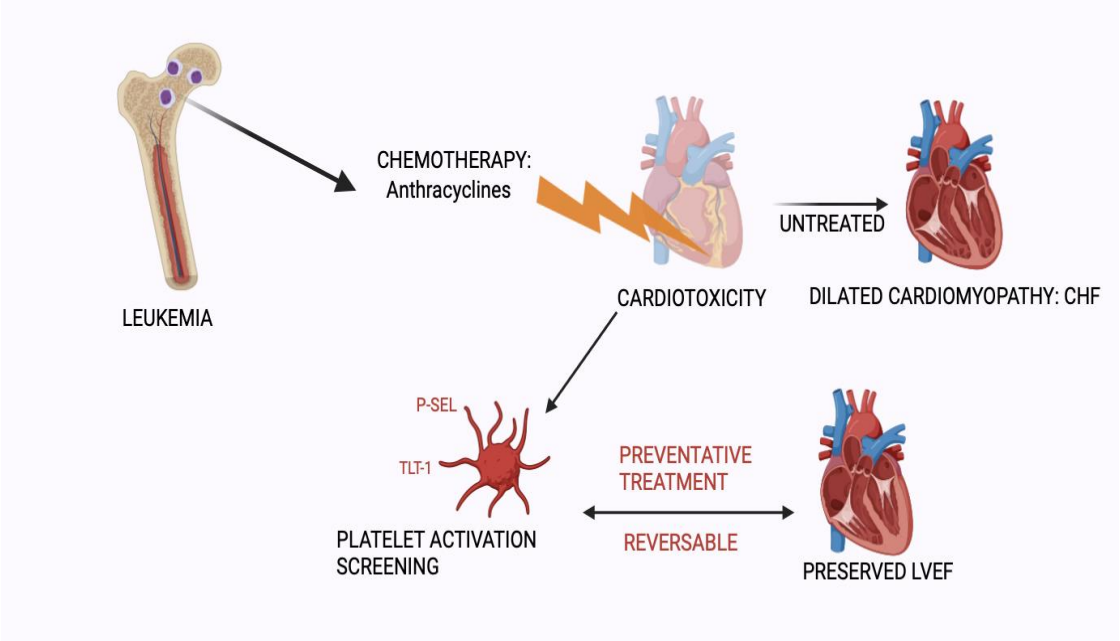


Figure 4.2: Schematic representation of anthracycline induced cardiotoxicity. Early screening for cardiomyopathy would allow for preventative treatment and preservation of left ventricular function. "Created with Biorender."

Patients usually present with a dilated, hypokinetic cardiomyopathy, with a decreased LVEF. The diagnostic criterion has been outlined as a decline in LVEF >10% points, with a final value<53%, however by the time patients present with these changes in cardiac function indexes in a chronic setting, the dilated cardiomyopathy is irreversible, and patients progress to congestive heart failure[52]. Moreover, the American society of clinical oncology does not recommend monitoring for cardiac toxicity in low-

risk patients, mainly due to burden of cost on patients, thus early progression may go unnoticed.

Diagnosis of anthracycline induced cardiotoxicity

Early detection is paramount to managing the risk and addressing complications associated with anthracycline-induced cardiotoxicity in clinical settings. This can be achieved by screening through cost efficient methods to determine which patients need vigilant monitoring, cardiac imaging (including ECG monitoring, and echocardiogram) and potential cardioprotective interventions. Previous studies have illustrated that Troponin I, being the gold standard for detecting cardiomyocyte injury would be a highly sensitive tool for determining whether there is cardiotoxicity and damage to cardiomyocytes[53–55].

The use and evidence for the efficacy of other biomarkers beyond troponin-1 is very limited. Many issues are present with using troponin as a biomarker for cardiotoxicity including the need for specialist consultation, repeated testing, and lack of evidence for the timing of troponin testing. Subsequently, recent studies have investigated the use of biomarkers such as C-reactive protein (CRP) and galectin-3 (Gal-3) to monitor signs of CHF[56]. CRP is a common marker of inflammation in the body, and gal-3 is a marker of fibrosis as well as inflammation. Levels of both these inflammatory markers are usually low at baseline and increase with blood post cellular injury. Although we might expect CRP to increase with anthracycline use in cancer patients, given the increase of cellular damage studies are failing to find an association between CRP levels and cardiotoxicity, with one study showing that baseline levels compared to 3 months after anthracycline administration showing no significant differences in levels[57].

Moreover, in both longer-term and shorter-term studies following anthracycline therapy, researchers fail to reveal any association between gal-3 and LVEF. Overall, research has not been able to reveal associations between these biomarkers and cardiovascular outcomes. Nonetheless, research in this area is not all negative. Increased levels of brain natriuretic peptide (BNP), for example, has been linked to lowered LVEF. Recent studies are looking into BNP as an early predictor of cardiotoxicity in patients undergoing anthracycline toxicity. In a study of cytotoxicity in patients undergoing anthracycline therapies, researchers found in all 11 patients which underwent a cardiac event in their study, all of them had at least 1 BNP value over 100 pg/ml. However, there is need for confirmation of these data on a larger cohort of patients[58,59].

Collectively, results and density of research in this field is very much lacking. In efforts to reduce costs, increase access, improve predictability, and improve outcomes, further research is needed into examining potential biomarkers that may detect signs of early cardiotoxicity and/or CHF.

Challenges in diagnosing CHF post anthracycline induced cardiotoxicity

Studies have found links between anthracycline dose, chest radiation, presence of standard cardiovascular risk factors, age at initial cancer diagnosis, sex and the risk of cardiotoxicity developing into CHF. However, to date there is no established risk assessment criterion or scoring system for the development of anthracycline induced CHF. Troponin I would be ideal for early screening and baseline detection of cardiomyocyte injury post anthracycline administration as it could serve as an indication to initiate cardioprotective treatment which prevents further myocardial damage[56,60,61]. However, there is a need for follow-up evaluation (second screening) in patients who

have increased levels of Troponin I, since initial cardiac injury is reversable in some patients and may not indicate progression to CHF, while other patients may have progression to CHF despite early Troponin I screen for cardiac injury. Early detection of progression to congestive heart failure will allow for preventative measures before it becomes irreversible.

In pediatric cohorts, studies have suggested that elevated cardiac troponins and natriuretic peptide levels during anthracycline exposure can be associated with the development of cardiac dysfunction in the first 3 years after treatment, but the association between these early findings and subsequent CHF is unclear[60,62]. Moreover, there can often be a latency period between the initial cardiotoxicity and onset of CHF, often leading to patients presenting at the hospital at the stage of clinical onset of symptoms (reduced LVEF and cardiac output), emphasizing the need for a second follow up screening tool.

Ideally, this evaluation would be follow-up echocardiograms since it is sensitive to changes in heart contractility and LVEF. Children's Oncology Group has developed the Long-Term Follow-Up Guidelines to screen Survivors of Childhood, Adolescent, and Young Adult Cancer, which are to: regularly monitor individuals at high risk for congestive heart failure (CHF) through echocardiography (high-risk criteria include exposure to anthracyclines of 250 mg/m2 or more, chest radiotherapy at 35 Gy or more, or a combination of lower-dose anthracyclines (≥ 100 mg/m2) and chest radiotherapy[51,56,63,64]. They also recommend initiating screening within 2 years of anthracycline exposure, repeating it at least every 5 years thereafter and to consider more frequent screening based on risk level and clinical suspicion[62,65,66]. Similarly, in adult

cancer patients, the screening mechanism for CHF development post anthracycline toxicity is echocardiography[67] (Figure 4.3).

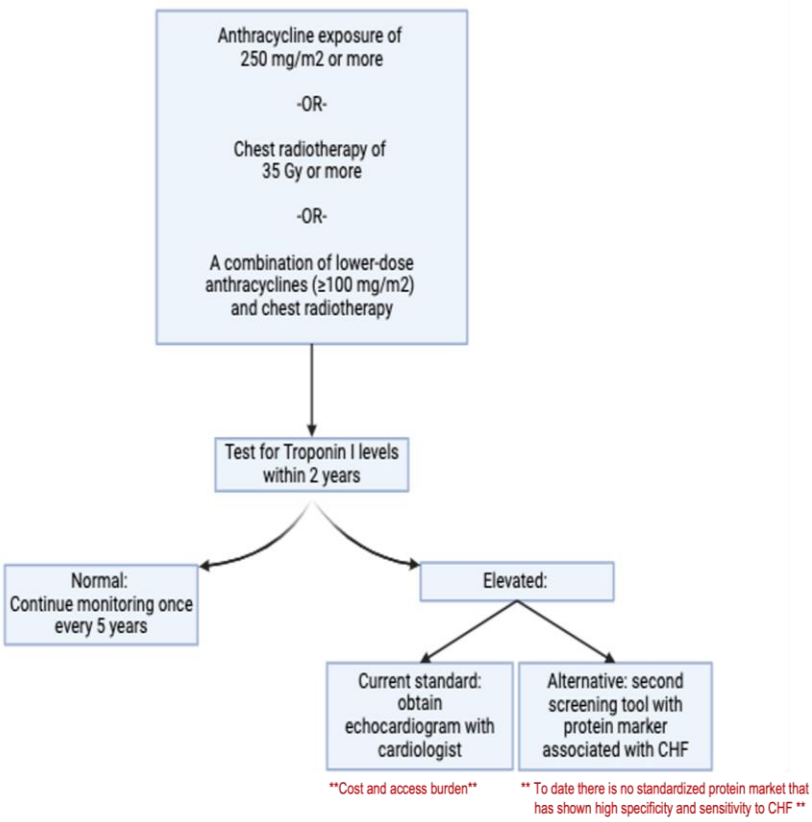


Figure 4.3: Challenges in diagnosing CHF post Anthracycline Induced Cardiotoxicity "Created with "Biorender"

From a global health perspective, an echocardiogram on average costs between 1000-3000 dollars. Screening requires specialist care, needing patients to be attended to by cardiologists, which is not cost efficient, specifically for those who lack access to health insurance and cannot afford the cost of echocardiography or cardiologist consultation fees. Thus, the development of an alternative secondary screening tool that is predictive for CHF development but more cost-efficient is necessary. To this end, an

alternative cost-efficient approach would be to screen for a protein marker that has been shown to have a clinical association with CHF and can be done in a laboratory setting. Platelets as dynamic body sensors.

Platelets are dynamic biomarkers. Well, known for their role in hemostasis[68], platelets have more recently been recognized as important mediators of the inflammatory response[69,70]. Platelets are constantly being produced and have a 7–10-day life cycle in the circulating plasma. Numerous physiological processes can alter the number of platelets that are produced, the circulation lifetime for differentiated platelets, and the chemical signatures of platelets and their extracellular products. As such, platelets provide a window to our state of health, which we can peer through by monitoring the numbers, functions and products of platelets[68].

Platelets are anuclear and are considered by many to be cellular fragments rather than true cells, however they maintain a discoid cellular shape and have mitochondria, RNA, and protein production machinery[68]. Over time, platelet aging is believed to lead to a reduction of available RNAs, but their mitochondrial content is thought to remain constant, such that the ratio of RNA to mitochondria should give insights to platelet age[71]. Platelet cellular functions are intact, including pinocytosis, so they are constantly sampling the plasma, offering protection for substances such as proteins or DNA that would normally be destroyed as naked entities in the plasma.

As platelets are constantly produced, they are also destroyed. Aged and senescent platelets lose sialic acid from their surface, exposing chemical signals that are recognized by Ashwell-Morell receptors on hepatocytes, leading to their clearance[72]. Platelets are also activated for reasons not only related to hemostasis, but also thrombosis,

inflammation and from signals released by cancerous cells. When platelets are activated appropriately or erroneously, they release markers of activation, such as CD154[73], P-selectin, and the triggering receptor expressed in myeloid cells (TREM) like transcript (TLT)-1[74,75]. Platelet activation also leads to fragmentation and generation of platelet derived microparticles[76–78].

As a result of these roles in sampling plasma and mediating inflammation, hemostasis, and thrombosis, and because both their production and survival are intricately tied to these processes, platelets carry signatures of health and disease. One example of platelet utility as a marker is there use in liquid biopsies and the detection of cancer. Many tumors release substances into the blood that are picked up by platelets[79]. Moreover, many tumors express tissue factor, which initiates the coagulation cascade interaction with factor VII, and increases thrombin generation and platelet activation[80]. Additionally, chemotherapeutic treatments can lead to platelet activation and release of platelet markers.

Platelets that have been exposed to the tumor microenvironment are considered to be educated by tumors and are called tumor-educated platelets[81]. One example of tumors educating platelets was demonstrated by evaluating circular RNAs from individuals with gastroenteropancreatic tumors. It has been demonstrated that the platelets had significant changes in 4983 circular RNAs showing a unique tumor signature and the potential to be predictive[82]). The interaction between the tumor microenvironment also changes platelet mRNA. mRNA sequencing of platelets from 283 patients allowed the discernment of patients with tumors that were localized, or metastasized, from those individuals that were healthy with incredible precision[83]. Monitoring platelets in patients can help us

understand cancer stages and direction of progression for a more precise treatment regimen.

Trem like transcript - 1, a platelet specific receptor, is a highly sensitive marker of cardiovascular disease.

