From the Department of Cardiology, Pulmonology, and Angiology at Heinrich Heine University Dusseldorf

S1P-receptor 1 Signaling reduces Arterial Thrombosis via Upregulation of Endothelial Thrombomodulin Expression

Dissertation

to obtain the academic title of Doctor of Philosophy (PhD) in Medical Sciences

from the Faculty of Medicine at Heinrich Heine University Dusseldorf

submitted by

Gabrielle Al Kassis

2026

As an inaugural dissertation printed by permission of the
Faculty of Medicine at Heinrich Heine University Düsseldorf
signed:
Dean: Prof. Dr. med. Nikolaj Klöcker
Examiner/s: Prof. Dr. Amin Polzin, PD Dr. Christian Buchbender

Zusammenfassung

Sphingosin-1-Phosphat (S1P) ist ein bioaktiver Lipidmediator, der eine wichtige und entscheidende Rolle im kardiovaskulären System spielt. Seine Beteiligung an der Blutgerinnung und Thrombusbildung bleibt jedoch umstritten. Thrombomodulin (TM) ist ein antithrombotischer Regulator, der von Endothelzellen (*endothelial cells,* ECs) exprimiert wird und eine zentrale Rolle in der Modulation der Gerinnung spielt. Der Einfluss von S1P auf die TM-Expression und seine Auswirkungen auf die Thrombosebildung sind bisher nicht vollstädig geklärt.

Wir stellten die Hypothese auf, dass S1P die Thrombozytenadhäsion und Thrombosebildung, durch eine Hochregulierung der endothelialen TM-Expression, reduziert. Daher untersuchten wir die Effekte von S1P auf die TM-Expression, die Thrombozytenadhäsion und -aktivierung *in vitro* und *ex vivo*, sowie die Thrombusbildung *in vivo*. Zusätzlich analysierten wir Sphingosinkinase-1-(SphK1)-defiziente Mäuse, um die physiologische Relevanz von endogenem S1P zu erforschen. Darüber hinaus untersuchten wir bei 74 Patienten mit kardiovaskulären Erkrankungen den Zusammenhang zwischen den S1P-Plasmaspiegeln und dem zirkulierenden Thrombin.

Unsere Ergebnisse zeigten, dass S1P die TM-Expression in ECs hochreguliert. Dieser Effekt wurde durch pharmakologische Inhibition des S1P-Rezeptor 1 (S1PR1) und der Phosphoinositid-3-Kinase (PI3K) abgeschwächt, was auf einen rezeptorvermittelten Signalweg hinweist. In Flusskammerexperimenten verringerte S1P die Thrombozytenadhäsion auf Endothelzellen. In Abwesenheit von Endothelzellen wurde die Thrombozytenaktivierung weder durch S1P, noch durch TM beeinflusst. Entsprechend zeigten SphK1-defiziente Mäuse, mit vermindertem S1P-Spiegel, eine reduzierte endothelilale TM-Expression und eine erhöhte arterielle Thrombusbildung *in vivo*. Eine Behandlung mit TM konnte diesen Effekt bei den Mäusen ausgleichen. Übereinstimmed damit, waren in einer Kohorte von 74 Patieneten mit kardiovaskulären Erkrankungen höhere S1P-Konzentrationen mit niedrigeren zirkulirenden Thrombinkonzentrationen assoziiert.

Zusammenfassend zeigte unsere Studie, dass S1P die Thrombusbildung durch einen endothelialen- und TM-abhängigen Mechanismus unterdrückt. Dieser Effekt wird durch die S1PR1- und PI3K-Signalwege vermittelt, die eine Hocheregulierung von TM fördern. Diese Erkenntnisse legen einen potenziellen therapeutischen Ansatz nahe, um dieThrombusbildung zu verhindern, ohne das Blutungsrisiko zu steigern.

Summary

Sphingosine-1-phosphate (S1P) is a bioactive lipid mediator that plays an important and critical role in the cardiovascular system. However, its involvement in coagulation and thrombus formation remains controversial. Thrombomodulin (TM), an antithrombotic regulator expressed by the endothelial cells (ECs), is a key modulator in thrombin activity. The influence of S1P on TM expression and its impact on thrombosis have not been fully elucidated.

We hypothesized that S1P reduces platelet adhesion and thrombus formation by upregulation of endothelial TM expression. Therefore, we evaluated S1P effects on TM-expression, platelet adhesion, activation *in vitro* and *ex vivo* as well as thrombus formation *in vivo*. In addition, we analyzed sphingosine kinase 1 deficient mice (SphK1$^{-/-}$) to explore the physiological relevance of endogenous S1P. In addition, we analyzed the association of plasma S1P levels and circulating thrombin in 74 patients with cardiovascular disease.

Our results demonstrate that S1P upregulates TM expression in ECs. This effect was blunted by pharmacological S1P receptor 1 (S1PR1)- and phosphoinositide 3-kinase (PI3K) inhibition, indicating a receptor-mediated signaling pathway. In flow chamber experiments, S1P reduced platelet adhesion on ECs. In the absence of ECs, platelet activation wasn´t affected either by S1P or TM. Consistently, SphK1$^{-/-}$, having reduced S1P level, showed decreased endothelial TM expression and increased arterial thrombus formation *in vivo*. So, TM treatment could rescue these mice. In line, in an all-comer cohort of 74 patients with cardiovascular disease, higher S1P concentrations were associated with lower circulating thrombin concentrations.

In conclusion, our study reveals that S1P suppresses thrombus formation through an endothelium- and TM- dependent mechanism. This effect is mediated by S1PR1 and PI3K signaling promoting TM upregulation. These findings suggest a future therapeutic approach to prevent thrombus formation without enhancing the bleeding risk.

List of abbreviations

AC: Adenyl Cyclase

ACD: acid citrate dextrose

ADP: adenosine diphosphate

Akt: Aktin

APC: Activated protein C

apoM: apolipoprotein M

ARIC: Atherosclerosis Risk in Communities

ATP: adenosine triphosphate

BMI: body mass index

BW: body weight

CAD: coronary artery disease

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

cGMP: cyclic guanosine monophosphate

CRP: C-reactive protein

CTLD: C-type lectin domain

DYN/cm²: dyne per square centimeter

ECs: endothelial cells

EGF: epidermal-growth-factor

EPCR: endothelial protein C receptor

ER: endoplasmic reticulum

eNOS: endothelial nitric oxide synthase

E-selectin: endothelial selectin

FACS: fluorescence activated cell sorting

FX: factor X

GFR: glomerular filtration rate

GPCRs: G protein-coupled receptors

GPIb/IX/V: platelets glycoprotein complex

GTP: guanosine triphosphate

$HbA1_c$ = glycated hemoglobin

HBSS: HEPES buffered saline solution

HDL: high density lipoproteins

HUVECs: human umbilical vein endothelial cells

i.p.: intraperitoneal

i.v.: intravenous

ICAM-1: intercellular adhesion molecule-1

IL: interleukin

IP3: inositol triphosphate

IRI: ischemia reperfusion injury

IVM: intravital microscopy

KLF: Kruppel-like factor

KO: knockout

LC-MS/MS: liquid chromatography-tandem mass spectrometry

LDL: low-density lipoprotein

LPPs: lipid phosphate phosphatases

MAPK: mitogen-activated protein kinase

MI: Myocardial infarction

MFI: mean fluorescence intensity

MMP: matrix metalloproteinases

MS: mass spectrometry

MW: molecular weight

NF- κB: nuclear factor kappa-light-chain-enhancer of activated B cells

OAC: oral anticoagulation

PAR-1: protease-activated receptor 1

PAR-4: protease-activated receptor 4

PARs: protease activated receptors

PBS: Dulbecco´s Phosphate Buffered Saline

PCI: percutaneous coronary intervention

PF4: Platelet factor 4

PGI2: prostacyclin

PI3K: phosphoinositide 3-kinase

PIP2: phosphatidylinositol-4,5-biphosphate

PIP3: phosphatidylinositol-3,4,5 triphosphate

PKC: protein kinase C

PL: Phospholipid

PPI: proton pump inhibitor

PRP: platelet rich plasma

P-selectin: platelet selectin

PSGL-1: P-selectin ligand-1

PTEN: phosphatase and tensin homolog

RT: room temperature

rTM: recombinant thrombomodulin

S1P: Sphingosine-1-phosphate

S1PR: sphingosine-1-phosphate receptor

Ser: Serine

SphK1: sphingosine kinase 1

SphK1$^{-/-}$: Sphingosine 1 kinase deficient mice

SphK2: sphingosine kinase 2

SPL: S1P Lyase

SPP: Sphingosine-1-phosphate phosphatase

SRM: selected reaction monitoring

sTM: soluble thrombomodulin

TAFI: thrombin-activable fibrinolysis inhibitor

TATC: Thrombin-Antithrombin Complex

TF: tissue factor

TG: triglycerides

Thr: threonine

TM: thrombomodulin

TM-AB: thrombomodulin neutralizing antibody

TNFα: Tumor necrosis factor α

Trap-6: thrombin receptor activating peptide-6

TxA_2: thromboxane A_2

VCAM-1: vascular cell adhesion molecule-1

VSMC: Vascular smooth muscle cell

VWF: von Willbrand factor

α-granules: alpha granules

δ-granules: dense granules

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 Sphingosine-1-phosphate (S1P)

1.1.1 Introduction

S1P (figure 1) is a signaling sphingolipid, synthetized by the enzymes sphingosine kinases 1 and 2 (SphK1 and SphK2), and plays multiple roles in regulating cardiovascular function [1]. S1P influences diverse cellular activities and biological processes such as cellular growth, proliferation, migration and apoptosis. These functions occur primarily through S1P binding to G protein-coupled receptors (GPCRs). There are five different S1P receptors (S1PRs): S1PR1, S1PR2, S1PR3, S1PR4 and S1PR5 [1].

Through both paracrine and endocrine signaling, S1P influences various biological processes, including the initiation of intracellular signaling pathways, regulation of immune cell trafficking (T- and B-cells migration), and vascular development. It also contributes to angiogenesis, skin cells proliferation and vascular stability and permeability [2].

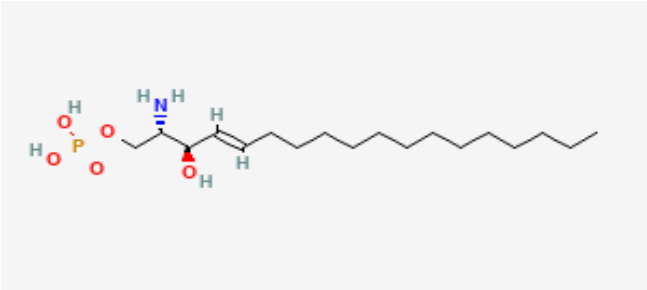

Figure 1: Sphingosine-1-phosphate structure.

Figure 1 represents S1P chemical structure. Its molecular formula is $C_{18}H_{38}NO_5P$.

1.1.2 S1P biosynthesis and location:

S1P predominantly occurs in the blood and the lymph. Lymph contains abundant S1P, forming a steep gradient between lymphoid tissues and circulation, which is critical for immune cell trafficking [3]. In the bloodstream, most S1P is transported by serum albumin increasing S1P solubility [4] and by apolipoprotein M (apoM) [5] enriched in high density lipoproteins (HDL) [6]. Its concentration in the plasma is 0.1 to 1.2 µM [1].

After S1P production, endothelial cells (ECs) release some of it in the plasma. Erythrocytes represent the principal source of plasma S1P, accounting for approximately 50 % of the circulating pool, with platelets also contributing [4]. There are other S1P sources playing a limited role in S1P maintenance like neutrophils and macrophages [4].

S1P is synthesized intracellularly through the breakdown of ceramide by ceramidases, leading to sphingosine production, which is subsequently phosphorylated by SphK to catalyze S1P generation. Intracellular S1P levels are maintained and regulated by S1P phosphatases and S1P lyase (SPL), leading to S1P irreversible degradation into hexadecenal and phosphoethanolamine. Or S1P can undergo reversible dephosphorylation catalyzed by sphingosine-1-phosphate phosphatases (SPPs) [7, 8].

In the extracellular environment [9], degradation of S1P is accomplished by lipid phosphate phosphatases (LPPs) isoforms (LPP1 and LPP3) [7, 10] and plays a crucial role in regulating S1P´s circulating level [8].

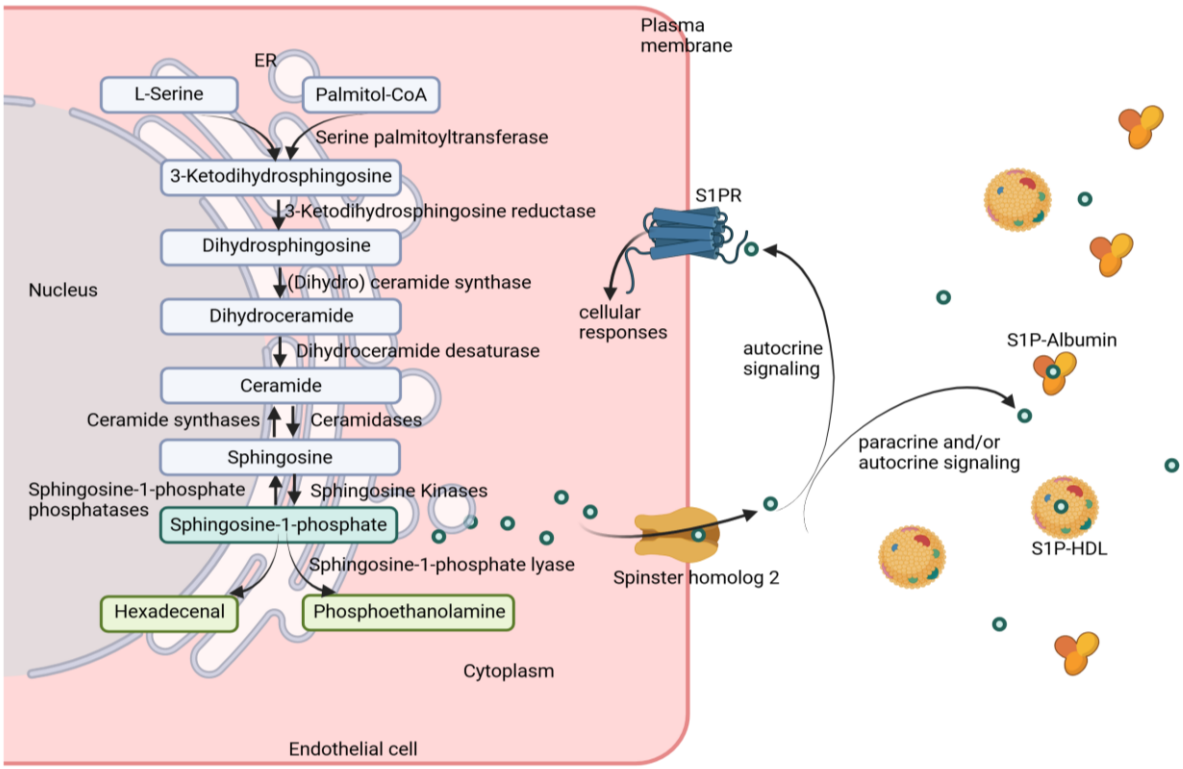

Figure 2: Sphingolipid metabolism and S1P transport.