Esponda et al. emphasized the importance of sTLT-1 in acute coronary syndrome (ACS). Among 53 patients enrolled, the 19 experiencing cardiogenic chest pain exhibited significantly higher mean plasma sTLT-1 levels (4.644 ng/mL ± 1.277 SEM) compared to controls (1.007 ng/mL ± 0.098 SEM), indicating its potential as a functional measure of atherothrombosis in ACS ($P < 0.05$)[84].

A second study investigating the role of soluble TREM-like transcript 1 (sTLT-1) in cardiovascular disease (CVD) highlight its significance as a biomarker for disease severity and progression. In a study involved 117 human subjects, plasma sTLT-1 levels were significantly elevated in both clinical (2342 ± 184pg/ml) and subclinical (1773 ± 118pg/ml) CVD cases compared to healthy controls (461 ± 57pg/ml). This elevation significantly correlated strongly with common risk factors for coronary artery disease (CAD). Additionally, ex vivo experiments demonstrated that sTLT-1 interacts with Fcɣ receptor I (FcɣRI) to activate inflammatory pathways, contributing to chronic inflammation and atherothrombosis[85].

Replicating the findings of Study 2, Study 3 reiterated the clinical relevance of sTLT-1 in CAD. Plasma sTLT-1 levels remained significantly elevated in clinical and subclinical CVD cases compared to controls, and strong correlations were observed with disease severity and risk factors for CAD[86]. A recent study by Moneam et al., published in 2024, further supports Esponda et als' results, where a cohort of 60 patients with ACS

were found to have elevated TLT-1 levels and a positive relationship with triglycerides and low-density lipoprotein (LDL)[87].

Overall, these studies underscore the clinical importance of sTLT-1 as a biomarker for CVD, providing valuable insights into its role in disease pathophysiology and progression (Table 1). With statistically significant associations observed between sTLT-1 levels and various aspects of CVD, including disease severity, risk factors, and clinical outcomes, sTLT-1 emerges as a promising target for diagnostic and therapeutic interventions in cardiovascular medicine. The above studies prompted the Washington laboratory to do a screening on 1510 patients with chronic heart disease to determine whether TLT-1 had a significant correlation with chronic cardiovascular events, interestingly s-TLT-1 levels could be correlated with congestive heart failure (CHF) in this cohort of patients[88].

PAPER / STUDY	YEAR	COHORT	SAMPLE	# PATIENTS	SUMMARY OF FINDINGS
1)Esponda et al	2014	ACS Patients	Plasma	19	A statistically significant difference exists between patients experiencing cardiogenic chest pain versus controls
2)Das et al	2019	CAD patients	Plasma	117	sTLT-1 levels are clinically related to CAD risk factors and correlates to disease severity and that the sTLT-1 interacts with $Fc\gamma R1$ to activate SYK pathway in macrophages
3)Shen et al	2021	CAD patients	Serum	83	sTLT-1 levels are is a marker of platelet activation and is postiively related to CAD
4)Bayron et al	2023	CAD patients	Plasma	1510	When patients were stratified based on sTLT-1 peak frequency distribution (544 pg/ml) a significant correlation with congestive heart failure was identified (OR =2.94; 1.040-8.282; p=0.042), which could be explained by LV dysfunction
5) Moneam et al.	2024	ACS	Serum	60	ACS is associated with increased level of sTLT-1, AND HAS A positive relationship with triglyceride (p=0.006), low density lipoprotein (LDL) (P=0.037) and neutrophil to lymphocyte ratio (NLR) (p=0.046)

Table 4.1: *Studies showing correlation between s-TLT-1 levels and cardiovascular disease.*

Potential use of Trem like transcript - 1 for CHF screening

The utilization of biomarker or protein screening (that can be done in a laboratory setting) for the early detection and treatment of congestive heart failure (CHF) in leukemia patients represents a pivotal advancement in cardiovascular care. A cornerstone feature of the pathophysiological progression of congestive heart failure is platelet dysfunction[89,90]. Patients with congestive heart failure are at increased risk of thromboembolisms, arrythmias and sudden cardiac death. Studies dated all the way back to 1970 have demonstrated greater platelet aggregation in patients with CHF. The altered hemodynamics in CHF, including stasis of blood flow, can lead to elevated platelet activation and aggregation. Several studies have demonstrated platelets have baseline activation during CHF, as shown by increased levels of procoagulant factors (tissue factor), platelet activation markers (s-P-Sel and s-TLT-1), plasma proteins that facilitate clot formation (fibrinogen)[91–94]. These data introduce the potential for a diagnostic screening criterion, where Troponin I detection indicate that there is cardiotoxicity, prompting a screening for a platelet activation marker (which would be sensitive to CHF), as a gold standard for early detection of cardiotoxicity and progression to congestive heart failure, respectively. Early diagnosis would allow for preventative treatment with medications such as B-Blockers, which have been shown to decrease incidence of LVEF dysfunction, heart failure and death[95].

The study by Bayrón-Marrero et al. underscores the potential of incorporating a biomarker and an early detection screening protein, specifically soluble Trem-Like Transcript-1 (s-TLT-1) into clinical practice (Table 1). Trem-Like Transcript -1 is a platelet-specific receptor that is stored in the platelet alpha granules and released onto the

platelet surface upon platelet activation[96]. Several studies have demonstrated that plasma levels of s-TLT-1 is a highly sensitive marker of platelet activation, even more so that s-P-sel, the most used marker of platelet activation[97]. Bayrón-Marrero et al analyzed data from 1510 patients with stable CAD and found that lower plasma sTLT-1 levels were significantly associated with left ventricular (LV) dysfunction (981.62 ± 1141 pg/mL vs. 1247.48 ± 1589 pg/mL; p = 0.003). Furthermore, stratification based on sTLT-1 levels revealed a significant association with congestive heart failure (OR = 2.94; 95% CI: 1.040-8.282; p = 0.042), particularly in patients with LV dysfunction, suggests its potential as a sensitive indicator of early cardiac impairment. More specifically, patients with s-TLT-1 levels exceeding 544 pg/mL exhibited a significant association with congestive heart failure, emphasizing the potential use of s-TLT-1 levels above 544 pg/mL as a warning signal for progression to congestive heart failure [88].

Early identification of cardiac dysfunction through s-TLT-1 measurement could enable timely intervention and tailored treatment strategies, thus mitigating the risk of CHF progression in leukemia patients. Integrating such a diagnostic screening criterion into routine monitoring protocols holds promise for enhancing patient outcomes, offering a proactive approach to CHF management in this vulnerable population. However, a study measuring s-TLT-1 levels in leukemia patients has not yet been done, further research and validation of s-TLT-1 and other diagnostic screening methods may usher in a new era of precision medicine, transforming the landscape of CHF detection and treatment in leukemia.

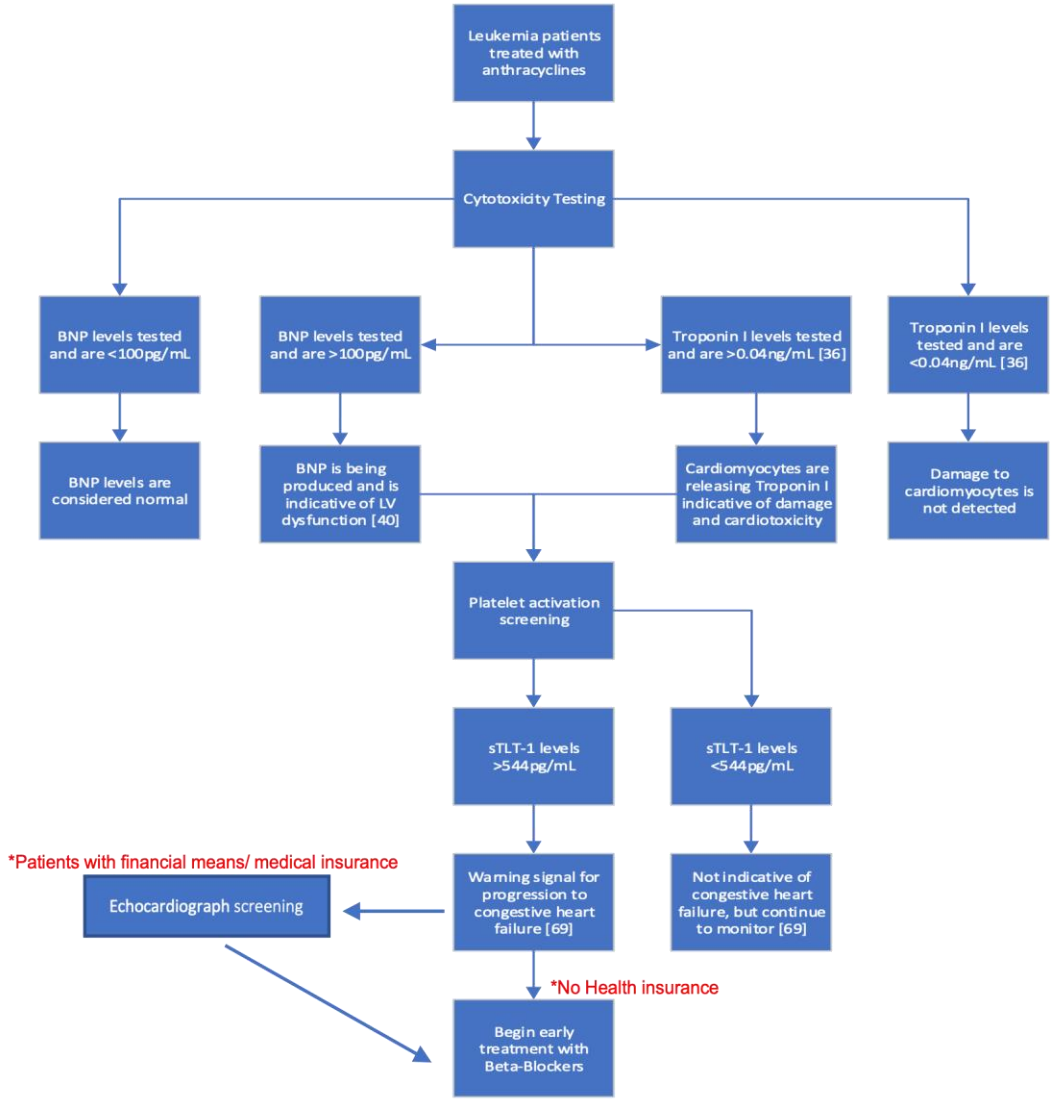


Figure 4.4: Cardiotoxicity Screening pathway

Conclusion: alternative approaches for diagnosing CHF in leukemia patients

The leading cause of mortality for cancer patients who are in remission is sequelae of aggressive treatment such as cardiac dysfunction. Using echocardiography, Troponin-I (Trop-I), and NT-proBNP for early CHF diagnosis presents challenges due to limited specificity and high costs. Echocardiography, while valuable, may lack conclusive evidence in early CHF stages, with compromised specificity due to

comorbidities. Similarly, Trop-I and NT-proBNP, though indicating myocardial stress, lack specificity, potentially leading to unnecessary investigations and costs. The economic burden extends beyond direct testing expenses, encompassing subsequent interventions and complications from false results. Missed or delayed CHF diagnosis can exacerbate symptoms and increase healthcare utilization.

This review has introduced s-TLT-1 screening as an alternative approach to CHF diagnosis, post initial Troponin-I (Trop-I) or NT-proBNP screening. It has been demonstrated in several studies that platelets can be used as biomarkers for progression of CVD, and that more specifically s-TLT-1, a platelet receptor, is highly sensitive to CVD, including CHF (Table 1). One of the pathophysiological features of congestive heart failure is dilated cardiomyopathy, altering cardiac contractility, leading to turbulent blood flow and increased platelet activation, thus increasing the risk of thrombus formation. This increased platelet activation could potentially be used as a "mirror" to classify stages of CHF progression. There are currently s-TLT-1 ELISAs for research use that could potentially be adapted for use in a clinical setting. Laboratories are currently working on developing a rapid testing kit to measure plasma s-TLT-1 levels. A rapid testing kit could substantially the cost of diagnosis compared to an echocardiogram, and easily be accessible in a standard clinical laboratory setting.

We propose the diagnostic protocol as demonstrated in Figure 4.4, where initial detection of cardiomyocyte injury through (Trop-I) or NT-proBNP elevation prompts regular screening for s-TLT-1 levels and when elevated above 544pg/ml the initiation of cardioprotective agents such as B-Blockers. Where possible echocardiogram is performed simultaneously to monitor ejection fraction and left ventricular function, but that

echocardiogram is not the determining factor for initiation of cardioprotective agents such as B-Blockers for those who do not have access to healthcare services. This review introduces a potential alternative approach to diagnosing CHF in leukemia patients receiving anthracyclines, however, validating the use of s-TLT-1, either alone or with other cardiovascular markers like Trop-I or NT-proBNP, with or without echocardiography, in a clinical cohort of patients, is necessary to confirm the sensitivity and specificity of this diagnostic approach.

Bibliography

1. Leukemia — Cancer Stat Facts. https://seer.cancer.gov/statfacts/html/leuks.html.

2. Chennamadhavuni, A., Lyengar, V., Mukkamalla, S. K. R. & Shimanovsky, A. Leukemia. (2023).

3. Leukemia Diagnosis, Symptoms and Treatments | DKMS. https://www.dkms.org/learn-more/blood-cancer/leukemia#:~:text=Chronic%20lymphocytic%20leukemia%20occurs%20when,changed%20lymphocytes%20%2D%20white%20blood%20cells.