The biosynthetic pathway of sphingolipids starts in the endoplasmic reticulum (ER), where L-serine condenses with palmitoyl-CoA to yield 3-ketodihydrosphyngosine. This molecule is subsequently reduced to dihydrosphingosine, which is then acylated by ceramide synthases to produce dihydroceramide. The latter is further desaturated by dihydroceramide desaturase to generate ceramide. Acting as a central precursor, ceramide gives rise to a variety of complex sphingolipids, including sphingomyelin (not mentioned in figure 2), and also contributes to the formation of S1P (adapted from [5]) and leads to S1P production. S1PR: Sphingosine-1-phosphate receptor, S1P: Sphingosine-1-phosphate, HDL: high density lipoproteins, ER: endoplasmic reticulum.

1.1.3 S1P receptors and functions

S1P binds to and activates a family of five S1P-specific GPCRs (table 1) [11]. Distinct subtypes of S1PRs are distributed across tissues: S1PR1, S1PR2 and S1PR3 are mainly found in the cardiovascular system, whereas S1PR4 is primarily associated with the lymphatic system and S1PR5 is predominantly expressed in the immune and nervous systems [1, 12]. As known, in cardiomyocytes, S1P signaling is largely driven by the high expression of S1PR1 [13], while cardiac fibroblasts predominantly expresses S1PR3 [14]. The vascular smooth muscle cells (VSMCs) exhibit elevated expression levels of S1PR2 and S1PR3, meanwhile the expression of S1PR1 remains comparatively low. Meanwhile S1PR1 is highly expressed in the ECs, followed by S1PR3, and rarely S1PR2 [1]. GPCR signaling occurs through cytoplasmic G proteins, which are composed of α-, β-, and γ-subunits. The structural and functional properties of these proteins are defined by α-subunit, leading to their categorization into G_i, G_s, G_q, and $G_{12/13}$ families. These G proteins may operate independently, antagonistically or synergistically [5]. Many signaling pathways are activated by these receptors after S1P activates them.

For example, S1PR1 exclusively couples with Gαi, which, upon receptor activation, inhibits adenylate cyclase (AC), leading to a reduction in cyclic adenosine monophosphate (cAMP) levels. Additionally, activation of Gαi also promotes signaling through protein kinase C (PKC) isoforms as well as ERK1/2 pathways [15]. In contrast, S1PR2 and S1PR3 can couple not only with Gαi but with Gαq also and with Gα12/13, triggering respectively phospholipase C (PLC) activation and RhoA exchange factor signaling [1, 16].

S1P can also lead to phosphoinositide 3-kinase (PI3K) activation. On the inner leaflet of the plasma membrane, G_i mediated activation of PI3K leads to the conversion of phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 leads to the phosphorylation and activation of aktin (Akt) promoting a variety of cellular responses and physiological processes such as angiogenesis and immune cell trafficking as previously mentioned [17-20]. For example, in ECs, activation of Gi and Akt-dependent phosphorylation of endothelial nitric oxide synthase (eNOS) results in full eNOS activation and subsequent nitric oxide (NO) production [20]. Through S1PR1 and S1PR3 signaling, S1P stimulates PI3K-Akt phosphorylation, thereby contributing to this pathway [7].

Besides its role by leading NO production and PI3K synthesis in the endothelium, S1P also influences the endothelial glycocalyx in different ways. Ye and colleagues [21] found that S1P prevents glycocalyx shedding and preserves its structure. This preservation is mainly supported and protected by the presence of S1PR1. Moreover, S1P suppresses the role of

matrix metalloproteinases (MMP), enzymes degrading the extracellular matrix components. Thus, S1P does not just maintain the endothelial glycocalyx but its treatment restores the glycocalyx via PI3K. Another study [22], showed that the presence of PI3K inhibitor (LY294002) abolished the rescuing effect of S1P on syndecan-1, heparan sulfate and chondroitin sulfate. This explains that PI3K pathway is required for S1P to stimulate the endothelial glycocalyx restoration.

To summarize, S1P underscore a crucial role in the regulation of cell proliferation by promoting cell cycle progression of different cell types through the S1PRs which is important for tissue growth, regeneration and development [23]. As it also modulates cell migration, by acting as chemoattractant for immune and ECs for the immune trafficking and vascular development [24] for better immune surveillance and wound healing. Moreover, S1P regulated vascular permeability by activating the S1PRs and enhancing the endothelial barrier integrity under certain conditions to prevent leakage [25]. S1P is a key regulator in angiogenesis, promoting ECs development and vessel maturation in coordination with growth factors [26].

Table 1: S1P signaling in different physiological functions (modified from [7, 27]).

Tissue	Receptors	Signaling pathways	Function
Vasculature	S1PR1	$Gi_{\beta\gamma}$/Ras/MAPK; Gi_{α}/Rho/Rac/GTPase-regulated Kinases	Reduces vascular permeability
		Gi/PI3K/Akt/Rac/eNOS activation	Promotes vascular stability, angiogenic growth and endothelial survival
		Gi/PLC/PKC/ intracellular Ca^{2+}	Induces lymph angiogenesis
		$G_{12/13}$/Rho/ROCK/PTEN	Elevates vascular permeability
		$G_{12/13}$/Akt-eNOS suppression	Maintains vascular barrier in anaphylaxis
	S1PR2	Gq/PLC/IP3/Ca^{2+} release	Induces vascular contraction
		$G_{12/13}$/Rac GTPase inhibition	Suppresses angiogenesis and morphogenesis
		Gq/G13/ RhoA/Stress fibers	Drives morphogenic and angiogenic remodeling
	S1PR3	RhoA/$ROCK_{1/2}$ signaling	Increases the endothelial permeability
		Gi/P13K/Akt/eNOS/NO release	Mediates vasorelaxation
		Gi/Rho/PLC/Ca^{2+} increase	Promotes vasoconstriction
Immune System	S1PR1	Akt/mTOR/TGFβ-Smad3 suppression	Regulates T-cell homeostasis
	S1PR2	$G_{12/13}$/Rho/ROCK	Regulates B cell survival
	S1PR5	$G_{12/13}$/Rho/ROCK	Restricts migration of immature oligodendrocytes
Nervous system	S1PR5	Gi/Akt	Enhances mature oligodendrocytes survival
	S1PR5	$G_{12/13}$/RhoA/Lim Kinase	Guides retinal ganglionic cell axonal growth and navigation
Heart	S1PR1	Gi/PI3K/Akt/Rac/eNOS	Protects cardiomyocytes and promotes coronary microvascular function [28].
	S1PR3	Gq/PLC/IP3/Ca^{2+} release	Modulates cardiac contractility
		Gi/PI3K/Akt $G_{12/13}$/Rho/ROCK	Contributes to ischemia-reperfusion injury (IRI) signaling and remodeling [29].

1.1.4 S1P role in the cardiovascular system and diseases

S1PR1 is predominantly present in cardiomyocytes, where it is essential for heart development, regulation of heart rate and pathological cardiac remodeling [30]. S1PR3, which shows the highest expression in cardiac tissue, together with other S1PRs subtypes, contributes to the control of cardiac contractility, calcium dynamics and additional regulatory processes [8].

Besides its role in cardiomyocytes, S1P supports the following roles:

1. Cardioprotection

In ischemia reperfusion injury (IRI), S1P contributes to the regulation of cell survival, vascular stability and inflammatory signaling. By influencing apoptotic and inflammatory pathway at the site of injury, it helps reduce endothelial and mitochondrial dysfunction [7]. IRI itself describes a pathological condition in which tissue damage arises from an initial restriction of blood flow, followed by the re-establishment of circulation and perfusion restoration [31].

2. Vascular tone and endothelial function:

Vascular ECs express high level of S1PR1, S1PR2 and S1PR3. These receptors are critically involved in maintaining the endothelium barrier function by activating cell-cell interactions. Moreover, S1P can cause vasodilation or vasoconstriction depending on the activated receptor. It is able to activate vasorelaxation by eNOS/NO in ECs and

vasoconstriction generated by RhoA/ROK pathways in VSMCs, affecting the vascular tone (figure 3) [6].

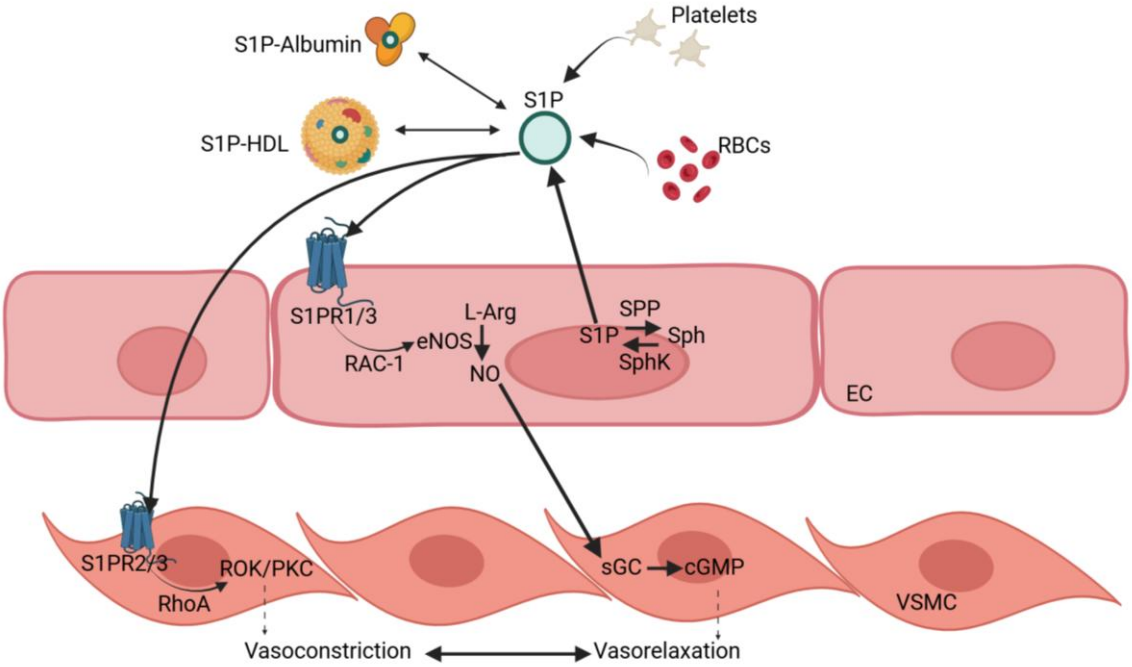

Figure 3: Schematic representation of S1P leading to the modulation of vascular tone.

In the bloodstream, S1P interact with S1PR1 and S1PR3 on ECs, triggering RAC-1 signaling that stimulates NO production. In VSMCs, NO facilitates vasorelaxation through cyclic guanosine monophosphate (cGMP) synthesis. On the other hand, S1P also activates S1PR2 and S1PR3 receptors on VSMCs, initiating the RhoA/ROK/PKC signaling cascade, which contributes vasoconstriction. The overall vascular effect of S1P is determined by the interplay between these contrasting mechanisms-vasoconstriction and vasodilation-which are influenced by factors such as subtype expression, S1P levels and the specific tissue environment (modified from [6]). S1PR: Sphingosine-1-phosphate receptor, S1P: Sphingosine-1-phosphate, EC: endothelial cell, HDL: high density lipoproteins, RBCs: red blood cells, L-Arg: L-Arginine, Sph: Sphingosine, SphK: Sphingosine kinase, eNO: endothelial nitric oxide, VSMC: vascular smooth muscle cells, cGMP: cyclic guanosine monophosphate, sGC: soluble guanylyl cyclase, PKC: protein kinase C.

3. Immune and inflammatory modulation

S1P restricts the circulation of lymphocytes, leading to new therapeutic opportunities for the treatment of immunosuppressive and inflammatory disorders. S1PR1 plays a key role in directing T cell trafficking during various stages of their development and activation. In addition, binding of S1P to S1PR1 enhances chemokine and cytokine production under conditions of tissue injury, thereby promoting immune cell infiltration and fibrosis [32].

1.1.5 Pathological implications

S1P receptors represent promising therapeutic targets for the prevention and management of major cardiovascular diseases, including hypertension, myocardial infarction (MI), stroke and heart failure [33].

For instance, signaling through the cardiac SphK1/S1P/S1PR1 axis modulates proinflammatory responses in cardiomyocytes, thereby contributing to improved post-MI remodeling and cardiac function [34]. In coronary artery disease (CAD) patients, elevated serum S1P levels correlate with the severity of arterial stenosis [8]. Conversely, in heart failure patients with reduced ejection fraction, plasma S1P concentrations are lower compared to those with mildly impaired left ventricle ejection fraction [8]. In the context of hypertension, S1P exerts blood pressure-lowering effects by stimulating S1PR1-dependent NO release [8].

Besides human tests, many mice studies showed the importance of S1P. Polzin and colleagues [35] found that increasing S1P levels in mice led to improved cardiac repair and remodeling following MI, showing a protective role for S1P in heart injury. In another study [36], IRI was conducted on SphK1-Knockout (KO) mice, having 41 % less S1P than normal mice. After inducing the injury, Sphk1-KO mice developed bigger infarcts in their hearts compared to normal mice. This study supported the conclusion that S1P, secreted from Sphk1, is cardioprotective and without it, the heart can develop more serious damage.

1.1.6 S1P clinical application

Nowadays S1P modulators are used as clinical treatment in case of multiple sclerosis. Based on the World Health Organization, "*multiple sclerosis affects function in cognitive, emotional, motor, sensory, or visual areas and occurs as a result of a person's immune system attacking their brain and spinal cord*" [37]. Thus, one of the key functions of S1PR1 is to help lymphocytes B and T exit the lymph nodes into the bloodstream [37]. Behrangi and colleagues [37] explained that Fingolimod (FTY720) as medication is not active in the original form. In the body FTY720 is phosphorylated by SphK2 to its active mode, Fingolimod-phosphate (FTY720-P). FTY720-P will bind to the S1PR1 and act as agonist meanwhile it is turning off the receptors, not by blocking it, but by removing it from the cell surface. In this case the lymphocytes cannot leave the lymph nodes, and they are trapped inside. This leads to less lymphocytes in the blood and less immune activity in the central nervous system (CNS). As clinical application, Fingolimod reduces the lymphocytes migration leading to less inflammation in the CNS. This makes Fingolimod a good treatment [37]. However, Fingolimod was approved in US in 2010 and in EU in 2011 [38]. Besides Fingolimod, there's different treatments like Ozanimod, Siponimod and Ponesimod.

1.2 Hemostasis

Physiological hemostasis is a tightly regulated process that depends on the coordinated interaction among platelets, ECs, immune cells and coagulation factors. These interactions lead to the formation of an initial platelet plug, followed by the development of a localized thrombus at the vascular injury site [39]. The clot functions to seal the site of injury, limiting blood loss and preventing further bleeding while tissue repair is initiated. As healing progresses, the clot undergoes gradual remodeling and eventually dissolves, allowing restoration of normal tissue structure at the damaged area [40]. Hemostasis is composed of several different steps, each of which is explained in the following sections.