4. Tiso, F. *et al.* Genetic diversity within leukemia-associated immunophenotype-defined subclones in AML. *Ann Hematol* 101, 571–579 (2022).

5. Alhmoud, J. F., Mustafa, A. G. & Malki, M. I. Targeting DNA Repair Pathways in Hematological Malignancies. *Int J Mol Sci* 21, 1–17 (2020).

6. Puiggros, A., Blanco, G. & Espinet, B. Genetic abnormalities in chronic lymphocytic leukemia: Where we are and where we go. *Biomed Res Int* 2014, (2014).

7. Taylor, J., Xiao, W. & Abdel-Wahab, O. Diagnosis and classification of hematologic malignancies on the basis of genetics. *Blood* 130, 410–423 (2017).

8. D'Arena, G. *et al.* Autoimmune cytopenias in chronic lymphocytic leukemia. *Clin Dev Immunol* 2013, (2013).

9. Acute myeloid leukaemia - Complications - NHS. https://www.nhs.uk/conditions/acute-myeloid-leukaemia/complications/.

10. Morrison, V. A. Infectious complications of chronic lymphocytic leukaemia: Pathogenesis, spectrum of infection, preventive approaches. *Best Pract Res Clin Haematol* 23, 145–153 (2010).

11. Moreira, J. *et al.* Infectious complications among individuals with clinical monoclonal B-cell lymphocytosis (MBL): A cohort study of newly diagnosed cases compared to controls. *Leukemia* 27, 136–141 (2013).

12. Acute myeloid leukaemia - Complications - NHS. https://www.nhs.uk/conditions/acute-myeloid-leukaemia/complications/.

13. Why People with Cancer Are More Likely to Get Infections | American Cancer Society. https://www.cancer.org/cancer/managing-cancer/side-effects/low-blood-counts/infections/why-people-with-cancer-are-at-risk.html.

14. Forconi, F. & Moss, P. Perturbation of the normal immune system in patients with CLL. *Blood* 126, 573–581 (2015).

15. Holdsworth, M. T., Raisch, D. W., Duncan, M. H., Chavez, C. M. & Leasure, M. M. Assessment of Chemotherapy-Induced Emesis and Evaluation of a Reduced-Dose Intravenous Ondansetron Regimen in Pediatric Outpatients with Leukemia. *http://dx.doi.org/10.1177/106002809502900103* 29, 16–21 (1995).

16. Chemotherapy-induced nausea and vomiting in acute leukemia and stem cell transplant patients: results of a multicenter, observational study | Haematologica. https://www.haematologica.org/article/view/3826.

17. Cheson, B. D., Frame, J. N., Vena, D., Quashu, N. & Sorensen, J. M. Tumor lysis syndrome: An uncommon complication of fludarabine therapy of chronic lymphocytic leukemia. *Journal of Clinical Oncology* 16, 2313–2320 (1998).

18. Cheson, B. D. *et al.* Tumor Lysis Syndrome in Chronic Lymphocytic Leukemia with Novel Targeted Agents. *Oncologist* 22, 1283–1291 (2017).

19. Epstein, R. S. *et al.* Cancer patients' perspectives and experiences of chemotherapy-induced myelosuppression and its impact on daily life. *Patient Prefer Adherence* 15, 453–465 (2021).

20. Bryer, E. & Henry, D. Chemotherapy-induced anemia: etiology, pathophysiology, and implications for contemporary practice. *International Journal of Clinical Transfusion Medicine* Volume 6, 21–31 (2018).

21. Skanland, S. S. & Mato, A. R. Overcoming resistance to targeted therapies in chronic lymphocytic leukemia. *Blood Adv* 5, 334–343 (2021).

22. Daver, N. *et al.* Secondary mutations as mediators of resistance to targeted therapy in leukemia. *Blood* 125, 3236–3245 (2015).

23. Yu, J. *et al.* Advances in targeted therapy for acute myeloid leukemia. *Biomark Res* 8, 1–11 (2020).

24. Chen, J., Odenike, O. & Rowley, J. D. Leukaemogenesis: more than mutant genes. *Nat Rev Cancer* 10, 23–36 (2010).

25. 6 Innovative Leukemia Treatment Options | MD Anderson Cancer Center. https://www.mdanderson.org/cancer-types/leukemia/leukemia-treatment.html.

26. CAR T-cell Therapy and Its Side Effects | American Cancer Society. https://www.cancer.org/cancer/managing-cancer/treatment-types/immunotherapy/car-t-cell1.html.

27. What is CAR T Cell Therapy? Know Before Treatment | MD Anderson Cancer Center. https://www.mdanderson.org/treatment-options/car-t-cell-therapy.html.

28. FDA investigating cancer risk linked to CAR-T cell therapy | BioPharma Dive. https://www.biopharmadive.com/news/fda-car-t-cancer-risk-investigation-lymphoma/700874/#:~:text=Both%20drugmakers%20and%20the%20FDA,can%20also%20cause%20secondary%20malignancies.

29. Esmaeilzadeh, A., Tahmasebi, S. & Athari, S. S. Chimeric antigen receptor -T cell therapy: Applications and challenges in treatment of allergy and asthma. *Biomedicine & Pharmacotherapy* 123, 109685 (2020).

30. Davila, M. L. *et al.* Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med* 6, (2014).

31. Frey, N. V. & Porter, D. L. Cytokine release syndrome with novel therapeutics for acute lymphoblastic leukemia. *Hematology* 2016, 567–572 (2016).

32. Xu, X. *et al.* Mechanisms of Relapse After CD19 CAR T-Cell Therapy for Acute Lymphoblastic Leukemia and Its Prevention and Treatment Strategies. *Front Immunol* 10, (2019).

33. Xu, X. *et al.* Challenges and Clinical Strategies of CAR T-Cell Therapy for Acute Lymphoblastic Leukemia: Overview and Developments. *Front Immunol* 11, 569117 (2021).

34. Stelljes, M. & Marks, D. I. Acute Lymphoblastic Leukemia in Adults. *The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies* 531–538 (2019) doi:10.1007/978-3-030-02278-5_71.

35. Przepiorka, D. *et al.* Risk Factors for Acute Graft-Versus-Host Disease After Allogeneic Blood Stem Cell Transplantation. *Blood* 94, 1465–1470 (1999).

36. Steele, M. & Narendran, A. Mechanisms of defective erythropoiesis and anemia in pediatric acute lymphoblastic leukemia (ALL). *Ann Hematol* 91, 1513–1518 (2012).

37. Michon, J. Incidence of anemia in pediatric cancer patients in Europe: results of a large, international survey. *Med Pediatr Oncol* 39, 448–450 (2002).

38. De Cicco, M. The prothrombotic state in cancer: Pathogenic mechanisms. *Crit Rev Oncol Hematol* 50, 187–196 (2004).

39. Bick, R. L. Coagulation abnormalities in malignancy: A review. *Semin Thromb Hemost* 18, 353–372 (1992).

40. Bachur, N. R. Anthracyclines. *Encyclopedia of Cancer* 57–61 (2002) doi:10.1016/B0-12-227555-1/00006-X.

41. Octavia, Y. *et al.* Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. *J Mol Cell Cardiol* 52, 1213–1225 (2012).

42. Zhang, S. *et al.* Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* 18, 1639–1642 (2012).

43. Curigliano, G. *et al.* Cardiotoxicity of anticancer treatments: Epidemiology, detection, and management. *CA Cancer J Clin* 66, 309–325 (2016).

44. Rayner, D. M. & Cutts, S. M. Anthracyclines. *Side Effects of Drugs Annual* 36, 683–694 (2023).

45. Curigliano, G. *et al.* Cardiotoxicity of anticancer treatments: Epidemiology, detection, and management. *CA Cancer J Clin* 66, 309–325 (2016).

46. Cardinale, D. & Cipolla, C. M. Chemotherapy-induced cardiotoxicity: importance of early detection. *Expert Rev Cardiovasc Ther* 14, 1297–1299 (2016).

47. Dhingra, R., Margulets, V. & Kirshenbaum, L. A. Molecular Mechanisms Underlying Anthracycline Cardiotoxicity: Challenges in Cardio-Oncology. *Cardio-Oncology: Principles, Prevention and Management* 25–34 (2017) doi:10.1016/B978-0-12-803547-4.00002-1.

48. Cardinale, D., Iacopo, F. & Cipolla, C. M. Cardiotoxicity of Anthracyclines. *Front Cardiovasc Med* 7, (2020).

49. Malik, A., Brito, D., Vaqar, S. & Chhabra, L. Congestive Heart Failure. *StatPearls* (2023).

50. Curigliano, G. *et al.* Cardiotoxicity of anticancer treatments: Epidemiology, detection, and management. *CA Cancer J Clin* 66, 309–325 (2016).

51. Melendez, G., Cardinale, D., Iacopo, F. & Cipolla, C. M. Cardiotoxicity of Anthracyclines. *Frontiers in Cardiovascular Medicine | www.frontiersin.org* 7, 26 (2020).

52. Plana, J. C. *et al.* Expert consensus for multimodality imaging evaluation of adult patients during and after cancer therapy: a report from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Eur Heart J Cardiovasc Imaging* 15, 1063–1093 (2014).

53. Cardinale, D., Biasillo, G., Salvatici, M., Sandri, M. T. & Cipolla, C. M. Using biomarkers to predict and to prevent cardiotoxicity of cancer therapy. *Expert Rev Mol Diagn* 17, 245–256 (2017).

54. Cardinale, D. *et al.* Left ventricular dysfunction predicted by early troponin I release after high-dose chemotherapy. *J Am Coll Cardiol* 36, 517–522 (2000).

55. O'Brien, P. J. Cardiac troponin is the most effective translational safety biomarker for myocardial injury in cardiotoxicity. *Toxicology* 245, 206–218 (2008).

56. Armenian, S. H. *et al.* Screening for cardiac dysfunction in anthracycline-exposed childhood cancer survivors. *Clin Cancer Res* 20, 6314 (2014).

57. Ky, B. *et al.* Early Increases in Multiple Biomarkers Predict Subsequent Cardiotoxicity in Patients With Breast Cancer Treated With Doxorubicin, Taxanes, and Trastuzumab. *J Am Coll Cardiol* 63, 809 (2014).

58. Lenihan, D. J. *et al.* The Utility of Point-of-Care Biomarkers to Detect Cardiotoxicity During Anthracycline Chemotherapy: A Feasibility Study. *J Card Fail* 22, 433–438 (2016).

59. Bhandari, B. & Cunningham, J. The Role of Brain Natriuretic Peptide as a Prognostic Marker for Sepsis. *Cureus* 12, (2020).

60. Lipshultz, S. E. *et al.* Predictive value of cardiac troponin T in pediatric patients at risk for myocardial injury. *Circulation* 96, 2641–2648 (1997).

61. Tzolos, E. *et al.* Dynamic Changes in High-Sensitivity Cardiac Troponin I in Response to Anthracycline-Based Chemotherapy. *Clin Oncol (R Coll Radiol)* 32, 292 (2020).

62. Lipshultz, S. E. *et al.* Changes in Cardiac Biomarkers During Doxorubicin Treatment of Pediatric Patients With High-Risk Acute Lymphoblastic Leukemia: Associations With Long-Term Echocardiographic Outcomes. *J Clin Oncol* 30, 1042–1049.

63. Lazăr, D. R. *et al.* Cardiotoxicity: A Major Setback in Childhood Leukemia Treatment. *Dis Markers* 2021, (2021).

64. Klein, K., de Haas, V. & Kaspers, G. J. L. Clinical challenges in de novo pediatric acute myeloid leukemia. *Expert Rev Anticancer Ther* 18, 277–293 (2018).

65. Mulder, R. L. *et al.* Recommendations for cardiomyopathy surveillance for survivors of childhood cancer: a report from the International Late Eff ects of Childhood Cancer Guideline Harmonization Group. *Review Lancet Oncol* 16, 123–159 (2015).

66. Children's Oncology Group. http://www.survivorshipguidelines.org/.

67. Tan, T. C. & Scherrer-Crosbie, M. Assessing the Cardiac Toxicity of Chemotherapeutic Agents: Role of Echocardiography. *Curr Cardiovasc Imaging Rep* 5, 403 (2012).

68. Desborough, M. J. R., Smethurst, P. A., Estcourt, L. J. & Stanworth, S. J. Alternatives to allogeneic platelet transfusion. *Br J Haematol* 175, 381–392 (2016).

69. Ferrer-Acosta, Y., González, M., Fernández, M. & Valance, W. A. Emerging Roles for Platelets in Inflammation and Disease. *J Infect Dis Ther* 2, (2014).

70. Semple, J. W., Italiano, J. E. & Freedman, J. Platelets and the immune continuum. *Nat Rev Immunol* 11, 264–274 (2011).

71. Supernat, A. *et al.* Transcriptomic landscape of blood platelets in healthy donors. *Sci Rep* 11, (2021).

72. Grozovsky, R. *et al.* The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nat Med* 21, 47–54 (2015).

73. Cognasse, F. *et al.* Platelets as Key Factors in Inflammation: Focus on CD40L/CD40. *Front Immunol* 13, (2022).

74. Smith, C. W. *et al.* TREM-like transcript 1: a more sensitive marker of platelet activation than P-selectin in humans and mice. *Blood Adv* 2, 2072 (2018).

75. Washington, A. V. *et al.* A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 104, 1042–1047 (2004).

76. Lundström, A. *et al.* Prognostic Value of Circulating Microvesicle Subpopulations in Ischemic Stroke and TIA. *Transl Stroke Res* 11, 708–719 (2020).

77. Boilard, E., Duchez, A. C. & Brisson, A. The diversity of platelet microparticles. *Curr Opin Hematol* 22, 437–444 (2015).

78. Panzer, S. *et al.* Plasma levels of P-selectin are determined by platelet turn-over and the P-selectin Thr715Pro polymorphism. *Thromb Res* 121, 573–579 (2008).

79. Klement, G. L. *et al.* Platelets actively sequester angiogenesis regulators. *Blood* 113, 2835–2842 (2009).

80. Hisada, Y. & Mackman, N. Tissue Factor and Cancer: Regulation, Tumor Growth, and Metastasis. *Semin Thromb Hemost* 45, 385–395 (2019).

81. Calverley, D. C. *et al.* Significant downregulation of platelet gene expression in metastatic lung cancer. *Clin Transl Sci* 3, 227–232 (2010).

82. Campolo, F. *et al.* Platelet-derived circRNAs signature in patients with gastroenteropancreatic neuroendocrine tumors. *J Transl Med* 21, 1–12 (2023).

83. Best, M. G. *et al.* RNA-Seq of Tumor-Educated Platelets Enables Blood-Based Pan-Cancer, Multiclass, and Molecular Pathway Cancer Diagnostics. *Cancer Cell* 28, 666–676 (2015).

84. Esponda, O. L., Hunter, R., Río, J. R. R. Del & Washington, A. V. Levels of soluble TREM-Like transcript 1 in patients presenting to the emergency department with chest pain. *Clinical and Applied Thrombosis/Hemostasis* (2015) doi:10.1177/1076029614547298.

85. Das, A. A. *et al.* Elevated level of circulatory sTLT1 induces inflammation through SYK/MEK/ERK signalling in coronary artery disease. *Clin Sci (Lond)* 133, 2283–2299 (2019).

86. Shen, L. *et al.* P-selectin (CD62P) and soluble TREM-like transcript-1 (sTLT-1) are associated with coronary artery disease: a case control study. *BMC Cardiovasc Disord* 20, (2020).

87. Hussein, Y. *et al.* Soluble triggering receptor expressed on myeloid cells like transcript-1 (sTLT-1) levels in patients with acute coronary syndrome. *International Journal of Clinical and Diagnostic Pathology* 7, 08–12 (2024).

88. Bayrón-Marrero, Z. *et al.* The Characterization and Evaluation of the Soluble Triggering Receptor Expressed on Myeloid Cells-like Transcript-1 in Stable Coronary Artery Disease. *Int J Mol Sci* 24, (2023).

89. Chung, I. & Lip, G. Y. H. Platelets and heart failure. *Eur Heart J* 27, 2623–2631 (2006).

90. Chung, I., Choudhury, A. & Lip, G. Y. H. Platelet activation in acute, decompensated congestive heart failure. *Thromb Res* 120, 709–713 (2007).

91. Stumpf, C. *et al.* Enhanced levels of CD154 (CD40 ligand) on platelets in patients with chronic heart failure. *Eur J Heart Fail* 5, 629–637 (2003).

92. Chin, B. S. P. *et al.* Prognostic value of interleukin-6, plasma viscosity, fibrinogen, von Willebrand factor, tissue factor and vascular endothelial growth factor levels in congestive heart failure. *Eur J Clin Invest* 33, 941–948 (2003).

93. Gibbs, C. R., Blann, A. D., Watson, R. D. S. & Lip, G. Y. H. Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: Effects of angiotensin-converting enzyme inhibitor and β-blocker therapy. *Circulation* 103, 1746–1751 (2001).

94. Varo, N. *et al.* Soluble CD40L. *Circulation* 108, 1049–1052 (2003).

95. Bosch, X. *et al.* Enalapril and Carvedilol for Preventing Chemotherapy-Induced Left Ventricular Systolic Dysfunction in Patients With Malignant Hemopathies: The OVERCOME Trial (preventiOn of left Ventricular dysfunction with Enalapril and caRvedilol in patients submitted to intensive ChemOtherapy for the treatment of Malignant hEmopathies). *J Am Coll Cardiol* 61, 2355–2362 (2013).

96. Branfield, S. & Washington, A. V. The enigmatic nature of the triggering receptor expressed in myeloid cells -1 (TLT- 1). *Platelets* 32, 753 (2021).

97. TREM-like transcript 1: a more sensitive marker of platelet activation than P-selectin in humans and mice - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6113608/#:~:text=In%20conclusion%2C%20TLT%2D1%20is,shell%20of%20thrombi%20in%20vivo.

CHAPTER 5

TLT-1-1, AS A REGULATOR OF HEPATIC REGENERATION

Abstract

Hepatectomy, a surgical procedure involving partial or complete liver removal, is essential for treating various liver conditions, including cancer and cirrhosis. Despite the liver's unique regenerative capacity, extensive resections or liver failure can lead to postresective liver failure, a significant risk of mortality. The 5-year survival rates post-resection vary based on factors such as disease stage and patient health.

This study addresses the intricate relationship between hepatic neoplasms, particularly liver cancer, and the critical process of hepatic regeneration following surgical interventions like hepatectomy. Liver cancer, ranking as the 6th most common worldwide, presents a significant global health burden with high mortality rates. In 2020, 905,700 people were diagnosed, and 830,200 died from liver cancer. Surgical resection or transplantation remains the primary curative options, but patients often face challenges such as cirrhosis, comorbidities, and increased mortality risks due to cancer metastasis.

Hepatectomy, a surgical procedure involving partial or complete liver removal, is essential for treating various liver conditions, including cancer and cirrhosis. Despite the liver's unique regenerative capacity, extensive resections or liver failure can lead to postresective liver failure, a significant risk of mortality. The 5-year survival rates post-resection vary based on factors such as disease stage and patient health. In this context, our study explores the role of TREM-Like Transcript-1 (TLT-1) in regulating platelet-mediated processes crucial for hepatic regeneration following a 70% hepatic resection in

mice. The findings contribute to understanding the mechanisms driving hepatic regeneration and provide insights into optimizing postoperative recovery and survival in patients with liver conditions, paving the way for potential therapeutic interventions.

Introduction

Liver cancer is the 6th most common cancer worldwide. In 2020, approximately 905,700 people were diagnosed with and 830,200 people died from liver cancer, globally.[1] The curative options for hepatic neoplasms, primary or metastatic, remain as either surgical resection or liver transplantation. However, patients with liver cancer typically have liver cirrhosis, comorbidities, or increased risk of mortality due to the nature of cancer being metastatic. Hepatectomy is the surgical procedure involving partial or complete removal of the liver. This procedure is necessary in cases of liver cancer (Hepatocellular carcinoma, adenoma, or metastatic colorectal cancer), cirrhosis and disease (cyst formations). Of all the systemic organs in the body, the liver is the only internal organ with the capacity to regenerate, allowing it to restore up to 70% of its volume. Survival post resection will vary from patient to patient depending on the extent of resection, underlying disease, and overall health of the patient. There is a general 5-year survival rate following resection ranging from 50% to 70%, depending on the condition and stage of disease. In patients with colorectal cancer complicated by metastasis in the liver there is a 5-year survival rate of 35.8% post resection[2].

Failure to regenerate is common with extensive resection or liver failure, leaving postresective liver failure as a risk of mortality[3,4]. There are currently no therapeutic interventions to optimize recovery after resection, understanding the mechanisms involved in hepatic regeneration would pave way for therapeutic interventions to

optimize patient recovery. Understanding the mechanisms driving hepatic regeneration would optimize post operative recovery and survival in such patients.

Liver parenchymal restoration is achieved by compensatory hypertrophy and hyperplasia of hepatocytes[5]. This restoration is a complex process facilitated by various cytokines, immune cells (platelets, leukocytes and Kupffer cells), and signaling pathways. Recent studies demonstrate that platelets mediate pathophysiological progression during liver inflammation, liver regenerative capacity, injury and cancer. Post hepatectomy, platelets accumulate in the space of Disse, interacting with hepatocytes[6] and vWF on the hepatic surface [7]. This triggers platelet activation and release of soluble mediators such as hepatocyte growth factor (HGF), insulin-like growth factor (IGF-1) PDGF, VEGF, 5-HT, ADP and ATP[8] and is essential for PI3K/AKT-dependent hepatocyte mitosis and an important initiator of liver regeneration[9] [10–12]. The successful regeneration of liver tissue post resection has been found to correlate with platelet accumulation and fibrinogen deposition. Patients without sufficient fibrinogen deposition have reduced hepatic regenerative potential and are associated with poor prognosis. Moreover, platelets have been shown to enhance Kupffer cell section of TNF-a and IL-6, L-6 drives the acute phase response, initiates cytoprotection and proliferation of hepatocytes via the IL-6-IL-6R interaction and gp130, activating Janus kinase (JAK)/signal transducer and transcription through STAT, MAPK and PI3K/AKT signaling pathways, all of which are required for hepatic regeneration.

Interestingly, present studies have demonstrated that liver regeneration is delayed when platelets and fibrinogen, a plasma protein that binds platelet receptors, are depleted in a rodent model, in support of this data, low plasma fibrinogen levels are associated

with liver dysfunction and mortality in patients, post hepatic resection[4]. This paves way for a novel theory, in which platelet/fibrinogen interactions is pivotal for hepatic regeneration, suggesting that there is a larger network of 'puzzle pieces' at play mediating the regenerative process, beyond the platelet-Kupffer cell/platelet hepatocyte interaction. However, the exact mechanisms, or link between these molecular and cellular interactions are unknown.

Upon activation, platelets release TREM-Like Transcript-1 (TLT-1) from their a-granules onto their surface. TLT-1 is a type 1 receptor that, like the integrin αIIbβ3, binds fibrinogen and facilitates platelet aggregation. However, although TLT-1 may assist in clot formation and hemostasis to arrest bleeding in a non-inflammatory mediated setting, TLT-1's main association is with regulating inflammatory-derived bleeding as demonstrated by increased hemorrhage after inflammatory treatments such as lipopolysaccharide LPS in the *treml1*$^{-/-}$ mice as compared to controls.TLT-1 has also been shown to regulate leukocyte activation and modulate sepsis induced acute inflammatory responses. The *treml1*$^{-/-}$ mouse model has been shown to have less fibrinogen deposition during an inflammatory response, correlating with an increased bleeding phenotype in the lungs, suggesting a role of this TLT-1 fibrinogen interaction during lung healing[13].

Taken together, all evidence points towards a platelet/fibrinogen/Kupffer (macrophage) pathophysiological mechanism underlying the hepatic regenerative capacity. Could TLT-1-, as a receptor for fibrinogen that interacts with leukocytes, be the linking factor, facilitating the transition from inflammatory response and homeostasis to hepatic regeneration post resection?

The goal of this proposal is to unravel the regenerative changes that lead to decreased fibrinogen deposition and decreased survival in the *treml1*$^{-/-}$ mice, as well as to characterize the TLT-1 Fibrinogen molecular interaction. We hypothesize that: TLT-1, being a ligand for fibrinogen, mediates fibrinogen deposition after liver resection and positively regulates post-surgical hepatic regeneration and survival.

Methods

PHx in mice

Mice aged between 10 and 12 weeks were divided into age-matched groups and underwent a two-thirds partial liver resection following established protocols with some adjustments. The surgery involved removing specific portions of the liver while preserving the gallbladder to prevent obstruction of the extrahepatic biliary tree. Sham surgeries were performed by gently manipulating the liver lobes without removing tissue. The procedures were conducted under deep surgical anesthesia induced by isoflurane using the Kent scientific SomnoSuite®Low-Flow Anesthesia System, and mice were administered 5 mg/kg carprofen for analgesia before surgery. In a sterile hood, mice were shaved on the abdomen and surgical area disinfected with 70% ethanol.

While under anesthesia a midline abdominal incision was made through the skin, then peritoneal layer. Retractors were used to expose the liver and we performed 70% hepatic resection (hepatectomy of left lateral, medial and caudate lobes, leaving the right lateral lobe), ligating the lobes with surgical sutures before resection at an initial time point (T=0) and resected the right lateral lobe at a second time point (T= 30 minutes, T=3 days or at T=7 days) post initial resection (T=0) in wild type and *treml1*$^{-/-}$ mice. Mice that were for survival studies (T=3, T=7) were sutured and left on heating pads to recover, for

pain management these mice were administered carprofen twice daily at a concentration of 5mg/kg. For three or seven-day surgeries, mice will be weighed at an initial time point and then on day three or seven, right lateral lobes of livers will be weighed on day 7 and a body mass ratio to regenerated right lateral lobe will be compared for t*reml1*$^{-/-}$ and control mice.

Collected livers were either fixed in 4% PFA and fixed or snap-frozen in liquid nitrogen for immunohistochemical analyses or protein quantification. This comprehensive approach allowed for the investigation of various parameters, including coagulation markers and liver tissue characteristics, to evaluate platelet adhesion, fibrinogen deposition, hepatic proliferative potential (KI-67) and proliferation (staining H & E for binucleated cells, mitoses, thickening of the hepatic plate, ductular reaction, and presence of inflammatory cells, and staining for F4/80 by immunofluorescence to quantify macrophage infiltration, by immunohistochemistry and survival as primary endpoints, t*reml1*$^{-/}$ and control mice were used for each time point (plasma sample collection and protein extraction for signaling).