1.2.1 Primary hemostasis

The primary hemostasis represents the physiological response to vascular injury and is driven by interactions between platelets, subendothelial adhesive proteins such as collagen and von Willebrand factor (VWF), along with circulating fibrinogen. It depends on intricate signaling cascades and the expression of distinct glycoproteins on the platelet surface, ultimately resulting in the formation and stabilization of the platelet plug reinforced by fibrin network.

1.2.1.1 Platelets

Platelets are small cells, having a discoid shape in the resting phase, produced by the megakaryocytes´ cytoplasm. Their production takes place in the bone marrow by thrombopoiesis or production of platelets. The hematopoietic cells are differentiated into polyploid megakaryocytes (50-1000 µm in diameter) followed by proplatelets formation, a long-branching cytoplasmic protrusions. Thrombopoiesis is driven by different transcription factors contributing to the redistribution of the organelles, granules and vesicular structures from the megakaryocytes to the proplatelets. Afterwards, the platelets will be released in the circulation system as the bone marrow is a highly vascularized tissue characterized by dense cellular organization [41].

Platelets are metabolically active anucleate cells, that communicate with their surroundings by engaging surface glycoprotein receptors with corresponding ligands. In adults, the normal platelet count range is 150 to 400 x 10^9/L [39]. These cells measure approximately 2 to 4 µm in diameter and circulate in the bloodstream with a lifespan of 7 to 10 days, after which they are eliminated in the spleen or in the liver [41].

The platelet-mediated response to endothelial or vascular injury proceeds through a sequence of steps, beginning with adhesion to the exposed sub-endothelium, followed by intracellular signaling, morphological changes, activation accompanied by granule secretion, and culminating in aggregation to close the damaged vessel and limit blood loss.

Beyond hemostasis, platelets contribute to normal physiology as well as to hemorrhagic, thrombotic, immune and inflammatory disorders [39]. They can exist in multiple functional states and interact with various cell types, including immune cells and VSMCs, thereby exerting diverse regulatory roles [42].

1.2.1.2 Platelet activation and adhesion

Usually in resting conditions prostacyclin (PGI2) and NO are secreted and released by the endothelium leading to platelet aggregation inhibition [43]. However, when vascular injury occurs, like a rupture of an atherosclerosis plaque, subendothelial collagen becomes exposed. In response, platelets migrate toward the injured vascular region and undergo activation, leading to the exposure of glycoprotein complex GPIb/IX/V on their surface. These glycoproteins facilitate platelets adhesion to the injured vessel wall through interaction with VWF produced by ECs [39]. After the formation of platelet-collagen bridge, the exposed collagen establishes direct binding with the platelet GPIa/IIa and GPVI receptors [43].

VWF molecule is formed by disulphide-linked multimers with up to 20000 kDa [44]. Once released into the plasma, the enzyme ADAMTS13 breaks down the molecular weight multimers into smaller fragments. This interaction reduces VWF ability to bind to platelet receptors and limits the spontaneous platelet aggregation [45]. The exposed collagen fibers on the endothelium surface bind to the platelets via GPIa/IIa and GPVI [43, 46], with the triple helical structure of collagen recognized by these receptors [47, 48]. Upon collagen binding, these receptors trigger intracellular metabolic cascade that activate GPIa/IIa, promoting platelet crosslinking through fibrinogen binding (figure 4).

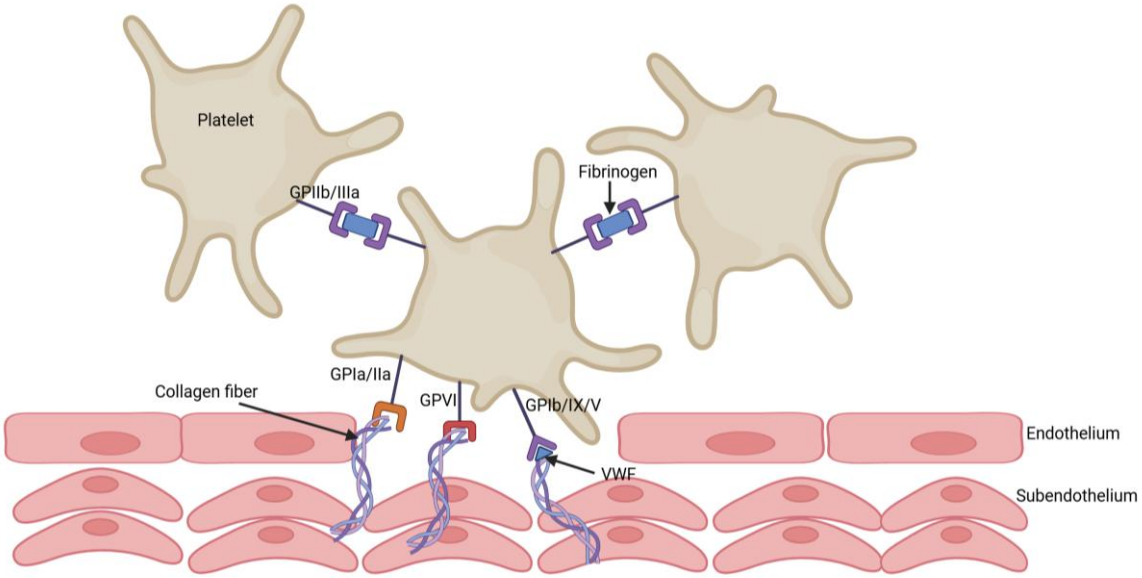

Figure 4: Schematic diagram of platelets adhesion on defect endothelium.

Subendothelial collagen fibers are exposed to the endothelial surface after an endothelial defect. Collagen fibers carry the VWF and platelets bind to collagen fibers and to VWF via glycoprotein Ia/IIa (GPIa/IIa) and glycoprotein Ib/IX/V (GPIb/IX/V). The resulting platelet activation leads to the activation of glycoprotein IIb/IIIa (GPIIb/IIIa). Fibrinogen fibers can bind to this receptor and this is the initial step of platelet activation and aggregation (modified from doctoral thesis of Dr. Marcel Benkhoff [49]).

The interaction between collagen and platelet receptors initiates platelet activation (figure 5), triggering release of various mediators such as adenosine diphosphate (ADP), serotonin, thrombin and epinephrine. These molecules activate GPCRs on platelets, leading to an increase in cytosolic calcium levels and initiation of intracellular signaling pathways. As a result, platelets undergo shape changes and integrin activation, enhancing their adhesive capacity. Within activation, platelets secrete the content of alpha granules (α-granules) and dense granules (δ-granules). ADP released from δ-granules binds to P2Y1 and P2Y12 receptors on the platelet surface, amplifying activation and promoting further ADP release [43].

Additionally, activated platelets produce and release thromboxane A2 (TxA2), which reinforces platelet recruitment and contributes to plug formation [46]. Thrombin, another potent agonist, converts fibrinogen to fibrin, thereby stabilizing the developing thrombus [41]. Platelet activation by thrombin occurs via protease-activated receptors PAR-1

and PAR-4, both of which are present on human and rodent platelets, however, PAR-1 is non-functional in murine platelets [50]. The activation process also induces cytoskeletal remodeling, resulting in platelet morphological transformation [51, 52].

Ultimately, these agonists converge on the activation of the GPIIba/IIIa receptor, which enables fibrinogen or VWF binding and thereby promotes platelet aggregation. This aggregation recruits more platelets to the injury site, culminating in thrombus formation [41, 43].

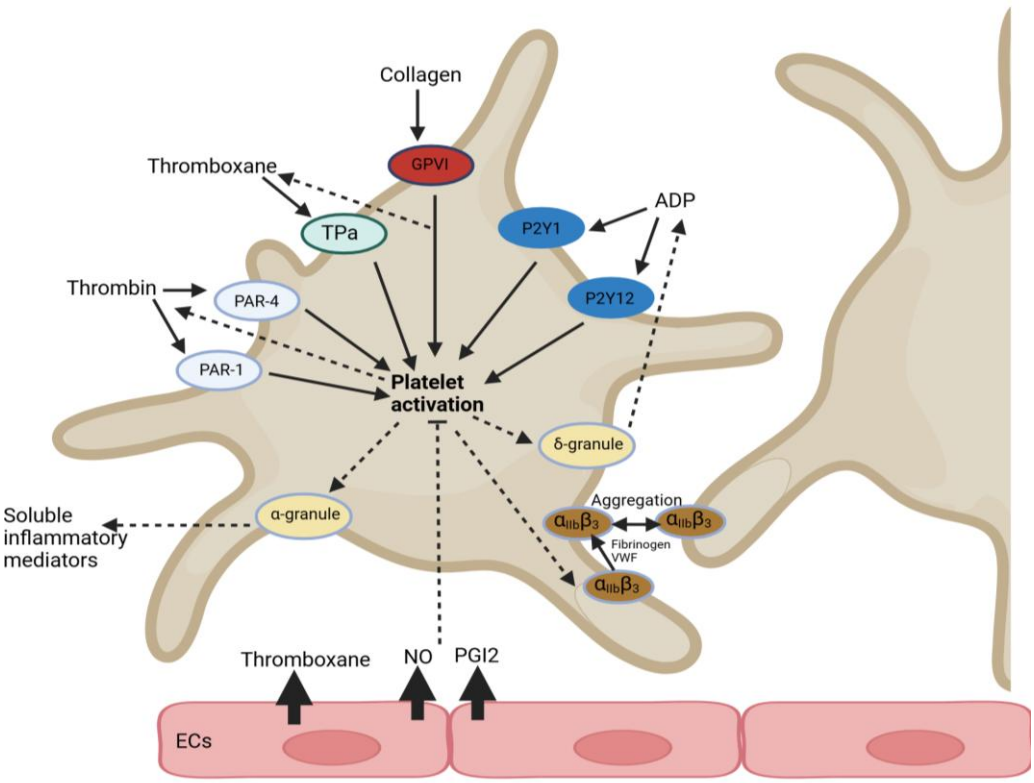

Figure 5: Schematic diagram of the platelet activation mechanism.

Platelet activation is forced via various mediators and metabolic pathways. Normally, PGI2 and NO are released by the endothelium, which inhibits the adhesion of platelets and prevents platelet activation. The release of soluble mediators from granules enhances platelet aggregation. Modified from [43]. ECs: endothelial cells, VWF: von Willbrand Factor, ADP: adenosine diphosphate, PAR-1: protease activated receptor 1, PAR-4: protease activated receptor 4, TPa: thromboxane proteinoid receptor alpha.

1.2.1.3 Platelet degranulation

Activated platelets function as secretory cells, releasing various substances from their storage granules to promote thrombosis, support vascular remodeling and recruit or activate other cells [41, 53]. This platelet's structure compromises three primary classes of granules: δ-granules, lysosomes, and the most abundant α-granules [43]. In addition, they release

bioactive molecules such as eicosanoids and extracellular vesicles. This secretion process, known as exocytosis, involves the fusion of intracellular granules with the plasma membrane, facilitated by specific proteins and calcium ions [54, 55].

1.2.1.3.1 Alpha granules

Platelet α-granules store a diverse range of proteins released during platelet activation, contributing to processes such as thrombosis, hemostasis, inflammation, immune defense and atherosclerosis [43]. These granules, measuring approximately 200-500 nm in diameter [56], can release over 300 soluble proteins and signaling molecules, as shown by recent secretome analysis [57, 58]. Each platelet contains around 50-80 α-granules, which hold a heterogeneous mix of membrane-associated proteins that are displayed on the platelet surface or secreted upon activation [53].

Among the key mediators released from α-granules are VWF [59], fibrinogen [60] and P-selectin [61]. VWF and fibrinogen are secreted into the surrounding environment, promoting thrombus formation. In contrast, P-selectin is carried to the platelet surface through membrane fusion, where it works alongside with other glycoproteins to support platelet aggregation via interactions with fibrin and other platelets [62].

Beyond hemostasis, α-granules play a significant role in innate immunity. This is primarily achieved through the modulation of platelets adhesion receptors involved in leukocyte interactions and the release of cytokines that influence immune cell behavior [43]. P-selectin, along with its ligand P-selectin glycoprotein ligant-1 (PSGL-1), is especially important for communication with monocytes, neutrophils and ECs. Additionally, α-granules store adhesion-related molecules, including platelet endothelial cell adhesion molecule-1 (PECAM-1), GPIIb/IIIa, and VWF [43].

1.2.1.3.2 Dense granule*s*

The second type of platelet granules are δ-granules, which each platelet containing approximately three to eight granules, each about 150 nm in diameter [63, 64]. These granules store small, non-protein molecules critical for enhancing platelet activity, including ATP, ADP and TxA2 [43]. Additionally, δ-granules contain histamine and significant levels of cations such as calcium [63, 64].

1.2.1.3.3 Lysosomes

Lysosomes represent the third and smallest subgroup of platelet granules, with each platelet containing between one and three [64]. They store a range of proteases, glycosidases, and other proteins with bactericidal properties [43]. In addition, lysosomal proteolytic enzymes play a role in thrombus remodeling [65]. Their secretion requires strong activation signals, such as high concentrations of collagen or thrombin [66].

1.2.2 Interaction between primary and secondary hemostasis

Although primary and secondary hemostasis are mostly described separately, they are interconnected during the coagulation process [65]. Both rely on finely regulated mechanisms, and any imbalance can result in either excessive bleeding or pathological thrombosis [39]. Thrombin, a central enzyme in the coagulation cascade, plays a vital role in activating platelets. Once activated, platelets provide a procoagulant platform that facilitates the interconnection of coagulation complexes. During this activation, the exposure of phosphatidylserine (a negatively charged phospholipid) increases on the platelet membrane from 0 to 12 % creating binding sites for coagulation proteins. Additionally, α-granules release clotting factors as factor V, further promoting the coagulation cascade known as secondary hemostasis [39].

1.2.3 Secondary hemostasis

Secondary hemostasis, also known as clot stabilization (figure 6), involves two distinct pathways-intrinsic and extrinsic- that lead to the formation of the prothrombin activation complex. Through its catalytic activity, the complex mediates the conversion of prothrombin into thrombin, which then transforms fibrinogen into fibrin fibers, stabilizing the platelet plug [66, 67].

The intrinsic pathway is triggered when factor XII (FXII) in the blood meets a damaged endothelial surface [40]. This initiates a cascade involving factors XI, IX and VIII, ultimately generating thrombin [68, 69].

In contrast, the extrinsic pathway is shorter and begins when tissue factor is released from injured endothelium. Tissue factor binds to circulating factor VII (FVII) and transforms it into its active form FVIIa. This complex activates factor X (FX) to FXa, merging both pathways at this point [66].

The common pathway starts with the activation of FX to FXa, a process facilitated by the tenase complex on activated platelets. FXa, along with cofactor FV, converts prothrombin to thrombin [66]. Thrombin subsequently converts fibrinogen into fibrin and activates additional coagulation factors including FXI, FV, FVIII and FXIII. The resulting fibrin subunits polymerize into strands, and with the cross-linking action FXIII, a stable fibrin mesh is formed, reinforcing the platelet plug [39, 67].