Immunofluorescent Staining

Cut OCT embedded tissue at 8 µm thickness, wash 3 times in PBS, 10 min for each wash. Add 200 µl blocking buffer per slide (gently, do not directly on tissue, but add to area without) and incubate for 1h at RT on an orbital shaker. Remove the blocking buffer by tapping the sections dry on tissues, rinse in PBS. Add 200 µl of primary antibody (Rabbit monoclonal [EPR17876] to CD41 or Proteintech Fibrinogen Beta Chain Polyclonal antibody Cat # 16747-1-AP) per slide (dilute 1:200 in blocking buffer for overnight incubation at 4° or dilute 1:100 in blocking buffer for 2h incubation at RT).

Wash slides 3x in PBS, 10min for each wash. Dilute secondary antibody in blocking buffer. Dilute Goat anti-rabbit Alexa 594 1:500 in this buffer and add 200 µl per slide and incubate for 2h at RT on an orbital shaker. Wash to remove secondary antibody . Mount slides with mounting medium containing DAPI (VECTASHIELD® Antifade Mounting Medium (H-1000-10). Carefully place a coverslip over the tissue, allow to dry for 24h in the dark before taking images.

Statistics

Image J was used for quantification of platelet and fibrinogen deposition while statistical analyses were performed in Prism. By combining t-tests and survival curves for mortality assessment, you are adopting a well-rounded statistical strategy that addresses the specific characteristics of your data. A t-test is well-suited for comparing means between two groups, making it appropriate for evaluating histological parameters (e.g., platelet accumulation and fibrinogen deposition) between the two mouse genotypes. This statistical test allows for the identification of significant differences in the observed histological features. This approach allows for a comprehensive exploration of both the immediate histological effects and the longer-term mortality outcomes associated with the different mouse genotypes in your study.

Results

In preliminary studies, we performed 70% hepatic resection (hepatectomy of left lateral, medial and caudate lobes) at an initial time point (T=0) and resected the right lateral lobe at a second time point (T= 30 minutes, or at T=7 days) post initial resection (T=0) in wild type and *treml1*$^{-/-}$ mice and evaluated platelet adhesion, fibrinogen deposition by immunohistochemistry and survival as primary endpoints.

TLT-1 regulates fibrinogen and platelet accumulation post PHx.

Immunoflourescent analysis demonstrates that *treml1-/-* mice initially have an increased platelet accumulation in the right lateral lobe of the liver 30 minutes post resection but at a seven-day time have decreased platelet accumulation compared to WT mice. While fibrinogen deposition in the hepatic tissue of *treml1-/-* mice compared to wildtype controls, is decreased for both 30 minute and 7-day time points, suggesting that TLT-1 plays a role in regulating fibrinogen deposition and platelet accumulation in hepatic tissue.

treml1$^{-/-}$ mice are more prone to mortality post PHx than WT mice

The 7-day recovery surgery suggests that *treml1-/-* mice are more susceptible to mortality over a 7-day time course (n=11, 4 dead: 36% Mortality) compared to wild type controls (n=11, 2 dead :18% Mortality), ≤ 0.0060.

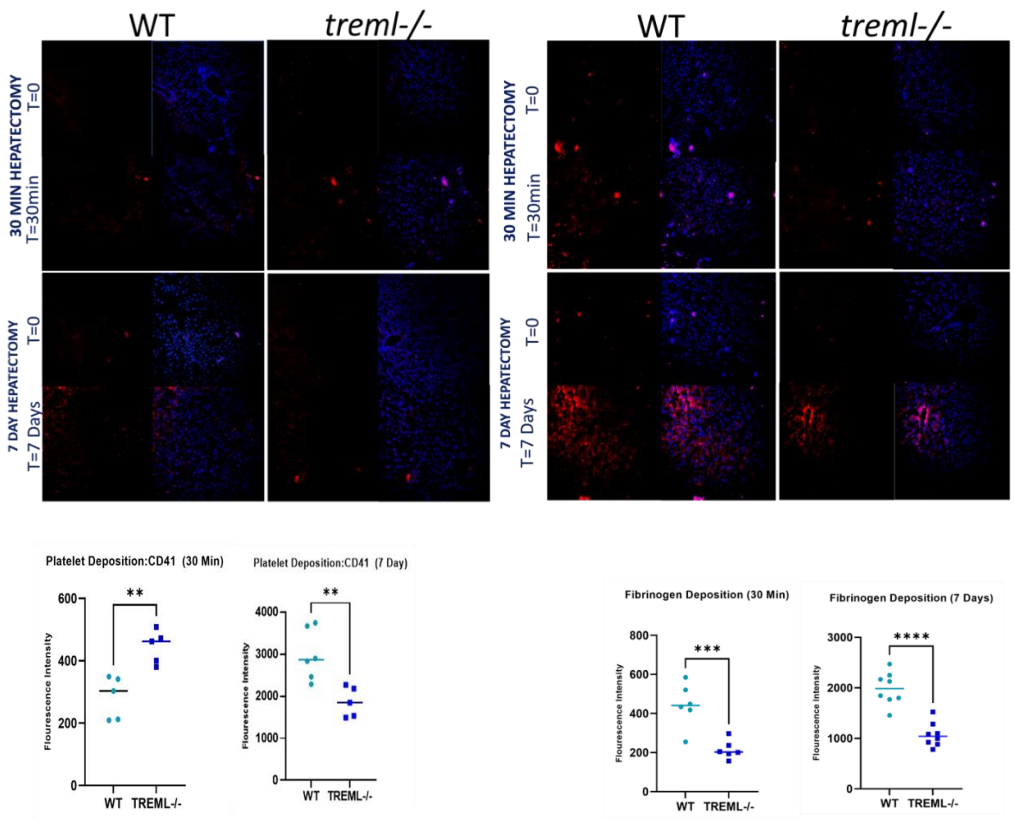


Figure 5.1: TLT-1 regulates fibrinogen and platelet accumulation post PHx. TLT-1 regulates fibrinogen and platelet accumulation post PHx.

(Platelet and fibrinogen staining with Alexa 594, DAPI = hepatocyte nuclei)

Quantification of platelets and fibrinogen in treml1-/- mice vs WT controls reveals that in treml1-/- mice initially have greater platelet accumulation in the acute phase response post injury but at the 7-day time point there are less platelets than in the WT mice, the initial acute phase platelet accumulation could be due to a compensatory mechanism and coagulation cascade activation post injury. The treml1-/- mice have less fibrinogen deposition in both the acute phase (30-minute time point) and 7 minute time point.

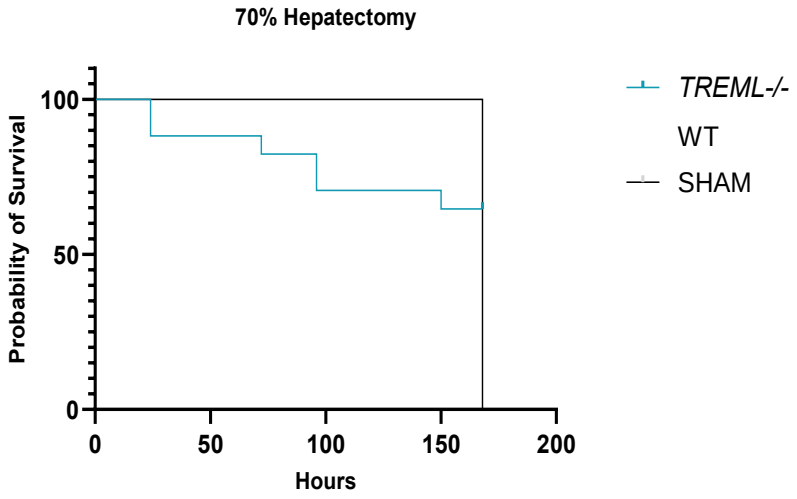


Figure 5.2: treml1$^{-/-}$ mice are more prone to mortality post PHx than WT mice, p ≤ 0.0060.

Survival curve for 7-day time point comparing WT vs *treml1*$^{-/-}$ mice, demonstrating that *treml1*$^{-/-}$ mice are more prone to mortality than WT mice, while SHAM controls all survive $p \leq 0.0060$.

Discussion

Recent studies have demonstrated that patients with low platelet counts have poor post operative recovery, moreover, those with low platelet counts had delayed post operative recovery of liver function, with studies strongly suggesting that activation of the coagulation pathways and platelet recruitment to be key elements promoting hepatic regeneration. [14] It has also been demonstrated that intrahepatic fibrinogen deposition promotes liver regeneration, and that low post operative plasma fibrinogen is associated with liver disease in a patient cohort. Platelet depletion in mice also delays regenerative capacity. Although PHx. has low mortality rates and advancement in surgery and post

operative care ensure that major resections have positive outcomes, there is still a percentage of patients who experience impaired hepatic function and delayed regenerative capacity.

TREM-Like Transcript-1 (TLT-1) is a type 1 receptor that, like binds fibrinogen and thus we wanted to investigate whether the interaction between platelets and fibrinogen though this receptor drives post operative fibrinogen deposition and promotes hepatic regeneration by using a 70% hepatic resection model. Our results support the idea that TLT-1 regulates platelet accumulation, subsequent fibrinogen deposition and survival. We propose that in the absence of TLT-1 the platelets are incapable of facilitating effective fibrinogen deposition to maintain homeostasis and repair, leading to increased morbidity and mortality.

The findings of our study provide valuable insights into the intricate relationship between platelet counts, hepatic regeneration, and postoperative outcomes following a 70% hepatic resection. Our investigation, employing wild type and treml1-/- mice, focused on platelet adhesion, fibrinogen deposition, and survival as primary endpoints. The results underscore the critical role of platelets and fibrinogen in hepatic tissue during the acute phase response, shedding light on potential factors influencing postoperative recovery.

Recent studies have underscored the significance of platelet counts in predicting postoperative outcomes. Our study aligns with this evidence, demonstrating that treml1-/- mice exhibit decreased platelet accumulation and fibrinogen deposition in hepatic tissue compared to wildtype controls. This observation suggests that TLT-1, a molecule associated with platelet function, plays a crucial role in regulating fibrinogen deposition

and platelet accumulation during the acute phase response. The initial increased platelet accumulation could be due to compensatory mechanisms, were platelets in the *treml1-/-* mice accumulate at the site of injury but are unable to bind fibrinogen as efficiently due to lack of TLT-1 receptors, thus recruiting more platelets to the site of injury. The implications of these findings extend beyond platelet biology, as they may have direct relevance to hepatic regeneration and postoperative recovery.

Furthermore, our investigation delves into the 7-day recovery period post-surgery, revealing an intriguing association between treml1 deficiency and increased susceptibility to mortality. Although the difference in mortality rates between treml1-/- mice and wild type controls did not reach statistical significance ($p=0.29$), the observed trend indicates a potential link between TLT-1 and postoperative survival. This finding highlights the need for further exploration into the molecular mechanisms underlying the observed mortality trends, as it may provide novel insights into factors influencing the long-term outcomes of hepatic resections.

The study also contributes to the existing body of knowledge regarding intrahepatic fibrinogen deposition and its role in promoting liver regeneration. Our results support previous research suggesting that fibrinogen deposition is a key element in hepatic regeneration, and alterations in this process may contribute to impaired postoperative recovery. The association between low postoperative plasma fibrinogen and liver disease in patients further emphasizes the clinical relevance of our findings.

Conclusion

In conclusion, our study underscores the importance of platelet function, specifically the role of TLT-1, in regulating fibrinogen deposition and platelet accumulation during hepatic regeneration. The observed mortality trends in treml1-/- mice over the 7-day recovery period also warrant further investigation into the molecular mechanisms governing postoperative survival. These findings contribute to the growing body of evidence linking platelet biology, fibrinogen deposition, and hepatic regeneration, providing valuable insights for improving postoperative outcomes, particularly in patients with low platelet counts

Bibliography

1. Rumgay, H. *et al.* Global burden of primary liver cancer in 2020 and predictions to 2040. *J Hepatol* 77, 1598–1606 (2022).

2. Nygård, I. E., Lassen, K., Kjaeve, J., Revhaug, A. & Wronski, M. Mortality and Survival Rates after Elective Hepatic Surgery in a Low-Volume Centre Are Comparable to Those of High-Volume Centres. *Article ID* 2012, (2012).

3. Lisman, T. & Porte, R. J. Blood Spotlight Mechanisms of platelet-mediated liver regeneration. (2016) doi:10.1182/blood-2016-04.

4. Groeneveld, D. *et al.* Intrahepatic fibrin(ogen) deposition drives liver regeneration after partial hepatectomy in mice and humans. (2019) doi:10.1182/blood-2018-08-869057.

5. Kholodenko, I. v & Yarygin, K. N. Cellular Mechanisms of Liver Regeneration and Cell-Based Therapies of Liver Diseases. (2017) doi:10.1155/2017/8910821.

6. Murata, S. *et al.* Platelets Promote Liver Regeneration in Early Period after Hepatectomy in Mice. doi:10.1007/s00268-006-0772-3.