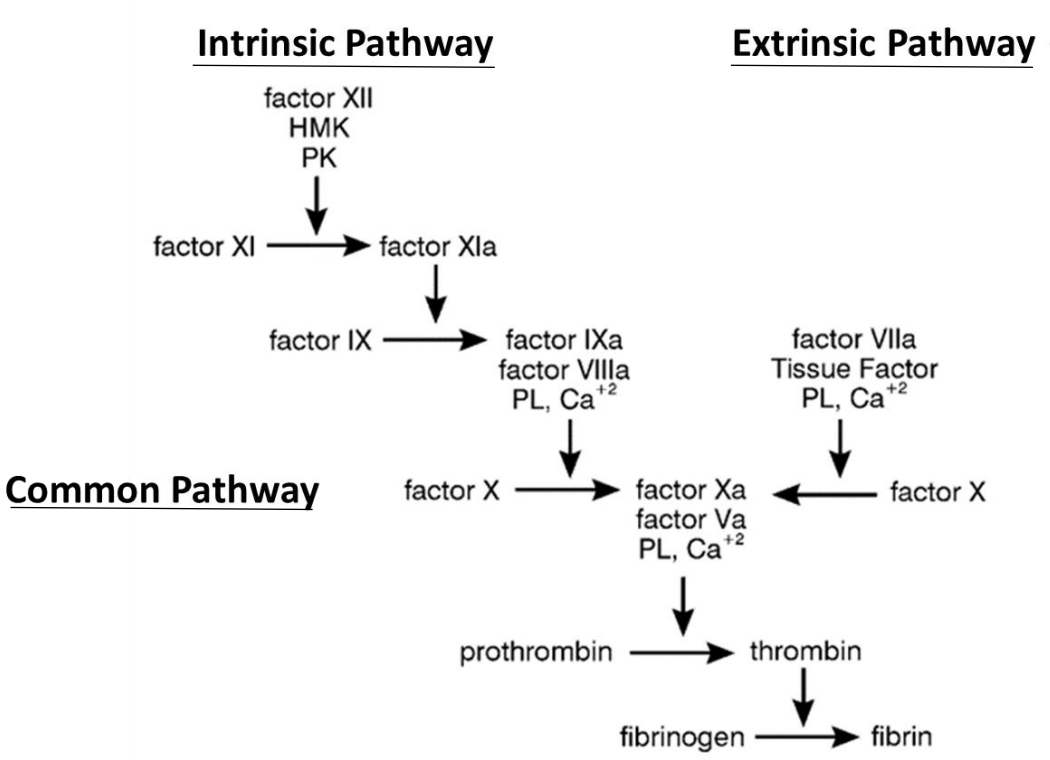

Figure 6: The secondary hemostasis coagulation cascade.

The secondary hemostasis starts on two different points, but the intrinsic and the extrinsic pathway come together at the stage of factor X and follow a common pathway (modified from [70]).

1.3 Endothelium:

The endothelium consists of a single layer of cells that line the inner luminal surface of blood vessels, functioning as both a structural and regulatory interface between the bloodstream and the vessel wall. It is essential for maintaining vascular hemostasis by inhibiting platelet and leukocyte adhesion, modulating vascular permeability and supporting normal blood flow [71]. Its central role involves regulating blood circulation and tissue perfusion by controlling the vessel diameter and vascular tone [72]. Moreover, the endothelium operates as a selective barrier that governs the passage of fluids, ions and macromolecules between the bloodstream and surrounding tissues. In response to injury, it recruits proinflammatory leukocytes and expresses adhesion molecules such as selectin, integrin and cytokines [72].

Platelet-endothelium interactions increase as a response to inflammation or infection. Platelets bind to endothelium, under low shear stress, via platelet GPIbα and endothelial VWF. At higher shear stress, especially in small venules, interactions involve endothelial P-selectin and PSGL1 or GP1bα, facilitating platelet rolling. Endothelial stress is characterized by elevated surface expression of E-selectin and P-selectin. Furthermore, activated platelets bearing CD40L trigger ECs to upregulate adhesion molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), as well as interleukin-8 (IL-8), thereby strengthening platelet-endothelium interactions [73].

Moreover, ECs express multiple S1PRs, notably S1PR1, S1PR2 and S1PR3. S1P/S1PR1 complex plays a crucial role in maintaining cytoskeletal remodeling [74], vascular stability, maturation and angiogenesis leading to blood vessels maintenance [18]. S1P signaling modulates the molecules adhesion in the endothelium, influencing leukocyte adhesion and transmigration during inflammatory responses [75].

To suppress platelet activation and thrombus formation, the luminal side of healthy ECs displays a range of anticoagulant molecules that preserve blood fluidity and inhibit clot development [72]. One key molecule in this regulation is thrombomodulin (TM).

1.4 Thrombomodulin (TM)

1.4.1 TM structure and domains

An essential role of the vascular endothelium is the prevention of intravascular thrombosis. TM, a glycoprotein expressed on the endothelial surface, is central to this anticoagulant mechanism, functioning as a thrombin cofactor and regulating processes of both coagulation and inflammation [72].

TM consists of five distinct domains (figure 7) [72, 76]:

- D1: NH2- terminal C-type lectin domain (CTLD).
- D2: a chain of six extracellular epidermal-growth-factors (EGF)-like repeats.
- D3: Extracellular serine/threonine (Ser/Thr)-rich region.
- D4: Transmembrane spanning region.
- D5: Short cytoplasmic tail.

Domain D1 regulates the inflammatory response and D2 and D3 mediate the anticoagulant and antifibrinolytic properties [72].

The six EGF-like repeats are functionally specialized. The observed activities were facilitated through the activation of PKC and the mitogen-activated protein kinase (MAPK) pathway. EGF repeats 5 and 6 bind to thrombin, altering its activity by preventing interaction with procoagulant substrates like FV and fibrinogen. Repeats 4 and 6 are critical for activating protein C, while repeats 3 to 6 are essential for activating thrombin-activable fibrinolysis inhibitor (TAFI) [77, 78].

The third domain of TM, rich in Ser and Thr residues, serves as the site for O-linked glycosylation and chondroitin sulfate attachment. This glycosaminoglycan enhances TM´s capacity to activate protein C by aiding thrombin neutralization and supports the interaction between platelet factor 4 (PF4) and protein C. Furthermore, chondroitin sulfate contributes to the stabilization of thrombin-TM complexes [77, 78].

TM also contains a conserved transmembrane domain succeeded by a short cytoplasmic tail where a cysteine residue enables TM multimerization. In ECs of human veins, thrombin binding to TM initiates signaling cascades, including GPCR-mediated pathways to activate eNOS3 [77].

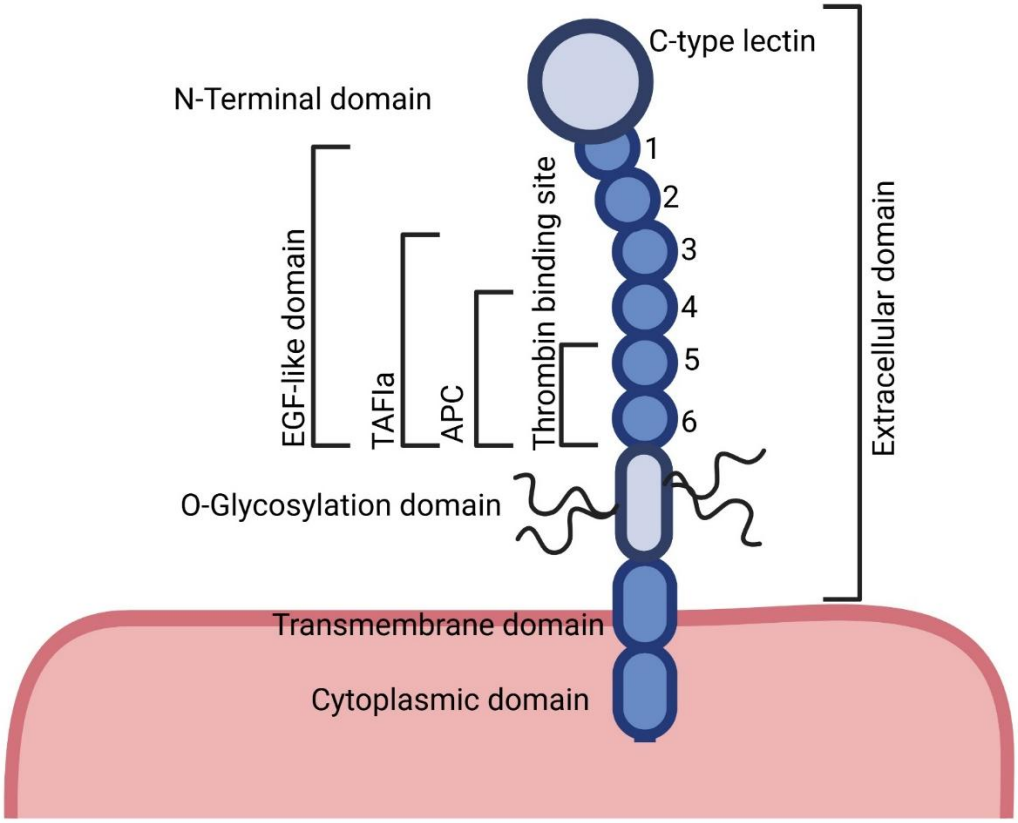

Figure 7: Structural organization of TM.

TM is composed of several structural domains: an N-terminal C-type lectin domain, a stalk containing six EGF-like repeats, a ser/thr rich segment that serves as a site of glycosylation, a transmembrane region and a short cytoplasmic tail. The EGF domains mediate thrombin binding, with repeats 4 to 6 being essential for protein C activation, while repeats 3 to 6 are necessary for TAFI activation (modified from [77]). EGF: epidermal-growth-factors, APC: activated protein C, TAFI: thrombin activable fibrinolysis inhibitor.

1.4.2 Physiological functions

TM has a crucial role in preserving vascular integrity through its regulation of blood coagulation, inflammation and fibrinolysis [72].

1.4.2.1 Anticoagulant mechanism

TM contributes to anticoagulation by altering thrombin activity and facilitating the activation of protein C. When thrombin binds to TM, its ability to act on procoagulant target (fibrinogen, platelets and coagulation factors V and VIII) is significantly reduced. This interaction not only limits thrombin´s procoagulant effect but also enhances its capacity to activate protein C [79, 80].

APC, supported by its cofactor protein S, reduces the thrombin generation through factors Va and VIIIa inactivation. In addition, the anti-inflammatory properties of TM may also stem from its high affinity to thrombin, allowing TM to act as a scavenger that neutralizes circulating thrombin [77].

1.4.2.2 Anti-inflammatory effect

TM exerts anti-inflammatory effects via both APC-dependent and APC-independent pathways [77].

In the APC dependent pathway (figure 8), TM promotes the activation of protein C, which then interacts with endothelial protein C receptor (EPCR). This complex activates PAR-1 via coupling to G-protein, leading to inhibition of kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, and consequently, suppression of inflammatory cytokine production. These signaling components, APC, EPCR and PAR-1, are organized in lipid rafts linked to caveolin-1. Upon binding of protein C to EPCR, the receptor dissociates from caveolin-1, shifting PAR-1 signaling from a proinflammatory toward anti-inflammatory profile. Furthermore, APC enhances endothelial barrier integrity by transactivating S1PRs [77, 81].

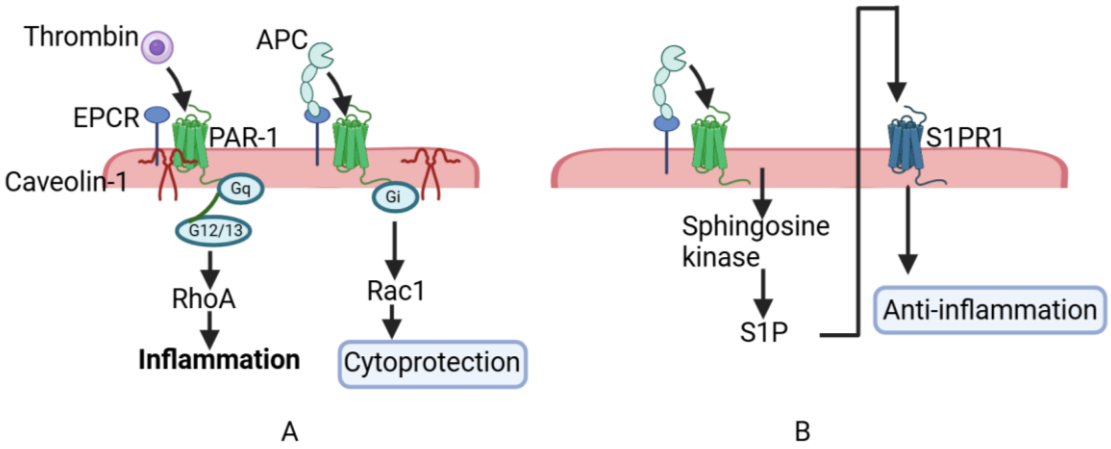

Figure 8: APC-dependent anti-inflammatory mechanism.

Suggested molecular mechanisms for anti-inflammatory effects of APC are illustrated as follows: (A) In the absence of protein C or APC, EPCR remains associated with caveolin-1 within lipid rafts, where thrombin cleavage of PAR-1 promotes proinflammatory signaling through coupling G12/13 and Gq proteins. When protein C binds to EPCR, the receptor detaches from caveolin-1, enabling thrombin to trigger a cytoprotective pathway via PAR-1 coupling to Gi proteins. (B) The binding of APC to EPCR allows APC to cleave PAR-1 and stimulate S1P production, which in turn activates S1PR1. This receptor activation strengthens endothelial barrier function and contributes to the anti-inflammatory actions of APC-EPCR complex (modified from [77]). EPCR: endothelial protein C receptor, PAR-1: protease activated receptor 1.

One of APC-independent mechanisms is when TM regulates fibrinolysis and inflammation through TAFI (figure 9). Once activated, TAFI removes lysine residues from C-terminal region of fibrin, thereby preventing plasminogen attachment to the clot and reducing fibrin degradation [77]. TAFI has an anti-inflammatory role too by deactivating proinflammatory mediators (C3a, C5a, bradykinin) [77, 81].