7. Kirschbaum, M. *et al.* Transient von Willebrand factor-mediated platelet influx stimulates liver regeneration after partial hepatectomy in mice. *Liver International* 37, 1731–1737 (2017).

8. Understanding the marvels behind liver regeneration. WIREs Developmental Biology,

8(3)10.1002/wdev.340/https://wires.onlinelibrary.wiley.com/doi/epdf/10.1002/wdev.340.

9. Gustafson, E. *et al.* Exposure of von Willebrand Factor on Isolated Hepatocytes Promotes Tethering of Platelets to the Cell Surface. *Transplantation* 103, 1630–1638 (2019).

10. Oe, H., Kaido, T., Mori, A., Onodera, H. & Imamura, M. Hepatocyte growth factor as well as vascular endothelial growth factor gene induction effectively promotes liver regeneration after hepatectomy in Solt-Farber rats. *Hepatogastroenterology* 52, 1393–1397 (2005).

11. Matsuo, R. *et al.* Platelets Strongly Induce Hepatocyte Proliferation with IGF-1 and HGF In Vitro. *J Surg Res* 145, 279–286 (2008).

12. Starlinger, P. *et al.* Perioperative von Willebrand factor dynamics are associated with liver regeneration and predict outcome after liver resection. *Hepatology* 67, 1516–1530 (2018).

13. Morales-Ortíz, J. *et al.* Platelet-derived TLT-1 is a prognostic indicator in ALI/ARDS and prevents tissue damage in the lungs in a mouse model. *Blood* (2018) doi:10.1182/blood-2018-03-841593.

14. Alkozai, E. M. *et al.* Immediate postoperative low platelet count is associated with delayed liver function recovery after partial liver resection. *Ann Surg* 251, 300–306 (2010).

CHAPTER 6

CONCLUSIONS /FUTURE DIRECTIONS

The exploration of platelet receptors and their intricate interactions with fibrinogen has uncovered significant complexities in hemostasis, inflammation, and disease pathology[1–4]. The Triggering Receptor Expressed on Myeloid Cells-like transcript 1 (TLT-1) characterized as a platelet-specific receptor, has revealed multifaceted roles in various physiological and pathological contexts, ranging from cardiovascular diseases to COVID-19 complications and hepatic regeneration post-hepatectomy.

TLT-1 as a regulator of fibrinogen deposition during pathophysiological progression.

Several studies have demonstrated that fibrinogen deposition is increased with pathophysiological progression of disease. Based on this knowledge, we have looked at inflammatory and regenerative models (chapters 3 and 4) to determine whether TLT-1 regulates fibrinogen deposition during disease progression[5–13]. In atherosclerosis, knock out mice for TLT-1 have a smaller lesion in the aortic sinus. There seems to be no significant differences in macrophage quantification within the lesion, moreover plasma quantification of fibrinogen by western blot demonstrates that the TLT-1 knock out mice produce similar quantities of fibrinogen when compared to a wild type control, and thus the decreased fibrinogen deposition post atherosclerosis or LPS challenged inflammatory response or hepatic regenerative model is not due to decreased fibrinogen production, but rather due to decreased deposition at the inflammatory site. TLT-1's involvement in

lesion progression and regulation of fibrinogen deposition within atherosclerotic plaques highlight its significance as a potential therapeutic target in cardiovascular diseases. The findings from human coronary artery analysis and murine models underscore TLT-1's contribution to atherosclerotic plaque formation and its potential implications for disease management.

In the context of hepatic regeneration post-hepatectomy, TLT-1 emerges as a key player in orchestrating platelet-mediated processes crucial for postoperative recovery. Understanding TLT-1's role in hepatic regeneration offers promising avenues for enhancing postoperative outcomes and patient survival, particularly in the management of liver conditions such as cancer.

The use of Trem like transcript- 1, a platelet specific receptor, as a biomarker

P-selectin and Trem like transcript 1 (TLT-1) are two important markers of platelet activation. These molecules are involved in various aspects of platelet activation and play crucial roles in thrombosis, inflammation, and vascular pathology[14–18]. Their detection in circulation serves as indicators of platelet activation and may have diagnostic and prognostic implications in various diseases

Potential use of Trem like Transcript- 1 as a biomarker for COVID-19

In chapter 3, TLT-1's impact on platelet activation has been studied in the context of thromboinflammation, particularly in COVID-19 patients, where its levels correlated with disease severity and mortality risk[19–22]. The identification of TLT-1 as a sensitive marker for platelet activation and disease severity therefore extends beyond cardiovascular disease, as previously demonstrated by Morales et al. in a study supporting TLT-1s role in the progression of ARDS, and now supported by an ICU

COVID-19 study, which also manifests as ARDS[23]. These data underscore its potential utility in prognostic evaluations and risk assessment models, offering insights into the development of targeted therapeutic strategies.

1. Potential use of Trem like Transcript- 1 for CHF screening post cardiotoxicity

Esponda et al. emphasized the importance of sTLT-1 in acute coronary syndrome (ACS). Among 53 patients enrolled, the 19 experiencing cardiogenic chest pain exhibited significantly higher mean plasma sTLT-1 levels (4.644 ng/mL ± 1.277 SEM) compared to controls (1.007 ng/mL ± 0.098 SEM), indicating its potential as a functional measure of atherothrombosis in ACS ($P < 0.05$)[24]

A second study investigating the role of soluble TREM-like transcript 1 (sTLT-1) in cardiovascular disease (CVD) highlight its significance as a biomarker for disease severity and progression. In a study involved 117 human subjects, plasma sTLT-1 levels were significantly elevated in both clinical (2342 ± 184 pg/ml) and subclinical (1773 ± 118 pg/ml) CVD cases compared to healthy controls (461 ± 57 pg/ml). This elevation significantly correlated strongly with common risk factors for coronary artery disease (CAD). Additionally, ex vivo experiments demonstrated that sTLT-1 interacts with Fcɣ receptor I (FcɣRI) to activate inflammatory pathways, contributing to chronic inflammation and atherothrombosis[25].

Replicating the findings of Study 2, Study 3 reiterated the clinical relevance of sTLT-1 in CAD. Plasma sTLT-1 levels remained significantly elevated in clinical and subclinical CVD cases compared to controls, and strong correlations were observed with disease severity and risk factors for CAD[15]. A recent study by Moneam et al., published in 2024, further supports Esponda et als' results, where a cohort of 60 patients with ACS

were found to have elevated TLT-1 levels and a positive relationship with triglycerides and low-density lipoprotein (LDL)[26].

Overall, as demonstrated below (table 1) these studies underscore the clinical importance of sTLT-1 as a biomarker for CVD, providing valuable insights into its role in disease pathophysiology and progression (Table 1). With statistically significant associations observed between sTLT-1 levels and various aspects of CVD, including disease severity, risk factors, and clinical outcomes, sTLT-1 emerges as a promising target for diagnostic and therapeutic interventions in cardiovascular medicine. The above studies prompted the Washington laboratory to do a screening on 1510 patients with chronic heart disease to determine whether TLT-1 had a significant correlation with chronic cardiovascular events, interestingly s-TLT-1 levels could be correlated with congestive heart failure (CHF) in this cohort of patients[27].

PAPER / STUDY	YEAR	COHORT	SAMPLE	# PATIENTS	SUMMARY OF FINDINGS
1)Esponda et al	2014	ACS Patients	Plasma	19	A statistically significant difference exists between patients experiencing cardiogenic chest pain versus controls
2)Das et al	2019	CAD patients	Plasma	117	sTLT-1 levels are clinically related to CAD risk factors and correlates to disease severity and that the sTLT-1 interacts with $Fc\gamma R1$ to activate SYK pathway in macrophages
3)Shen et al	2021	CAD patients	Serum	83	sTLT-1 levels are is a marker of platelet activation and is postiively related to CAD
4)Bayron et al	2023	CAD patients	Plasma	1510	When patients were stratified based on sTLT-1 peak frequency distribution (544 pg/ml) a significant correlation with congestive heart failure was identified (OR =2.94; 1.040-8.282; p=0.042), which could be explained by LV dysfunction
5) Moneam et al.	2024	ACS	Serum	60	ACS is associated with increased level of sTLT-1, AND HAS A positive relationship with triglyceride (p=0.006), low density lipoprotein (LDL) (P=0.037) and neutrophil to lymphocyte ratio (NLR) (p=0.046)

Table 4.1: Studies showing correlation between s-TLT-1 levels and cardiovascular disease.

The utilization of biomarker or protein screening (that can be done in a laboratory setting) for the early detection and treatment of congestive heart failure (CHF) in

leukemia patients represents a pivotal advancement in cardiovascular care. A cornerstone feature of the pathophysiological progression of congestive heart failure is platelet dysfunction[28,29]. Patients with congestive heart failure are at increased risk of thromboembolisms, arrythmias and sudden cardiac death. Studies dated all the way back to 1970 have demonstrated greater platelet aggregation in patients with CHF. The altered hemodynamics in CHF, including stasis of blood flow, can lead to elevated platelet activation and aggregation. Several studies have demonstrated platelets have baseline activation during CHF, as shown by increased levels of procoagulant factors (tissue factor), platelet activation markers (s-P-Sel and s-TLT-1), plasma proteins that facilitate clot formation (fibrinogen)[30–33]. These data introduce the potential for a diagnostic screening criterion, where Troponin I detection indicate that there is cardiotoxicity, prompting a screening for a platelet activation marker (which would be sensitive to CHF), as a gold standard for early detection of cardiotoxicity and progression to congestive heart failure, respectively. Early diagnosis would allow for preventative treatment with medications such as B-Blockers, which have been shown to decrease incidence of LVEF dysfunction, heart failure and death[34].

Anthracyclines, widely used in hematological tumors, face limitations due to severe cardiotoxicity, causing progressive cardiomyopathy and, in severe cases, congestive heart failure. Heart failure incidence is significantly higher in cancer patients treated with anthracyclines for more than 5 years. Early detection of left ventricular dysfunction and cardiotoxicity is crucial to mitigate progression to congestive heart failure (CHF) in this population. There are currently no standardized methods for early detection of CHF in anthracycline induced cardiotoxicity. Using echocardiography,

Troponin-I (Trop-I), and NT-proBNP for early congestive heart failure (CHF) diagnosis presents challenges due to limited specificity and high costs.

Bayrón-Marrero et al. underscores the potential of incorporating a biomarker and an early detection screening protein, specifically soluble Trem-Like Transcript-1 (s-TLT-1) into clinical practice (Table 1). Trem-Like Transcript -1 is a platelet-specific receptor that is stored in the platelet alpha granules and released onto the platelet surface upon platelet activation[35]. Several studies have demonstrated that plasma levels of s-TLT-1 is a highly sensitive marker of platelet activation, even more so that s-P-sel, the most used marker of platelet activation[36]. Bayrón-Marrero et al analyzed data from 1510 patients with stable CAD and found that lower plasma sTLT-1 levels were significantly associated with left ventricular (LV) dysfunction (981.62 ± 1141 pg/mL vs. 1247.48 ± 1589 pg/mL; $p = 0.003$). Furthermore, stratification based on sTLT-1 levels revealed a significant association with congestive heart failure (OR = 2.94; 95% CI: 1.040-8.282; $p = 0.042$), particularly in patients with LV dysfunction, suggests its potential as a sensitive indicator of early cardiac impairment. More specifically, patients with s-TLT-1 levels exceeding 544 pg/mL exhibited a significant association with congestive heart failure, emphasizing the potential use of s-TLT-1 levels above 544 pg/mL as a warning signal for progression to congestive heart failure [27].

Early identification of cardiac dysfunction through s-TLT-1 measurement could enable timely intervention and tailored treatment strategies, thus mitigating the risk of CHF progression in leukemia patients. Integrating such a diagnostic screening criterion into routine monitoring protocols holds promise for enhancing patient outcomes, offering a proactive approach to CHF management in this vulnerable population. However, a

study measuring s-TLT-1 levels in leukemia patients has not yet been done, further research and validation of s-TLT-1 and other diagnostic screening methods may usher in a new era of precision medicine, transforming the landscape of CHF detection and treatment in leukemia patients.

Introduction: The classic and immune platelet bibliography:

1. Kaushansky, K. Thrombopoiesis. *Semin Hematol* 52, 4–11 (2015).
2. KICKLER, T. S. Platelet biology – an overview. *Transfusion Alternatives in Transfusion Medicine* 8, 79–85 (2006).
3. Broos, K., Feys, H. B., De Meyer, S. F., Vanhoorelbeke, K. & Deckmyn, H. Platelets at work in primary hemostasis. *Blood Rev* 25, 155–167 (2011).
4. Historical overview of the role of platelets in hemostasis and thrombosis | Haematologica. https://www.haematologica.org/article/view/1986.
5. Stalker, T. J., Newman, D. K., Ma, P., Wannemacher, K. M. & Brass, L. F. Platelet Signaling. *Handb Exp Pharmacol* 210, 59 (2012).
6. Li, X., Sim, M. M. S. & Wood, J. P. Recent Insights into the Regulation of Coagulation and Thrombosis. *Arterioscler Thromb Vasc Biol* 40, E119–E125 (2020).
7. Wahed, A. & Dasgupta, A. Essentials of Coagulation. *Hematology and Coagulation* 231–261 (2015) doi:10.1016/B978-0-12-800241-4.00015-2.
8. Nishikawa, M., Tanaka, T. & Hidaka, H. Ca2+ -calmodulin-dependent phosphorylation and platelet secretion. *Nature* 287, 863–865 (1980).
9. Starikova, E. A., Mammedova, J. T. & Porembskaya, O. Ya. Thrombin in the Crossroad Hemostasis and Inflammation. *J Evol Biochem Physiol* 59, 1710–1739 (2023).
10. Ballantyne, J. Serology | Overview. *Encyclopedia of Forensic and Legal Medicine* 53–63 (2005) doi:10.1016/B0-12-369399-3/00320-7.