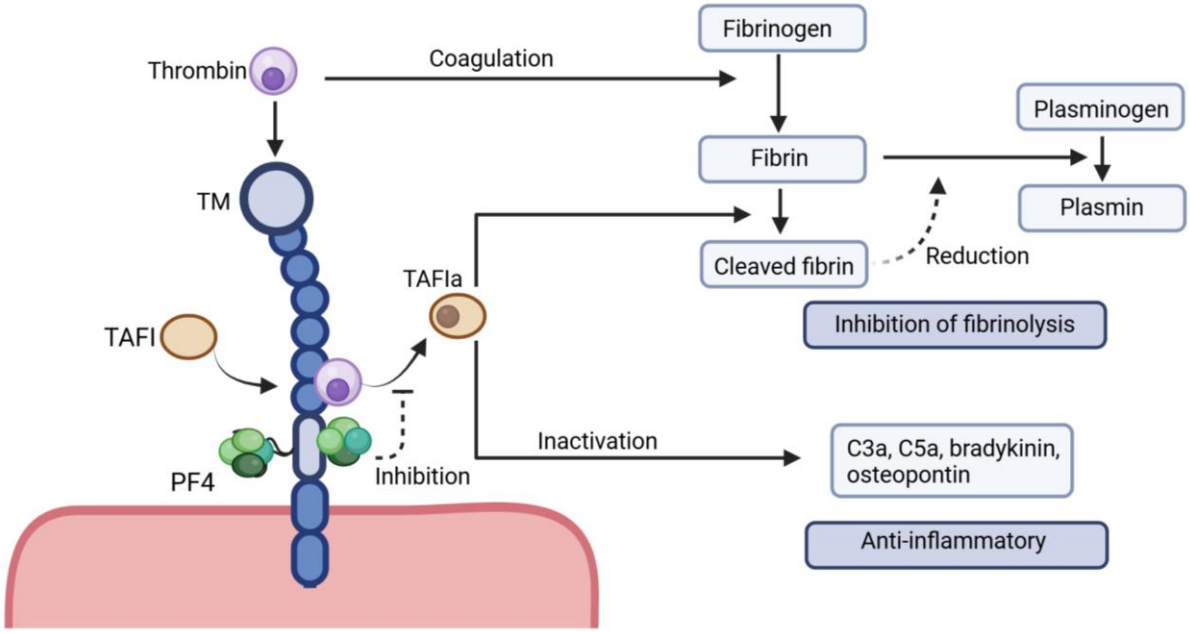

Figure 9: TAFI-mediated regulation of inflammation and fibrinolysis.

TAFI exhibits anti-inflammatory activity by inactivating key proinflammatory mediators-such as C3a, C5a, bradykinin and osteopontin- by C-terminal residues removal. Additionally, TAFI impairs fibrinolysis by removing C-terminal lysine from fibrin, thereby downregulating plasmin generation. PF4 further influences this balance by inhibiting TAFI activation while promoting protein C activation through the thrombin-TM complex. TM: thrombomodulin, TAFI: thrombin-activable fibrinolysis inhibitor (modified from [77]).

1.4.2.3 Interaction between inflammation and coagulation

A systemic inflammation leads to the activation of coagulation cascade and *vice versa* (figure 10) [77]. Inflammatory stimuli, such as endotoxins and cytokines like interleukin-6 (IL-6), upregulate the tissue factor (TF) expression on ECs, monocytes and macrophages, initiating coagulation. TF forms a complex with FVIIa, which activates FX. FXa, in association with FVa, assembles the prothrombinase complex, leading to the conversion of prothrombin to thrombin, thereby promoting fibrin generation and platelet activation [72].

At the same time, inflammation impairs natural anticoagulants like antithrombin and protein C. Conversely, coagulation components such as thrombin, fibrin and TM influence inflammatory pathways. Thrombin, via protease-activated receptors (PARs), stimulates cytokine release (e.g. IL-6, IL-8), leukocyte adhesion and endothelial activation [77].

TM plays a counter-regulatory role. When thrombin binds to TM, its activity shifts from procoagulant to anticoagulant. This complex, along with EPCR, activates protein C [72]. APC then partners with protein S to form a complex that inhibits coagulation by inactivating

factors Va and VIIIa. This results in the suppression of inflammation by inhibiting cytokine release and preventing activation of the NF- κB signaling pathway in monocytes [77].

Finally, thrombin-TM complex also serves to activate TAFI, which not only limits fibrinolysis but also inactivates inflammatory mediators (C3a and C5a) [77, 81].

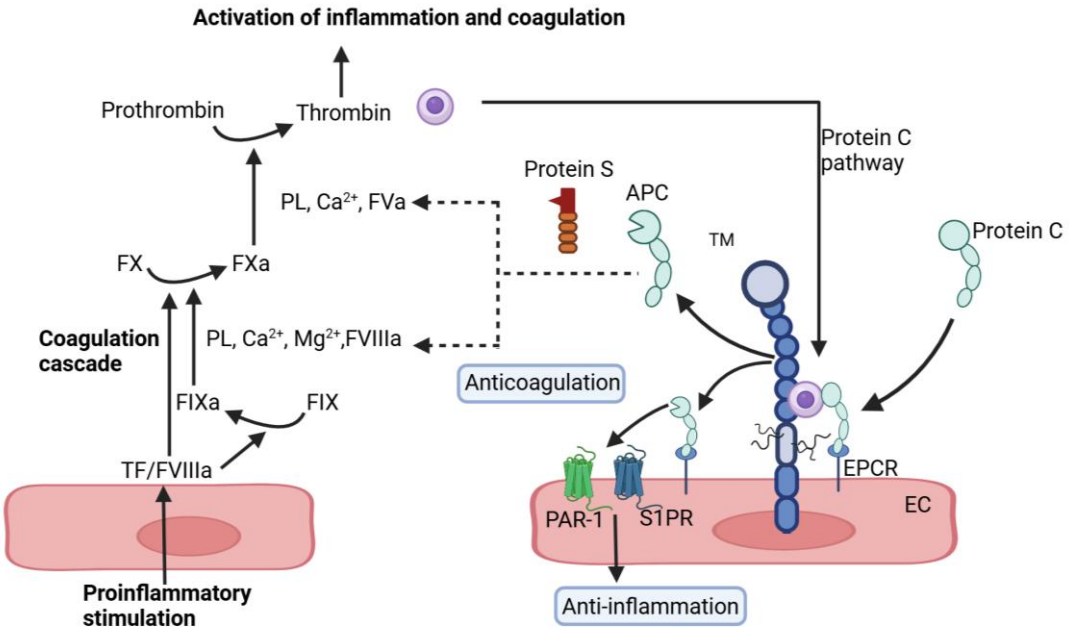

Figure 10: Interplay between inflammation and coagulation.

TF initiates the coagulation cascade, culminating in the assembly of the FXa-FVa complex that drives thrombin generation. Thrombin, in turn, facilitates fibrin generation and activates platelets, monocytes and ECs through PAR signaling, thereby amplifying both coagulation and inflammation in a positive feedback loop. In contrast, a negative feedback mechanism occurs when thrombin binds to TM, to form a complex for protein C activation. APC, together with protein S, suppresses the coagulation by inactivating factors Va and VIIIa, while also exerting anti-inflammatory effect through APC-EPCR-mediated PAR signaling. This, thrombin, TM and protein C-EPCR system is central to the regulation and interplay between coagulation and inflammation (modified from [77]). TF: tissue factor, PL: phospholipid.

1.4.3 Regulation of TM

Oxidation of methionine-388 located on the fifth EGF-like repeat domain can nearly abolish TM activity, resulting in diminished protein C activation and a subsequent reduction in its anticoagulant function [82]. TM can also be glycosylated at specific Ser/Thr residues, with the addition of chondroitin sulfate chains enhancing its anticoagulant function [77]. During

inflammation or endothelial stress, TM can be detached from the cell surface, generating a soluble form that circulates in plasma. Although this soluble TM (sTM) retains partial anticoagulant activity, it also serves as a biomarker of endothelial injury. This process is called TM shedding [72, 83].

Beyond these structural changes, TM expressions are dynamically influenced by intracellular signaling. The PI3K/Akt pathway plays a significant role in promoting TM expression, especially under stress conditions, by enhancing ECs survival and maintaining vascular integrity. Conversely, TM itself can regulate PI3k/Akt signaling through its effect on phosphatase and tensin homolog (PTEN), a phosphatase that negatively controls PI3k/Akt pathway. TM modulates PTEN phosphorylation leading to Akt negative regulation [82, 84]. This shows a bidirectional interaction between PI3K/Akt and TM.

1.4.4 TM and cardiovascular diseases

Under normal physiological conditions, expression of TM is localized on the luminal surface of coronary arteries. But this expression is reduced in atherosclerosis. Consequently, recombinant TM (rTM) has been proposed as a therapeutic strategy to slow disease development and progression [79]. In healthy individuals, higher circulating sTM levels are linked to a reduced risk of coronary heart disease, whereas low plasma concentrations may indicate endothelial injury [83, 85].

However, interpretation of sTM levels remains complex, as elevated concentrations may arise either from enhanced expression on intact ECs or from the release of TM fragments following endothelial damage. While the first scenario implies a protective effect, the second reflects vascular injury. Therefore, sTM measurements should always be taken in consideration in the patient´s vascular history and background [79, 83].

1.4.5 Therapeutic applications

Soluble form of rTM maintains the capacity to bind to thrombin and APC, thereby reducing leukocyte accumulation and suppressing tumor necrosis factor α (TNFα) expression [77]. As mentioned in 1.4.4.(TM and cardiovascular diseases), rTM is used to limit atherosclerosis. Additional studies showed that rTM is used in endothelial dysfunction, especially under proinflammatory conditions [79]. For example, Thrombomodulin alpha, also known as ART-123 (brand name Recomodulin®), is a rTM that was approved in Japan on January 25, 2008. It is used for treating disseminated intravascular coagulation or DIC [86].

Statins are primarily used to lower cholesterol. But also, they exert beneficial pleiotropic effects on the vascular endothelium by upregulating TM expression, enhancing both anticoagulant and anti-inflammatory responses [72].

1.5 Aims of the thesis

S1P is a bioactive lipid mediator known to regulate vascular function, inflammation and endothelial integrity. TM is a glycoprotein, expressed on the ECs surface, with anticoagulant properties that are essential for preserving hemostatic equilibrium. So, both are important endothelial factors.

This thesis aims to examine the potential regulatory influence of S1P on TM expression in ECs. The study explores the involvement of specific signaling pathways, particularly S1PR1 and PI3K, in mediating this effect. Additionally, it examines the functional impact of S1P-induces TM modulation on platelet adhesion and activation. Using SphK1 knockout (KO) mice, the physiological relevance of endogenous S1P in regulating TM expression and thrombus formation is assessed. Finally, the study evaluates whether TM supplementation can counteract the pro-thrombotic phenotype in the context of reduced S1P levels.

To complement the murine studies and enhance the translational relevance, this thesis also includes investigations in human subjects. Therefore, S1P levels will be analyzed in patients with various types of cardiovascular diseases to evaluate the potential correlation between S1P, TM expression and thrombotic risk.

By integrating both animal and human data, the study aims to detect the role of S1P in modulating TM expression and its potential implications for thrombosis prevention.

2 Materials and methods:

2.1 Laboratory tools and equipment

2.1.1 Chemicals

Table 2: Chemicals used in the study.

Chemicals	Company
Calcein	Sigma Aldrich
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) powder	Sigma Aldrich
Fe-(II)-chloride	Merck
Growth medium (2 % FCS)	Promocell
LY294002	Sigma Aldrich
HEPES	Roth
HEPES buffered saline solution	Lonza
Human Thrombomodulin (BDCA-3) Recombinant Protein, PeproTech®	Thermo Fisher
HUVEC medium	Lonza
Ketamine	Zoetis
Magnesium chloride hexahydrate ($MgCl_2x6H2O$)	Merck
NaCl 0.9 %	B.Braun, Melsungen
PBS: Dulbecco´s Phosphate Buffered Saline without calcium and magnesium	Sigma Aldrich
Penicillin/Streptomycin	Thermo Fisher
Potassium chloride (KCl)	KMF optiChem
Sodium bicarbonate ($NaHCO_3$)	Sigma Aldrich
Sodium chloride (NaCl)	VWR
Sodium phosphate monobasic monohydrate ($NaH_2PO_4xH_2O$)	Sigma Aldrich
Sphingosine-1-phosphate	Enzo lifescience
TRAP-6	Sigma Aldrich
Trypsin/EDTA	Promocell
Trypsin-neutralizing solution	Thermo Fischer
W146	Tocris
Xylazine	Bayer AG

2.1.2 Antibodies

Table 3: Antibodies used in this study.

Antibodies	Company
CD41-61 - APC-Cy7	Emfret Analytics
CD62P - BV421	SantaCruz Biotech
TM antibody	Abcam

2.1.3 Utensils

Table 4: Utensils used in this study.

Utensils	Company
15 ml falcon	Greiner
21 G butterfly needle	Safety Multifly-Needle, Sarstedt
Ascentis Express C18 main column	Supelco
Centrifuge	Eppendorf
Citrate tubes	Fischer Scientific
Eppis	Eppendorf
FACS tubes	Sarstedt
Fibronectin-coated 12-well plates	Cellstar
Fibronectin-coated flow chambers	Sigma Aldrich
Flask	Cellstar
guard cartridge	Supelco
Human thrombomodulin ELISA Kit	Abcam
Mouse THBD/CD1141 Thrombomodulin ELISA Kit	Life Span Bioscience
Pasteur pipettes	Central warehouse
Pipette tips	Eppendorf
Pipettes	Eppendorf

2.1.4 Devices

Table 5: Devices used in this study.

Devices	Company
BD FACSVerse™	BD Biosciences, Crystal Lakes (USA)
Cell culture incubator	Heracell 240, Thermo electron corporation
Mass spectrometry	Applied Biosystems
Nikon Eclipse TE2000-U microscope	Nikon
Plate reader	BMG LABTECH FLUOstar Omega
QTRAP 6500	Applied Biosystems
Vanquish Flex UHPLC system	Thermo Fisher Scientific
Zeiss Axiotech microscope	Zeiss Axiotech

All devices used corresponded to the usual laboratory standard.

2.1.5 Software

Table 6: Software used in this study.

Software	Company
Analyst software	AB Sciex
Bioflux-Montage™	Bioflux
Flow-Jo™	BD Biosciences, Franklin Lakes (USA)
GraphPad Prism	GraphPad Software Inc., San Diego (USA)
Omega	FLUOstar Omega

2.2 Methods

2.2.1 Cell culture

The culture of human umbilical vein endothelial cells (HUVECs) is for studying ECs function in a well-controlled *in vitro* environment and conditions. HUVECs closely model the behavior of vascular ECs in the human body. They provide a versatile platform to study endothelial function, vascular health and testing therapeutic strategies.

HUVECs (Promocell) were seeded into a flask filled with Growth Medium (2 % FCS) heated at 37°C in water bath, including 1 % penicillin/streptomycin. The flasks were incubated at 37°C in the incubator. The cells were split every three days to fibronectin-coated 12-well plates. After three days of culture, the HUVECS were trypsinized for subculture. For the splitting, the cells were rinsed with 5 ml of HEPES buffered saline solution (HBSS). Next, HBSS was aspirated completely and 1.5 ml Trypsin/EDTA was added to the cells. Then the cells were incubated for 1.5 minutes (min) at 37°C until they were detached. After that the cells were dispersed with an empty 1 ml pipette tip until the cells were separated. The culture was neutralized by adding 3 ml trypsin-neutralizing solution and 7 ml of HUVEC medium. The cells were transferred into 15 ml falcon tube and centrifuged at 900 g for 3 min at 37°C. The supernatant was discarded, and the pellet was resuspended with HUVEC medium. Experiments were conducted with cell density of 80 %. When the culture is ready, an incubation with S1P (1 µM) was performed for 24 hours (h). When required, S1PR1 antagonist W146 (10 µM) or PI3K inhibitor LY294002 (20 µM) were added 15 min prior to S1P application. After 24 h, TM-expression and TM level in cell media were measured by human thrombomodulin ELISA Kit. Therefore, cells and cell media were prepared according to the manufacturer's protocol.

2.2.2 Platelet adhesion

The goal of platelet adhesion or Bioflux experiment is to observe and quantify how platelets interact with various surfaces under shear stress. It helps bridge the gap between static *in vitro* studies and complex *in vivo* conditions by offering a controlled, reproducible and physiologically relevant environment to study platelet adhesion and function.

In this study, HUVECs were seeded onto fibronectin-coated flow chambers. Laminar flow with 2 DYN/cm² for 72 h was used to ensure homogeneous distribution of cells. After washing with PBS without calcium and magnesium, S1P (1 µM), W146 (10 µM) and thrombomodulin neutralizing antibody (TM-AB) (dilution 1:1000, ab109189) were added. Citrate-anticoagulated whole blood of healthy volunteers was stained with calcein (4 µM) and perfused through the capillary for ten min at 10 DYN/cm². Pictures were taken using the Nikon Eclipse TE2000-U microscope and results were calculated using Bioflux-

Montage™ software. Results were given in platelet-covered areas in percent normalized on untreated controls.

2.2.3 Human blood samples

To start, a flow cytometry experiment was conducted to test the platelet reactivity under different experimental conditions. For this we tested P-selectin expression on healthy volunteers' platelets.

Then, we carried out a prospective, exploratory, single-center translational study involving 74 patients diagnosed with cardiovascular diseases. The study followed an all-comers approach. Eligible participants were adults aged 18 years or older with at least one cardiovascular disease and the capacity to formally provide written informed consent. Exclusion criteria comprised malignancies or coagulation disorders. All participants provided informed consent for enrollment. The study protocol was approved by the Institutional Ethics Committee (Medical Faculty, Heinrich Heine University, Dusseldorf) and adhered with the ethical standards of the World Medical Association Declaration of Helsinki. Blood sampling was performed following the manufacturer's guidelines for the Human Thrombin-Antithrombin Complex (TATC) ELISA Kit to determine TATC levels. Plasma S1P concentrations were detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.2.4 Flow Cytometry

Flow cytometry is a technique based on sorting and detecting cells regarding their chemical and physical properties. These cells are detected by fluorescent dyes coupled with specific antibodies. These antibodies will bind to specific structures on the target cells.

This protocol helps to study the markers' expression on the platelet surface or their release. It leads to understanding how this expression changes in different healthy conditions, diseases and therapies. So, any abnormal marker expression indicates platelet disorder.

After collecting the blood in citrate tubes, the human blood was centrifuged for 10 min, 270 g at room temperature (RT) to produce platelet rich plasma (PRP). In different 1.5 ml tubes, 25 µl of thrombocytes medium are added to 100 µl of PRP. The samples were divided into three groups: control samples, samples incubated with S1P and others incubated with TM, at 37°C for 6 min. Then, thrombin receptor activating peptide-6 (Trap-6) was used to induce platelet activation with a concentration of 10 µM. After resuspending, the samples were incubated at 37°C for 6 min. The stimulated PRP was stained with antibodies mix, diluted 1:100 in 1xPBS Dulbecco´s Phosphate Buffered Saline solution. Anti-CD41-61 and anti-CD62P antibodies were used to determine platelets population and P-selectin expression respectively. Each sample is stained with 25 µl of the antibodies mix and incubated at 37°C

for 20 min in the dark. In FACS tubes, 10 µl of each sample was added to 990 µl of 1xPBS and measured with BD FACSVerse™ flow cytometer. Data were processed and analysed using Flow-Jo™ software. Results were given as mean fluorescence intensity (MFI) or positive cells percentage.

HEPES Tyrodes Buffer (Thrombocytes medium):

- 113 mM sodium chloride (NaCl) (MW: 58,44 g/mol)
- 12 mM sodium bicarbonate ($NaHCO_3$) (MW: 84,01 g/ml)
- 2,9 mM potassium chloride (KCl) (MW: 74,56 g/mol)
- 0,36 mM sodium phosphate monobasic monohydrate ($NaH_2PO_4xH_2O$) (MW: 137,99 g/mol)
- 1 mM magnesium chloride hexahydrate ($MgCl_2x6H_2O$) (MW: 203,31 g/mol)
- 5 mM HEPES (MW: 238,3 g/mol)
- pH=7,4 decreased with acid citrate dextrose (ACD) buffer.

P-selectin gating and expression:

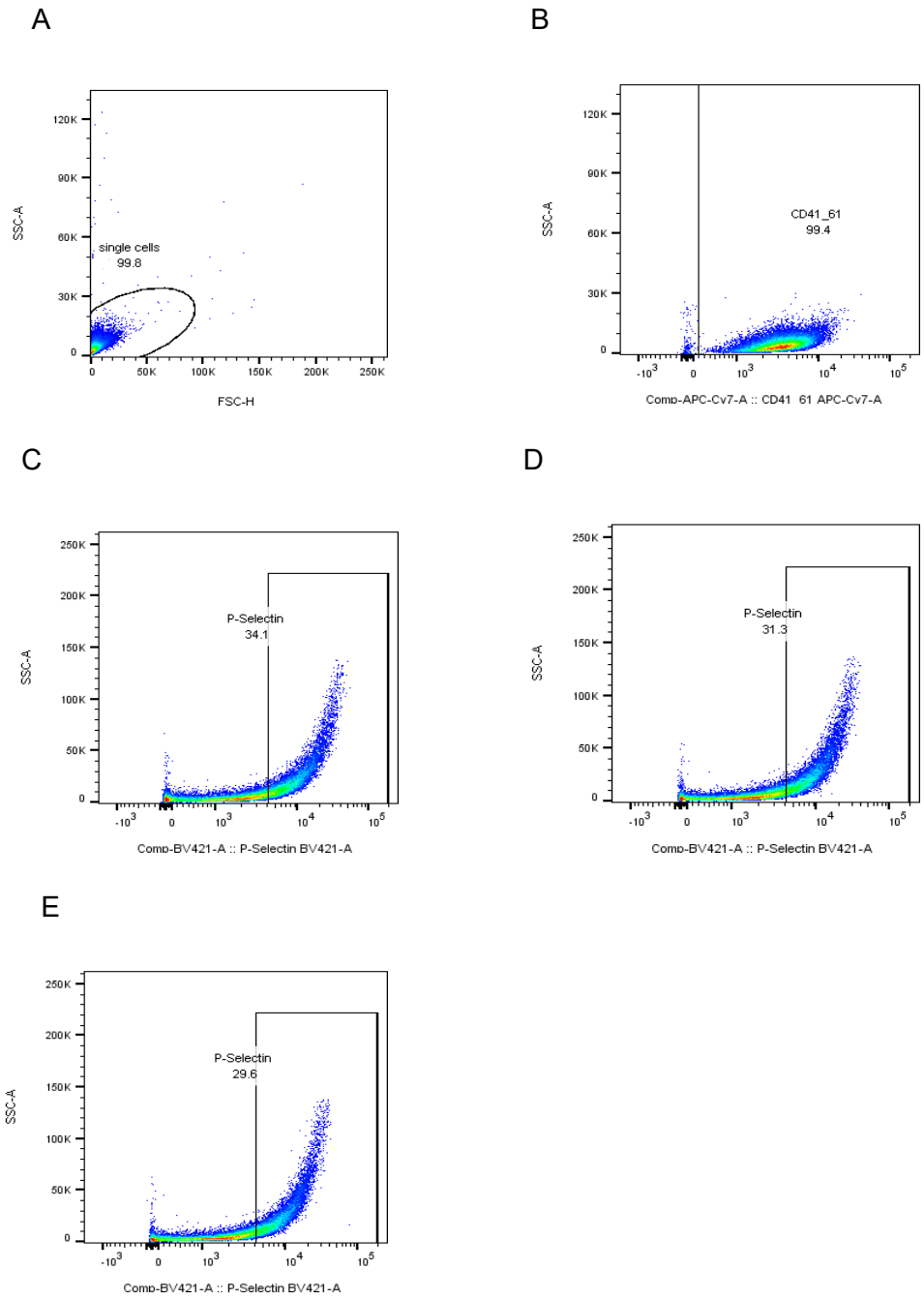

Figure 11: Human platelet P-selectin expression after Trap-6 stimulation under different experimental conditions.

The gating strategy begins with the identification of the overall cell population (A), followed by gating the platelet population CD41_61 (B). Within the gated platelets, P-selectin expression is analyzed. (C), (D) and (E) illustrate P-selectin expression on human platelets after Trap-6 stimulation. (C) a control sample stimulated with Trap-6 only (34.1 %). (D) a sample pre-incubated with S1P and then stimulated with Trap-6 (31.3 %). (E) a pre-incubated sample with TM and then stimulated with Trap-6 (29.6 %).

2.2.5 Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

Sphingolipid or S1P quantification in human plasma was conducted based on the method by Peng.et al. [87], with some adjustments. For this test a Vanquish Flex UHPLC system equipped was used with an Ascentis Express C18 main column (150 mm × 2.1 mm, 2.7 µm) and fitted with a guard cartridge (50 mm × 2.1 mm, 2.7 µm) maintained at 60°C. Two solvents were used A and B. Solvent A made of acetonitrile/water (6:4, v:v) containing 10 mM ammonium formate, 0.1 % formic acid and 5 mM phosphoric acid. Solvent B is consisted of isopropanol/acetonitrile (9:1; v:v) with the same additives of solvent A. Chromatographic separation was performed at a flow rate of 0.5 ml/min using 25 min long gradient (initial (30 % B), 0.0–2.0 min (hold 30 % B), 2.0–3.0 min (30–56.1 % B), 3.0–4.0 min (56.1–58.3 % B), 4.0–5.5 min (58.3–60.2 % B), 5.5–7.0 min (60.2–60.6 % B), 7.0–8.5 min (60.6–62.3 % B), 8.5–10.0 min (62.3–64.0 % B), 10.0–11.5 min (64.0–64.5 % B), 11.5–13.0 min (64.5–66.2 % B), 13.0–14.5 min (66.2–66.9 % B), 14.5–15.0 min (66.9–100.0 % B), 15.0–19.0 min (hold 100 % B), 19.0 min (5 % B), 19.0–22.0 min (hold 5 % B), 22.0 min (30 % B), 22.0–25.0 min (hold 30 % B)).

The LC was interfaced with a QTRAP 6500 and mass spectrometry (MS) working in a positive electrospray ionization mode. The source parameters included: curtain gas at 30 units, source temperature at 25°C, ion source gas I at 40 units, ion source gas II at 65 units, and medium collision gas. Additional MS settings were ion spray voltage at +5500 V, declustering potential at +100 V, entrance potential at +10 V, and exit potential at +13 V. Targeted analysis was carried out using scheduled selected reaction monitoring (SRM) with unit resolution for both Q1 and Q3, a 2 min detection window, and a cycle time of 0.5 seconds. Data was acquired using Analyst software (version 1.7.2; AB Sciex), and results were processed and manually integrated using Skyline [88]. These measurements were kindly conducted by Dr.Philipp Wollnitzke, from Prof. Bodo Levkau research group (Institute for Molecular Medicine III-HHU-Dusseldorf).

2.2.6 Animals

Male C57BL/6J wildtype mice (12-15 weeks old) were received from Janvier Labs (Saint-Berthevin, France). Sphingosine-1-kinase deficient mice ($SphK1^{-/-}$) were kindly provided by Prof. Bodo Levkau research group (Institute for Molecular Medicine III-HHU-Dusseldorf). All animals were housed under 12-hour day-night-rhythm with unrestricted access to standard chow and water (ad libitum). To reduce potential cofounding factors, animals were randomized for experimental procedures and not analyzed in blocks. Investigators were blinded to group assignments. Due to the exploratory nature of the study, no formal size calculation was performed. Some mice received S1P via tail vein injection at a dose of 38 ng/g [89] and were randomly assigned to control or treated groups.

2.2.7 Murine TM determination

Aortic mice TM-expression was determined via Mouse THBD/CD141 Thrombomodulin Elisa Kit. Isolation and lysis of the aorta for TM determination were carried out following the manufacturer's protocol. Aortic TM-expression was calculated as mass of TM per mass of aorta.

2.2.8 *In vivo* arterial thrombus formation

In our study, this method was first used to compare untreated C57BL/6J mice with those that received S1P treatment (38 ng/g, 16 h before the experiment). We then compared C57BL/6J mice with SphK1$^{-/-}$ mice, examining both groups either with or without rTM (Human Thrombomodulin (BDCA-3) Recombinant Protein) treatment. After five days of daily treatment with rTM (0.4 g/kg, i.p.), arterial thrombosis was experimentally induced through vessel injury of the carotid artery and observed under intravital microscopy (IVM).

The mouse is injected i.p. with ketamine (100 mg/kg BW) with a maximum of 10 ml/kg according to GV-Solas guidelines. After the mouse is fully anesthetized, the mouse is injected with X488 antibody-platelets specific. Placed on a warm plate at 37°C, an X-ray film is placed under the artery and separated from other tissues. An 10 % Fe-(III)-chloride-soaked tissue is applied to the left vessel wall of the carotid artery for three min. The mouse is transferred directly under the microscope on a heated plate after removing the paper. The clot formation is observed using a Zeiss Axiotech microscope (100-watt HBO mercury lamp). The IVM experiments were kindly conducted by Dr. Maike Barçik, from Prof. Dr. Amin Polzin research group (Cardiovascular Research Laboratory-HHU-Dusseldorf).

2.2.9 Tail bleeding assay

Mice were anesthetized using ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW), then positioned prone on a 37°C heating plate. A five mm portion of the tail tip was excised, and the tail was immediately submerged in a tube filled with pre-warmed (37°C) isotonic saline solution (NaCl 0.9 %). Blood loss was allowed to occur for 30 min. To quantify bleeding, haemoglobin concentration in the saline was determined spectrophotometrically at 550 nm, following established protocols [90]. Total blood loss was calculated based on a standard curve generated from known volumes of lysed mouse blood.

2.2.10 Statistical analysis

Statistical analyses were performed using SPSS©-Software (IBM) and GraphPad-Prism© (GraphPad Software Inc.). Continuous variables following a normal distribution were evaluated using the t-test, whereas those not normally distributed were analyzed with the Mann-Whitney-U-test. For comparisons involving three or more groups, ANOVA and mixed-effects analysis were applied as appropriate. Comparisons between single groups were conducted using Tukey's multiple comparison test. P values below 0.05 were defined significant.

2.3 Ethics committee approval

All mice experiments were approved by *Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV, 81-02.04.2020.A408)* and in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123) and 2010/63/EU.

3 Results

3.1 Part 1: TM expression depending on S1PR1 signaling pathway

3.1.1 Cell culture

We began by culturing HUVECs and treating them with 1 µM S1P for 24 h (figure 12a), followed by washing steps as described in “2.2.1 Cell culture” in the “2.2 Methods” previously. TM surface expression was quantified using ELISA. Three groups were compared to each other: untreated control, only S1P treated and S1P+W146 treated cells.