11. Renschler, A. S. Fibrinogen (fibrin) degradation products/d-dimer. *Clinical Veterinary Advisor: The Horse* 933 (2011) doi:10.1016/B978-1-4160-9979-6.00393-7.

12. Chapin, J. C. & Hajjar, K. A. Fibrinolysis and the control of blood coagulation. *Blood Rev* 29, 17 (2015).

13. Mandel, J., Casari, M., Stepanyan, M., Martyanov, A. & Deppermann, C. Beyond Hemostasis: Platelet Innate Immune Interactions and Thromboinflammation. *Int J Mol Sci* 23, (2022).

14. Dib, P. R. B. *et al.* Innate immune receptors in platelets and platelet-leukocyte interactions. *J Leukoc Biol* 108, 1157–1182 (2020).

15. Guo, L. & Rondina, M. T. The Era of Thromboinflammation: Platelets Are Dynamic Sensors and Effector Cells During Infectious Diseases. *Front Immunol* 10, (2019).

16. Cicchinelli, S. *et al.* PAMPs and DAMPs in Sepsis: A Review of Their Molecular Features and Potential Clinical Implications. *Int J Mol Sci* 25, (2024).

17. Clemetson, K. J. & Clemetson, J. M. Platelet receptors. *Platelets* 169–192 (2019) doi:10.1016/B978-0-12-813456-6.00009-6.

18. Semple, J. W., Italiano, J. E. & Freedman, J. Platelets and the immune continuum. *Nat Rev Immunol* 11, 264–274 (2011).

19. Sonmez, O. & Sonmez, M. Role of platelets in immune system and inflammation. *Porto Biomed J* 2, 311–314 (2017).

20. Ludwig, N., Hilger, A., Zarbock, A. & Rossaint, J. Platelets at the Crossroads of Pro-Inflammatory and Resolution Pathways during Inflammation. *Cells* 11, (2022).

21. Hardy, A. R. *et al.* P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* 105, 3552–3560 (2005).

22. Konopatskaya, O. *et al.* PKCα regulates platelet granule secretion and thrombus formation in mice. *Journal of Clinical Investigation* 119, 399–407 (2009).

23. Zaid, Y., Senhaji, N., Naya, A., Fadainia, C. & Kojok, K. PKCs in thrombus formation. *Pathologie Biologie* 63, 268–271 (2015).

24. Harper, M. T. & Poole, A. W. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost* 8, 454–462 (2010).

25. Flevaris, P. *et al.* Two distinct roles of mitogen-activated protein kinases in platelets and a novel Rac1-MAPK–dependent integrin outside-in retractile signaling pathway. *Blood* 113, 893 (2009).

26. Smyth, S. S. *et al.* G-protein-coupled receptors as signaling targets for antiplatelet therapy. *Arterioscler Thromb Vasc Biol* 29, 449–457 (2009).

27. Bye, A. P., Unsworth, A. J. & Gibbins, J. M. Platelet signaling: a complex interplay between inhibitory and activatory networks. *Journal of Thrombosis and Haemostasis* 14, 918–930 (2016).

28. Perdomo, J. & Leung, H. H. L. Immune Thrombosis: Exploring the Significance of Immune Complexes and NETosis. *Biology (Basel)* 12, 1332 (2023).

29. Kornfeld, M. & Munsayac, J. R. Antiplatelet Medications. *Inpatient Anticoagulation* 47–65 (2022) doi:10.1002/9781118067178.ch3.

30. Fedan, J. S. Thrombosis-Embolism☆. *Reference Module in Biomedical Sciences* (2016) doi:10.1016/B978-0-12-801238-3.99531-X.

31. Shanti, R. M. & Aziz, S. R. Management of the Anticoagulated Patient. *Current Therapy in Oral and Maxillofacial Surgery* 203–207 (2011) doi:10.1016/B978-1-4160-2527-6.00026-8.

32. Risman, R. A., Kirby, N. C., Bannish, B. E., Hudson, N. E. & Tutwiler, V. Fibrinolysis: an illustrated review. *Res Pract Thromb Haemost* 7, 100081 (2023).

33. Sharp, A. A. Pathological fibrinolysis. *Br Med Bull* 20, 240–245 (1964).

34. Maron, B. A. & Loscalzo, J. The role of platelets in fibrinolysis. *Platelets, Second Edition* 415–430 (2006) doi:10.1016/B978-012369367-9/50783-7.

35. Venugopal, A. Disseminated intravascular coagulation. *Indian J Anaesth* 58, 603 (2014).

Introduction: Fibrinogen bibliography

1. White, N. J., Gonzalez, E., Moore, E. E. & Moore, H. B. Fibrinogen. *Trauma Induced Coagulopathy* 101–116 (2023) doi:10.1007/978-3-030-53606-0_8.
2. Fibrinogen - StatPearls - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK537184/#:~:text=Fibrinogen%20is%20a%20340kDa%20hexameric,or%20function%20of%20circulating%20fibrinogen.
3. ASTRUP, T. Fibrinolysis in the organism. *Blood* 11, 781–806 (1956).
4. Jackson, D. P., Beck, E. A. & Charache, P. Congenital Disorders of Fibrinogen. *Fed Proc* 24, 703–706 (2019).
5. Collen, D., Tytgat, G. N., Claeys, H. & Piessens, R. Metabolism and Distribution of Fibrinogen. I. FIBRINOGEN TURNOVER IN PHYSIOLOGICAL CONDITIONS IN HUMANS. *Br J Haematol* 22, 681–700 (1972).
6. Wahed, A. & Dasgupta, A. Essentials of Coagulation. *Hematology and Coagulation* 231–261 (2015) doi:10.1016/B978-0-12-800241-4.00015-2.
7. Gervois, P. *et al.* Negative Regulation of Human Fibrinogen Gene Expression by Peroxisome Proliferator-activated Receptor α Agonists via Inhibition of CCAAT Box/Enhancer-binding Protein β*. *Journal of Biological Chemistry* 276, 33471–33477 (2001).

8. Luyendyk, J. P., Schoenecker, J. G. & Flick, M. J. The multifaceted role of fibrinogen in tissue injury and inflammation. *Blood* 133, 511 (2019).
9. Zhang, J. Z. & Redman, C. Fibrinogen assembly and secretion. Role of intrachain disulfide loops. *J Biol Chem* 271, 30083–30088 (1996).
10. Zhang, J. Z. & Redman, C. M. Role of interchain disulfide bonds on the assembly and secretion of human fibrinogen. *Journal of Biological Chemistry* 269, 652–658 (1994).
11. Redman, C. M. & Xia, H. Fibrinogen biosynthesis: Assembly, intracellular degradation, and association with lipid synthesis and secretion. *Ann N Y Acad Sci* 936, 480–495 (2001).
12. Risman, R. A., Kirby, N. C., Bannish, B. E., Hudson, N. E. & Tutwiler, V. Fibrinolysis: an illustrated review. *Res Pract Thromb Haemost* 7, 100081 (2023).
13. Chapin, J. C. & Hajjar, K. A. Fibrinolysis and the control of blood coagulation. *Blood Rev* 29, 17 (2015).
14. Sharp, A. A. Pathological fibrinolysis. *Br Med Bull* 20, 240–245 (1964).
15. Maron, B. A. & Loscalzo, J. The role of platelets in fibrinolysis. *Platelets, Second Edition* 415–430 (2006) doi:10.1016/B978-012369367-9/50783-7.
16. Venugopal, A. Disseminated intravascular coagulation. *Indian J Anaesth* 58, 603 (2014).
17. Asakura, H. Classifying types of disseminated intravascular coagulation: clinical and animal models. *J Intensive Care* 2, (2014).

Introduction: Trem-like transcript 1 – platelet specific receptor Bibliography

1. Guo, L. & Rondina, M. T. The Era of Thromboinflammation: Platelets Are Dynamic Sensors and Effector Cells During Infectious Diseases. *Front Immunol* 10, 2204 (2019).
2. Gremmel, T., Frelinger, A. L. & Michelson, A. D. Platelet Physiology. *Semin Thromb Hemost* 42, 191–204 (2016).
3. Delabranche, X., Helms, J. & Meziani, F. Immunohaemostasis: a new view on haemostasis during sepsis. *Ann Intensive Care* 7, 117 (2017).
4. Gattis, J. L. *et al.* The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. *J Biol Chem* 281, 13396–13403 (2006).
5. Valance Washington, A., Quigley, L. & McVicar, D. W. Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. *Blood* 100, 3822–3824 (2002).
6. Allcock, R. J. N., Barrow, A. D., Forbes, S., Beck, S. & Trowsdale, J. The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. *Eur J Immunol* 33, 567–577 (2003).
7. Barrow, A. D. *et al.* Cutting Edge: TREM-Like Transcript-1, a Platelet Immunoreceptor Tyrosine-Based Inhibition Motif Encoding Costimulatory Immunoreceptor that Enhances, Rather than Inhibits, Calcium Signaling via SHP-2. *The Journal of Immunology* 172, 5838–5842 (2004).

8. Virmani, R., Farb, A. & Kolodgie, F. D. Histopathologic alterations after endovascular radiation and antiproliferative stents: similarities and differences. *Herz* 27, 1–6 (2002).

9. Washington, A. V. *et al.* A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. *Blood* 104, 1042–1047 (2004).

10. Colonna, M. TREMs in the immune system and beyond. *Nat Rev Immunol* 3, 445–453 (2003).

11. Hamburger, A. E., West, A. P. & Bjorkman, P. J. Crystal structure of a polymeric immunoglobulin binding fragment of the human polymeric immunoglobulin receptor. *Structure* 12, 1925–1935 (2004).

12. Valance Washington, A., Quigley, L. & McVicar, D. W. Initial characterization of TREM-like transcript (TLT)-1: A putative inhibitory receptor within the TREM cluster. *Blood* (2002) doi:10.1182/blood-2002-02-0523.

13. Bouchon, A., Dietrich, J. & Colonna, M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 164, 4991–4995 (2000).

14. Bouchon, A., Facchetti, F., Weigand, M. A. & Colonna, M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410, 1103–1107 (2001).

15. Dowal, L., Yang, W., Freeman, M. R., Steen, H. & Flaumenhaft, R. Proteomic analysis of palmitoylated platelet proteins. *Blood* 118, e62–e73 (2011).

16. Ferrer-Acosta, Y., González, M., Fernández, M. & Valance, W. A. Emerging Roles for Platelets in Inflammation and Disease. *J Infect Dis Ther* 2, (2014).

17. Yoon, S. H., Lee, Y. D., Ha, J., Lee, Y. & Kim, H. H. TLT-1s, alternative transcripts of triggering receptor expressed on myeloid cell-like transcript-1 (TLT-1), Inhibits the triggering receptor expressed on myeloid cell-2 (TREM-2)-mediated signaling pathway during osteoclastogenesis. *J Biol Chem* 287, 29620–29626 (2012).

18. Washington, A. V. *et al.* TREM-like transcript-1 protects against inflammation-associated hemorrhage by facilitating platelet aggregation in mice and humans. *J Clin Invest* 119, 1489–1501 (2009).

19. Morales-Ortíz, J. *et al.* Platelet-derived TLT-1 is a prognostic indicator in ALI/ARDS and prevents tissue damage in the lungs in a mouse model. *Blood* 132, 2495–2505 (2018).

20. Fong, K. P. *et al.* Deciphering the human platelet sheddome. *Blood* 117, e15-26 (2010).

21. Smith, C. W. *et al.* TREM-like transcript 1: a more sensitive marker of platelet activation than P-selectin in humans and mice. *Blood Adv* 2, 2072 (2018).

22. Tadokoro, S. *et al.* Talin binding to integrin beta tails: a final common step in integrin activation. *Science* 302, 103–106 (2003).

23. Hodivala-Dilke, K. M. *et al.* Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest* 103, 229–238 (1999).

24. Phillips, D. R. & Scarborough, R. M. Clinical pharmacology of eptifibatide. *Am J Cardiol* 80, 11B-20B (1997).

25. Scarborough, R. M. Development of eptifibatide. *Am Heart J* 138, 1093–1104 (1999).

26. Adgey, A. A. J. An overview of the results of clinical trials with glycoprotein IIb/IIIa inhibitors. *Am Heart J* 135, (1998).

27. Dai, A. *et al.* The Structure of a Full-length Membrane-embedded Integrin Bound to a Physiological Ligand. *J Biol Chem* 290, 27168 (2015).

28. Fibrinogen-binding properties of the human platelet glycoprotein IIb-=IIIa complex: a study using crossed-radioimmunoelectrophoresis - PubMed. https://pubmed.ncbi.nlm.nih.gov/6286013/.

29. Litvinov, R. I., Farrell, D. H., Weisel, J. W. & Bennett, J. S. The Platelet Integrin αIIbβ3 Differentially Interacts with Fibrin Versus Fibrinogen. *J Biol Chem* 291, 7858 (2016).