S1P treatment resulted in a significant 25 % increase in TM expression (figure 12b) compared to untreated controls ($p=0.0051$). To assess the involvement of S1PR1, a parallel group was pre-incubated with the selective S1PR1 antagonist W146 followed by S1P treatment. In this group, the S1P-induced upregulation was completely abolished, restoring TM levels to baseline and showing a significant difference compared to only S1P treatment ($p=0.0126$). These findings indicate that TM upregulation is mediated specifically via S1PR1-dependent signaling, with robust statistical support ($n=11$, one-way Anova followed by Tukey´s multiple test, $p<0.05$).

To determine whether S1P promotes TM shedding or enhances only its endothelial surface expression, TM levels were measured in the cell culture supernatant (figure 12c). No significant differences were observed between control and only S1P treatment, and between only S1P and S1P+W146 treated HUVECs groups (respectively $p=0.5796$ and $p=0.7830$) ($n=11$, one-way Anova followed by Tukey´s multiple test, $p>0.05$). This demonstrates that S1P selectively increases surface associated TM without inducing its release into the extracellular environment, thereby enhancing endothelial antithrombotic potential without altering sTM levels.

Next, we investigated the downstream signaling pathway linking S1PR1 to TM regulation. Since PI3K is a well-known S1PR1 effector, we employed the PI3K inhibitor LY294002 (figure 12d). Four groups were compared: untreated cells, treated only with S1P, treated only with LY294002, and S1P+ LY294002 treated cells. Consistent with earlier results, S1P alone significantly increased the TM expression relative to controls ($p=0.0078$) ($n=6$, two-way Anova followed by Tukey's multiple comparisons test, $p<0.05$). Meanwhile, LY294002 alone reduced TM expression below the control levels ($p=0.0193$). Importantly, the combined S1P+ LY294002 group displayed significantly lower TM expression compared to S1P alone ($p=0.0006$), but no difference compared to LY294002 alone ($p=0.9762$). This indicated that PI3K activity plays a crucial role in S1P-induced TM upregulation.

We further tested the role of MMP by using the inhibitor GM6001 (figure 12e). As before, S1P treatment increased TM expression compared to untreated control mice ($p=0.0081$) ($n=6$, two-way Anova followed by Tukey's multiple comparisons test, $p<0.05$). GM6001 alone did not significantly alter TM levels relative to control ($p=0.4653$). However, co-treatment with S1P and GM6001 yielded expression levels comparable to GM6001 alone ($p>0.9999$) and significantly lower than only S1P treatment ($p=0.0397$). These results show that MMP activity is also necessary for the S1P-mediated increase in TM expression, identifying MMPs as crucial mediators of the S1P-S1PR1-TM signaling axis.

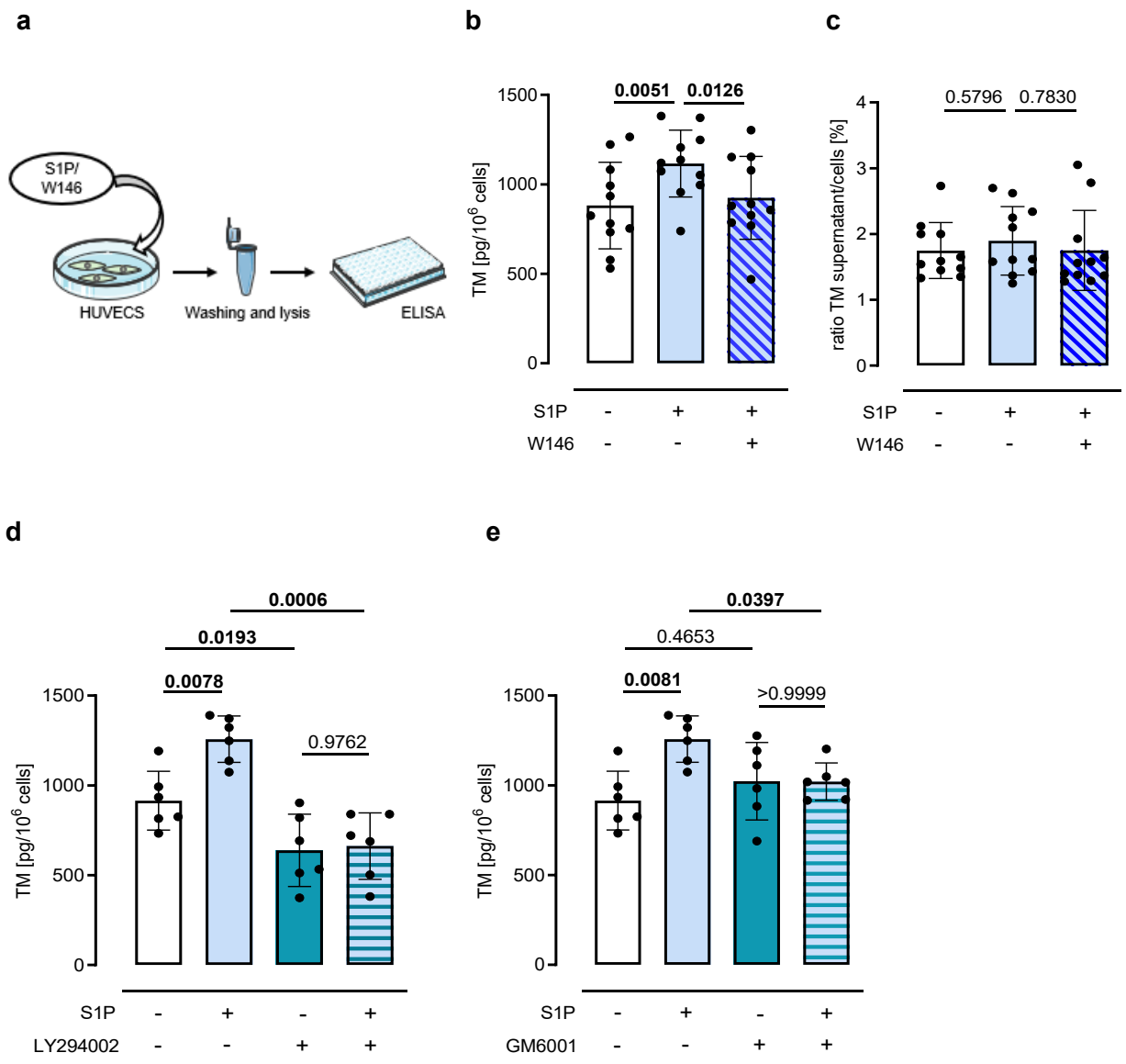

Figure 12: S1PR1 pathway regulates endothelial TM expression.

a HUVECS treatment with S1P and/or W146 for 24 h. **b** S1P increases significantly the endothelial TM expression meanwhile W146 blunted this effect by inhibiting S1PR1 (n=11, one-way ANOVA followed by Tukey's multiple comparisons test). **c** TM expression in the supernatant remains unchanged following S1P and W146 treatment (n=11, one-way ANOVA followed by Tukey's multiple comparisons test). **d** LY294002-mediated PI3K inhibition suppresses TM expression upregulated by S1P (n=6, two-way ANOVA followed by Tukey's multiple comparisons test). **e** TM upregulation by S1P is suppressed by GM6001 as MMP inhibitor (n=6, two-way ANOVA followed by Tukey's multiple comparisons test).

3.1.2 Flow chamber

To investigate platelet adhesion to ECs in the presence of S1P, we performed flow chamber assays using three platelet groups: untreated control, only S1P treated, and S1P+W146 treated. Platelets were perfused over HUVECs monolayers and adhesion was quantified (figure 13a).

In the first experimental series (figure 13b), ECs pre-treated with S1P showed a significant reduction in platelet adhesion compared to controls ($p=0.0094$) ($n=7$, one-way ANOVA followed by Tukey's multiple comparisons test, $p<0.05$), consistent with the observed S1P inducing an increase in TM expression. In contrast, co-treatment with S1P and the S1PR1 antagonist W146 restored adhesion levels to near control values, with significantly higher adhesion compared to S1P-only treatment ($p=0.0051$). These results show that the anti-adhesive effect of S1P is mediated specifically via S1PR1.

In a second experimental series (figure 13c), we compared three groups: untreated control, S1P-treated and S1P+TM-AB-treated platelets. Consistent with the first round, S1P treatment alone significantly reduced platelet adhesion compared to control ($p=0.0060$) ($n=6$, one-way ANOVA followed by Tukey's multiple comparisons test, $p<0.05$). However, addition of TM-AB abolished this effect. Platelets in the S1P+TM-AB group adhered to levels significantly higher than S1P only ($p=0.0409$) but showed results near to the control values. This indicates that TM is the key mediator of the anti-adhesive action of S1P. Moreover, S1P does not act directly on platelets but rather enhances endothelial TM, which in turn reduces platelet adhesion.

Fluorescence microscopy (figures 13d) visually confirmed the lower platelet adhesion in S1P treated conditions compared to the control where we can see more platelets adhered identified by the green color. As it shows how this effect is abolished by the presence of W146 even in the presence of S1P.

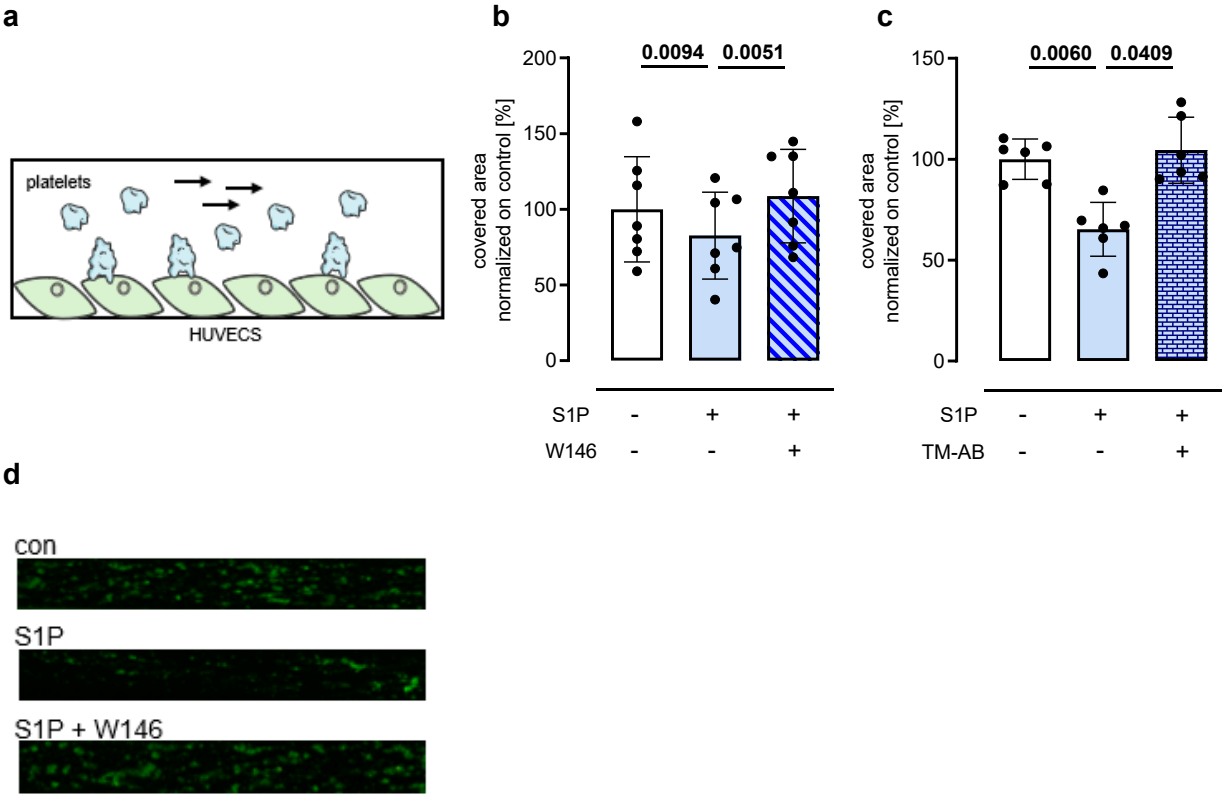

Figure 13: S1PR1 pathway modulates platelet adhesion to ECs via TM dependent mechanisms in flow chamber.

a Evaluation of platelet adhesion in flow chamber with treated HUVECS. **b** Platelet adhesion is reduced in presence of S1P, while this effect is blunted by W146 as S1PR1 inhibitor (n=7, one-way ANOVA followed by Tukey's multiple comparisons test). **c** To further examine the TM-dependency of these effects, we assessed platelet adhesion to ECs in the presence of TM-AB. The antibody inhibited the anti-adhesive effect of S1P (n=6, one-way ANOVA followed by Tukey's multiple comparisons test). **d** Fluorescence imaging of platelet adhesion is detected with the green color under flow conditions.

3.1.3 Flow cytometry and aggregation

To establish a reference profile of platelet reactivity and validate our assays, we proceeded with *ex vivo* experiments. Flow cytometry and aggregation experiments were performed on human PRP (figure 14a). Three experimental groups were included: untreated controls, PRP treated with S1P only, and PRP treated with TM only.

Flow cytometry analysis assessed platelet activation through P-selectin expression following TRAP-6 stimulation (figure 14b). Platelet treated with S1P only did not show a difference compared to untreated controls (p=0.2534). Whereas only TM treatment

significantly reduced P-selectin expression compared to the untreated control group (p=0.0443) (n=7, one-way ANOVA followed by Tukey's multiple comparisons test, p<0.05).

In parallel, aggregation assays did not reveal any significant differences among the three groups (figure 14c). The comparison between control and S1P-treated platelets yielded p= 0.7085, control versus only TM p=0.2662 and only S1P versus only TM p=0.8123 (n=7, one-way ANOVA followed by Tukey's multiple comparisons test).

Taken together, these results demonstrate that S1P does not affect platelet activation or aggregation in the absence of ECs, indicating that its anti-thrombotic effect is largely endothelium dependent. As S1P is not in direct contact with the platelets. In contrast, TM directly reduces TRAP-6-induced P-selectin expression, suggesting a direct effect on platelet activation markers, while aggregation response and cascade remain unaffected even in absence of ECs.