30. Huber, W. *et al.* Determination of kinetic constants for the interaction between the platelet glycoprotein IIb-IIIa and fibrinogen by means of surface plasmon resonance. *Eur J Biochem* 227, 647–656 (1995).

31. Washington, A. V., Esponda, O. & Gibson, A. Platelet biology of the rapidly failing lung. *Br J Haematol* 188, 641–651 (2020).

32. Ye, F., Liu, J., Winkler, H. & Taylor, K. A. Integrin αIIbβ3 in a Membrane Environment Remains the Same Height after Mn2+ Activation when Observed by Cryo-electron Tomography. *J Mol Biol* 378, 976 (2008).

33. Giomarelli, B. *et al.* Inhibition of thrombin-induced platelet aggregation using human single-chain Fv antibodies specific for TREM-like transcript-1. *Thromb Haemost* 97, 955–963 (2007).

34. Manfredi, B. *et al.* The Characterization of Monoclonal Antibodies to Mouse TLT-1 Suggests That TLT-1 Plays a Role in Wound Healing. *Monoclon Antib Immunodiagn Immunother* 37, 78 (2018).

35. Jessica, M. CONTROLS EARLY THROMBUS FORMATION AND STABILITY BY FACILITATING AIIBB3 OUTSIDE-IN SIGNALING IN MICE. *Int J Adv Res (Indore)* (2018) doi:10.21474/ijar01/7469.

36. Glembotsky, A. C. *et al.* Downregulation of TREM-like transcript-1 and collagen receptor α2 subunit, two novel RUNX1-targets, contributes to platelet dysfunction in familial platelet disorder with predisposition to acute myelogenous leukemia. *Haematologica* 104, 1244–1255 (2018).

37. Gros, A. *et al.* Single platelets seal neutrophil-induced vascular breaches via GPVI during immune-complex-mediated inflammation in mice. *Blood* 126, 1017–1026 (2015).

38. Barrachina, M. N. *et al.* GPVI surface expression and signalling pathway activation are increased in platelets from obese patients: Elucidating potential anti-atherothrombotic targets in obesity. *Atherosclerosis* (2019) doi:10.1016/j.atherosclerosis.2018.12.023.

39. Classen, A., Lloberas, J. & Celada, A. Macrophage Activation: Classical Vs. Alternative. *Methods in Molecular Biology* 531, 29–43 (2009).

40. Kopec, A. K. *et al.* Thrombin promotes diet-induced obesity through fibrin-driven inflammation. *Journal of Clinical Investigation* (2017) doi:10.1172/JCI92744.

41. Goerge, T. *et al.* Inflammation induces hemorrhage in thrombocytopenia. *Blood* 111, 4958–4964 (2008).

42. Lefrançais, E. *et al.* The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature* 544, 105–109 (2017).

43. Ortiz-Muñoz, G. *et al.* Cystic fibrosis transmembrane conductance regulator dysfunction in platelets drives lung hyperinflammation. *J Clin Invest* 130, 2041–2053 (2020).

44. Branfield, S. & Washington, A. V. Control the Platelets, Control the Disease: A Novel Cystic Fibrosis Hypothesis. *J Thromb Haemost* 18, 1531 (2020).

45. Derive, M. *et al.* Soluble TREM-like transcript-1 regulates leukocyte activation and controls microbial sepsis. *J Immunol* 188, 5585–5592 (2012).

46. Shi, R. *et al.* Expression level of 12-amino acid triggering receptor on myeloid cells-like transcript 1 derived peptide alleviates lipopolysaccharide-induced acute lung injury in mice. *Int J Mol Med* 41, 2159–2168 (2018).

47. Schmoker, A. M. *et al.* Defining the TLT-1 interactome from resting and activated human platelets. *J Proteomics* 215, (2020).

48. Xu, C. *et al.* Regulation of T cell receptor activation by dynamic membrane binding of the CD3epsilon cytoplasmic tyrosine-based motif. *Cell* 135, 702–713 (2008).

49. Duchardt, E., Sigalov, A. B., Aivazian, D., Stern, L. J. & Schwalbe, H. Structure induction of the T-cell receptor zeta-chain upon lipid binding investigated by NMR spectroscopy. *Chembiochem* 8, 820–827 (2007).

50. Aivazian, D. & Stern, L. J. Phosphorylation of T cell receptor zeta is regulated by a lipid dependent folding transition. *Nat Struct Biol* 7, 1023–1026 (2000).

51. Paddock, C. *et al.* Residues within a lipid-associated segment of the PECAM-1 cytoplasmic domain are susceptible to inducible, sequential phosphorylation. *Blood* 117, 6012–6023 (2011).

52. Doi, Y. *et al.* Normal development of mice and unimpaired cell adhesion/cell motility/actin-based cytoskeleton without compensatory up-regulation of ezrin or radixin in moesin gene knockout. *J Biol Chem* 274, 2315–2321 (1999).

53. Kawaguchi, K., Yoshida, S., Hatano, R. & Asano, S. Pathophysiological Roles of Ezrin/Radixin/Moesin Proteins. *Biol Pharm Bull* 40, 381–390 (2017).

54. Boulaftali, Y. *et al.* Platelet ITAM signaling is critical for vascular integrity in inflammation. *J Clin Invest* 123, 908–916 (2013).

55. Derive, M. *et al.* Soluble Trem-like Transcript-1 Regulates Leukocyte Activation and Controls Microbial Sepsis. *The Journal of Immunology* (2012) doi:10.4049/jimmunol.1102674.

56. Gattis, J. L. *et al.* The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. *Journal of Biological Chemistry* (2006) doi:10.1074/jbc.M600489200.

Conclusion Bibliography

1. Mandel, J., Casari, M., Stepanyan, M., Martyanov, A. & Deppermann, C. Beyond Hemostasis: Platelet Innate Immune Interactions and Thromboinflammation. *Int J Mol Sci* 23, (2022).
2. Broos, K., Feys, H. B., De Meyer, S. F., Vanhoorelbeke, K. & Deckmyn, H. Platelets at work in primary hemostasis. *Blood Rev* 25, 155–167 (2011).
3. Starikova, E. A., Mammedova, J. T. & Porembskaya, O. Ya. Thrombin in the Crossroad Hemostasis and Inflammation. *J Evol Biochem Physiol* 59, 1710–1739 (2023).
4. KICKLER, T. S. Platelet biology – an overview. *Transfusion Alternatives in Transfusion Medicine* 8, 79–85 (2006).
5. Collen, D., Tytgat, G. N., Claeys, H. & Piessens, R. Metabolism and Distribution of Fibrinogen. I. FIBRINOGEN TURNOVER IN PHYSIOLOGICAL CONDITIONS IN HUMANS. *Br J Haematol* 22, 681–700 (1972).
6. Hantgan, R. R., Stahle, M. C. & Lord, S. T. Dynamic Regulation of Fibrinogen: Integrin αIIbβ3 Binding. *Biochemistry* 49, 9217 (2010).
7. Gogstad, G., Brosstad, F., Krutnes, M., Hagen, I. & Solum, N. Fibrinogen-Binding Properties of the Human Platelet Glycoprotein IIb–IIIa Complex: A Study Using Crossed-Radioimmunoelectrophoresis. *Blood* 60, 663–671 (1982).
8. White, N. J., Gonzalez, E., Moore, E. E. & Moore, H. B. Fibrinogen. *Trauma Induced Coagulopathy* 101–116 (2023) doi:10.1007/978-3-030-53606-0_8.
9. Cerit, L. Fibrinogen and Atherosclerosis. *Arq Bras Cardiol* 108, 189 (2017).

10. Jackson, D. P., Beck, E. A. & Charache, P. Congenital Disorders of Fibrinogen. *Fed Proc* 24, 703–706 (2019).

11. Pieters, M., Wolberg, A. S. & Alisa Wolberg, C. S. Fibrinogen and fibrin: An illustrated review. *Res Pract Thromb Haemost* 3, 161–172 (2019).

12. Renschler, A. S. Fibrinogen (fibrin) degradation products/d-dimer. *Clinical Veterinary Advisor: The Horse* 933 (2011) doi:10.1016/B978-1-4160-9979-6.00393-7.

13. Luyendyk, J. P., Schoenecker, J. G. & Flick, M. J. The multifaceted role of fibrinogen in tissue injury and inflammation. *Blood* 133, 511 (2019).

14. Smith, C. W. *et al.* TREM-like transcript 1: a more sensitive marker of platelet activation than P-selectin in humans and mice. *Blood Adv* 2, 2072 (2018).

15. Shen, L. *et al.* P-selectin (CD62P) and soluble TREM-like transcript-1 (sTLT-1) are associated with coronary artery disease: a case control study. *BMC Cardiovasc Disord* 20, (2020).

16. Shen, L. *et al.* P-selectin (CD62P) and soluble TREM-like transcript-1 (sTLT-1) are associated with coronary artery disease: A case control study. *BMC Cardiovasc Disord* 20, 1–7 (2020).

17. Lipshultz, S. E. *et al.* Changes in Cardiac Biomarkers During Doxorubicin Treatment of Pediatric Patients With High-Risk Acute Lymphoblastic Leukemia: Associations With Long-Term Echocardiographic Outcomes. *J Clin Oncol* 30, 1042–1049.

18. Yu, J. *et al.* Advances in targeted therapy for acute myeloid leukemia. *Biomark Res* 8, 1–11 (2020).

19. Landi, A. & De Servi, S. The burden of thrombotic complications in critically ill patients with COVID-19: charting the uncharted. *Intern Emerg Med* 15, 893–895 (2020).

20. COVID-19-associated acute respiratory distress syndrome versus classical acute respiratory distress syndrome (a narrative review) - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8816697/.

21. Ragab, D., Salah Eldin, H., Taeimah, M., Khattab, R. & Salem, R. The COVID-19 Cytokine Storm; What We Know So Far. *Front Immunol* 11, (2020).

22. Islam, H., Chamberlain, T. C., Mui, A. L. & Little, J. P. Elevated Interleukin-10 Levels in COVID-19: Potentiation of Pro-Inflammatory Responses or Impaired Anti-Inflammatory Action? *Front Immunol* 12, 677008 (2021).

23. Morales-Ortíz, J. *et al.* Platelet-derived TLT-1 is a prognostic indicator in ALI/ARDS and prevents tissue damage in the lungs in a mouse model. *Blood* (2018) doi:10.1182/blood-2018-03-841593.

24. Esponda, O. L., Hunter, R., Río, J. R. R. Del & Washington, A. V. Levels of soluble TREM-Like transcript 1 in patients presenting to the emergency department with chest pain. *Clinical and Applied Thrombosis/Hemostasis* 21, 30–34 (2015).

25. Das, A. A. *et al.* Elevated level of circulatory sTLT1 induces inflammation through SYK/MEK/ERK signalling in coronary artery disease. *Clin Sci (Lond)* 133, 2283–2299 (2019).

26. Hussein, Y. *et al.* Soluble triggering receptor expressed on myeloid cells like transcript-1 (sTLT-1) levels in patients with acute coronary syndrome. *International Journal of Clinical and Diagnostic Pathology* 7, 08–12 (2024).

27. Bayrón-Marrero, Z. *et al.* The Characterization and Evaluation of the Soluble Triggering Receptor Expressed on Myeloid Cells-like Transcript-1 in Stable Coronary Artery Disease. *Int J Mol Sci* 24, (2023).

28. Chung, I. & Lip, G. Y. H. Platelets and heart failure. *Eur Heart J* 27, 2623–2631 (2006).

29. Chung, I., Choudhury, A. & Lip, G. Y. H. Platelet activation in acute, decompensated congestive heart failure. *Thromb Res* 120, 709–713 (2007).

30. Stumpf, C. *et al.* Enhanced levels of CD154 (CD40 ligand) on platelets in patients with chronic heart failure. *Eur J Heart Fail* 5, 629–637 (2003).

31. Chin, B. S. P. *et al.* Prognostic value of interleukin-6, plasma viscosity, fibrinogen, von Willebrand factor, tissue factor and vascular endothelial growth factor levels in congestive heart failure. *Eur J Clin Invest* 33, 941–948 (2003).

32. Gibbs, C. R., Blann, A. D., Watson, R. D. S. & Lip, G. Y. H. Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: Effects of angiotensin-converting enzyme inhibitor and β-blocker therapy. *Circulation* 103, 1746–1751 (2001).

33. Varo, N. *et al.* Soluble CD40L. *Circulation* 108, 1049–1052 (2003).

34. Bosch, X. *et al.* Enalapril and Carvedilol for Preventing Chemotherapy-Induced Left Ventricular Systolic Dysfunction in Patients With Malignant Hemopathies: The OVERCOME Trial (prevention of left Ventricular dysfunction with Enalapril

and carvedilol in patients submitted to intensive Chemotherapy for the treatment of Malignant hemopathies). *J Am Coll Cardiol* 61, 2355–2362 (2013).

35. Branfield, S. & Washington, A. V. The enigmatic nature of the triggering receptor expressed in myeloid cells -1 (TLT- 1). *Platelets* 32, 753 (2021).

36. TREM-like transcript 1: a more sensitive marker of platelet activation than P-selectin in humans and mice - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6113608/#:~:text=In%20conclusion%2C%20TLT%2D1%20is,shell%20of%20thrombi%20in%20vivo.