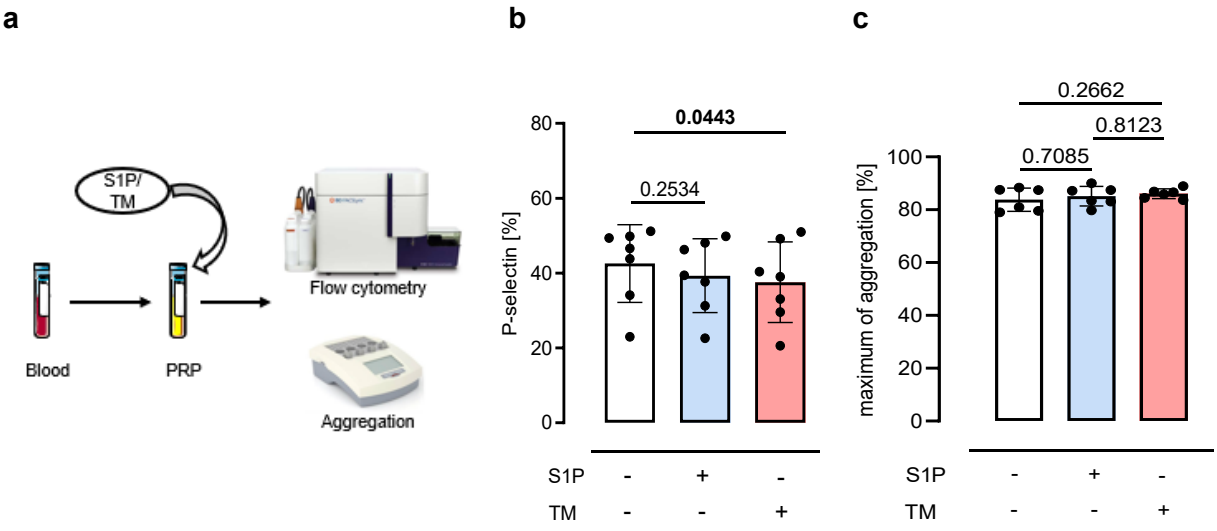

Figure 14: TM attenuates P-selectin expression.

a To investigate how S1P affects platelets independently of ECs, flow cytometry and aggregation experiments are conducted using human PRP. **b** P-selectin expression on platelets was significantly reduced following TRAP-6 stimulation after incubation with TM (n=7, one-way ANOVA followed by Tukey's multiple comparisons test). **c** Incubation with S1P or TM did not alter the platelet aggregation cascade induced by TRAP-6 (n=7, one-way ANOVA followed by Tukey's multiple comparisons test).

3.2 Part 2: TM affecting the thrombus formation

After completing the first part of the study with optimized results, we proceeded with *in vivo* experiments using male C57BL/6J wildtype mice (12-15 weeks old). Two groups were included: untreated control group and a group injected with 38 ng/g of S1P, 16 h before conducting the experiments (figure 15).

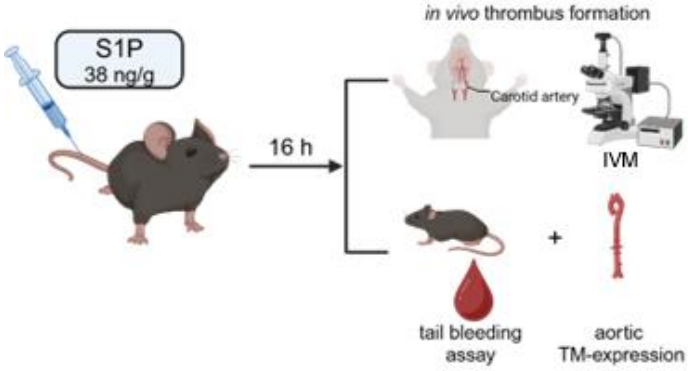

Figure 15: Mice conducted experiments illustration.

Two groups of mice, untreated and treated with 38 ng/g of S1P 16 h before the experiment, were tested by IVM to test the first time of occlusion and the thrombus size. As the blood loss was tested by tail bleeding assay and finally the aortic TM expression tested by ELISA.

3.2.1 Aortic TM expression

To evaluate aortic TM expression (figure 16), an ELISA test was conducted based on the protocol instructions. ELISA analysis revealed significantly higher TM levels in the aortic endothelium of S1P-treated mice in comparison to untreated controls (p=0.0359) (n=5, unpaired t-test, p<0.05). These mice results confirm again that S1P upregulates effectively the TM expression.

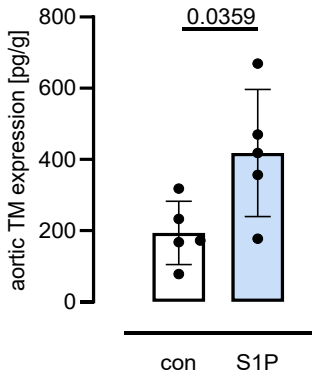

Figure 16: Upregulated aortic TM expression driven by S1P.

TM expression is higher in mice treated with 38 ng/g of S1P 16 h before the tests than in the untreated mice (n=5, unpaired t-test).

3.2.2 Occlusion observations

Under IVM, the time to first carotid artery occlusion (figure 17a) was measured in untreated control C57BL/6J mice and others treated with S1P. In control mice, artery occlusion occurred within 20 min, whereas in S1P-treated mice no occlusion was observed during the 60 min monitoring period (p<0.001) (n=5, unpaired t-test, p<0.05).

Moreover, the analysis of thrombus size in these two mice groups (figures 17b/c) showed markedly a larger thrombus formation, approximately 500 μm^2, in control mice than in S1P-treated mice, approximately 200 μm^2 (p<0.001) (n=5, unpaired t-test, p<0.05).

Together these data demonstrate that S1P strongly inhibits carotid artery thrombus formation, both by preventing vessel occlusion and reducing thrombus size.

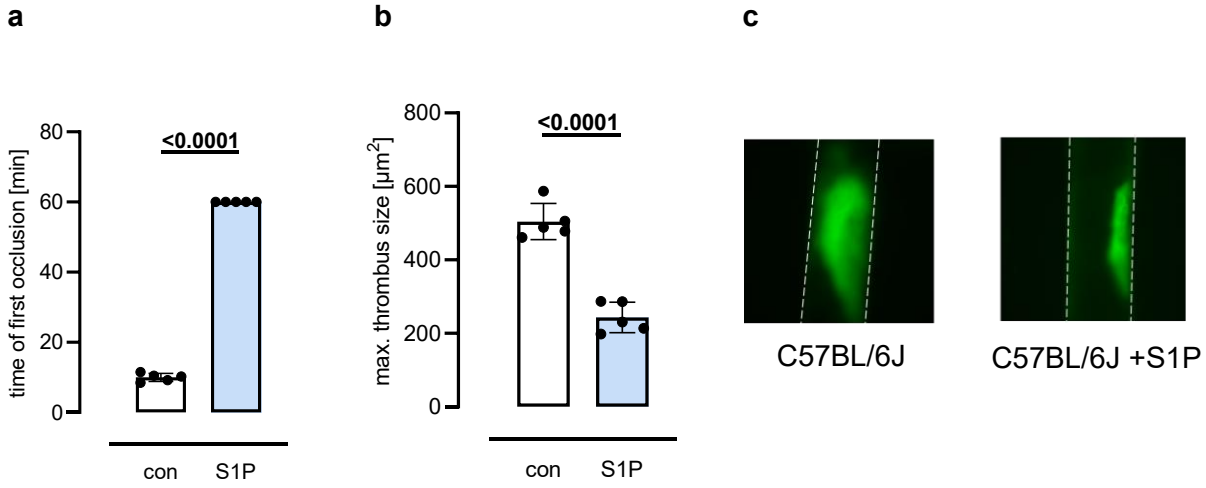

Figure 17: S1P treatment reduces thrombus formation.

a S1P treatment significantly abolished carotid arterial occlusion during the one-hour observation period, indicating S1P antithrombotic effect (n=5, unpaired t-test). **b** S1P treated mice exhibited significantly smaller thrombus than in the control mice (n=5, unpaired t-test). **c** Exemplary images of thrombus size formation in C57BL/6J wildtype mice untreated and treated with S1P.

3.2.3 Tail bleeding

To assess potential effects on hemostasis and bleeding risk, we measured blood loss by tail bleeding method for 30 min (figure 18). Same mice groups as in the previous experiments were compared. No difference was detected between untreated control mice and S1P-treated mice (p=0.8743) (n=5, unpaired t-test, p>0.05).

This indicates that S1P´s antithrombotic effect does not impair normal hemostatic function and does not lead to any bleeding risk, meanwhile based on the previous tests it inhibits thrombus formation.

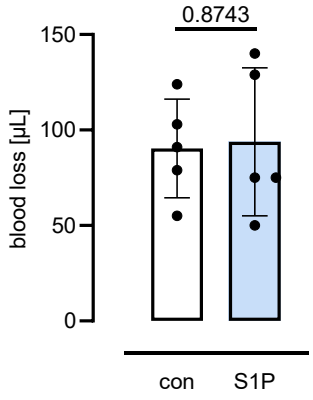

Figure 18: S1P treatment do not affect the blood flow.

Bleeding tail in mice did not show any difference (n=5, unpaired t-test) between untreated and treated mice with S1P showing that S1P has no effect on bleeding risk and blood flow.

3.2.4 TM aortic expression in C57BL/6J and SphK1$^{-/-}$ mice

To further investigate the role of endogenous S1P, we examined SphK1$^{-/-}$ mice, having about 70 % less plasma S1P levels in comparison to C57BL/6J wildtype mice.

ELISA (figure 19a) revealed significantly reduced aortic TM expression in SphK1$^{-/-}$ mice compared to C57BL/6J wildtype mice ($p<0.001$) ($n_{SphK1-/-}= 11$ and $n_{C57BL/6J}= 11$, unpaired t-test, $p<0.05$).

Furthermore, IVM (figure 19b) demonstrated that in C57BL/6J wildtype mice, the absence of TM led to occlusion within 10 min, whereas the treatment with TM completely prevented the carotid occlusion ($p<0.001$). In contrast, SphK1$^{-/-}$ mice lacking TM developed occlusion in less than 10 min, earlier than untreated C57BL/6J controls ($p=0.0062$). Administration of rTM delayed but did not fully prevent the occlusion in SphK1$^{-/-}$ mice, in contrast to the complete protection observed in C57BL/6J mice with TM treatment ($p<0.001$) ($n_{C57BL/6J}=6$, $n_{C57BL/6J+TM}=4$, $n_{SphK1-/-}=6$, $n_{SphK1-/-+TM}=4$, two-way ANOVA followed by Tukey's multiple comparisons test).

In summary, the absence of TM accelerated the thrombus formation with shorter time of first occlusion, compared to wildtype mice that received rTM. Administration of rTM to SphK1$^{-/-}$ mice restored thrombus formation resistance compared to SphK1$^{-/-}$ mice lacking S1P and rTM. These results confirm the TM´s central role in mediating thrombus formation and artery occlusion inhibition, and S1P role in vascular protection.

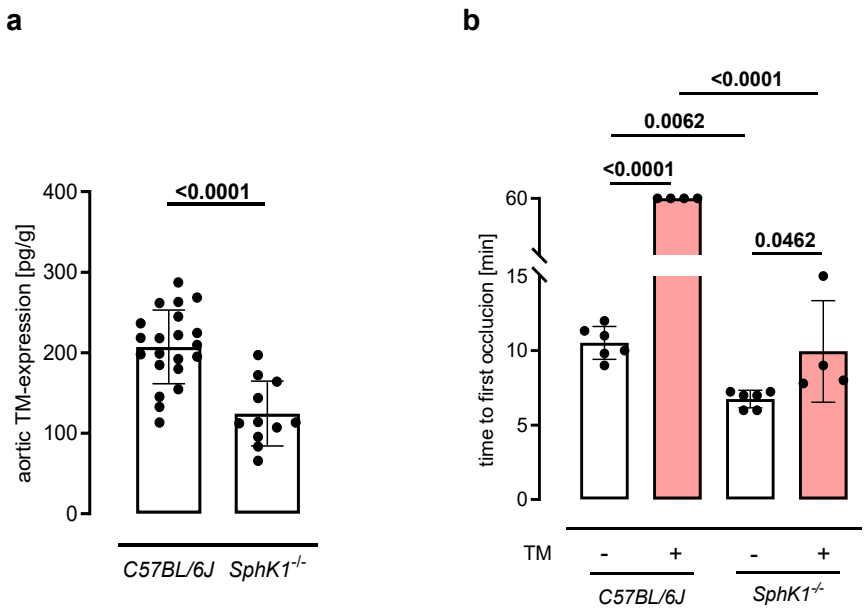

Figure 19: S1P and TM together inhibit the thrombus formation.

a Sphk1$^{-/-}$ mice exhibited significantly less TM expression than C57BL/6J mice ($n_{SphK1-/-}$= 11 and $n_{C57BL/6J}$= 11, unpaired t-test). **b** TM inhibited carotid artery thrombus formation in C57BL/6J mice. In contrast Sphk1$^{-/-}$ mice, with low S1P level, showed a thrombus formation, which was reversed by TM treatment ($n_{C57BL/6J}$=6, $n_{C57BL/6J+TM}$=4, $n_{SphK1-/-}$=6, $n_{SphK1-/-+TM}$=4, two-way ANOVA followed by Tukey's multiple comparisons test).

3.3 Part 3: S1P and thrombin expression in human plasma

To dig more into this research topic and study the correlation between S1P and thrombin level in different cardiovascular diseases, we tested patients' plasma. In a cohort of 74 patients (table 7) with various cardiovascular diseases, we checked the S1P concentrations in the patient's plasma and the thrombin plasma concentration (figure 20).

Plasma S1P concentrations showed a modest but statistically significant negative correlation with TATC levels (Pearson r = -0.2356, p = 0.0433). This relationship supports the concept that higher circulation of plasma S1P is associated with reduced thrombin generation, consistent with the anti-thrombotic effects observed *in vitro* and *in vivo* previously.

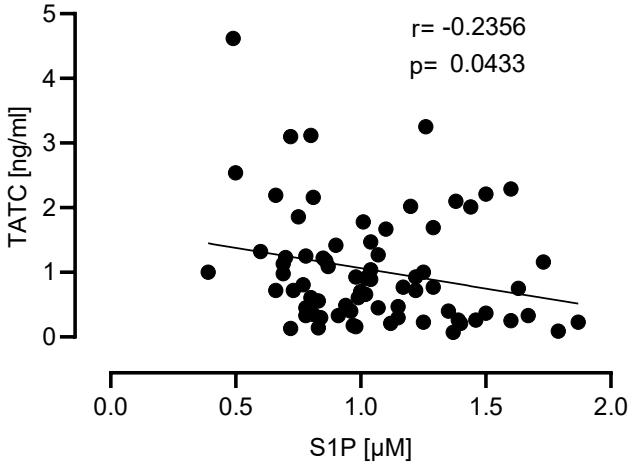

Figure 20: Negative correlation between S1P and thrombin levels in human plasma.

In a cohort of 74 patients (males and females) with different cardiovascular diseases, plasma S1P level showed a negative correlation with plasma thrombin concentration measured by TATC (Persons r = -0.2356, p=0.0433).

Table 7: Patients´characteristics.

Demographics	**n = 74**
Age – years (mean ± SD)	72.5 ± 13.4
Male Gender – no. (%)	24 (32.4 %)
BMI (kg/m²)	26.55 ± 5.47
Morbidities	
Arterial Hypertension - no. (%)	44 (59,5 %)
Atrial Fibrillation – no. (%)	36 (48.5 %)
Dyslipidemia - no. (%)	14 (18.9 %)
Diabetes mellitus - no. (%)	32 (43.2 %)
Coronary artery disease - no. (%)	44 (59.5 %)
Prior myocardial infarction - no. (%)	10 (13.5 %)
Prior PCI – no. (%)	20 (27.0 %)
Medication	
Aspirin – no. (%)	21 (28.4 %)
P2Y12 – no. (%)	9 (12.2 %)
OAC – no. (%)	38 (51.4 %)
ACE inhibitor – no. (%)	20 (27.0 %)
Beta Blockers – no. (%)	50 (67.6 %)
Diuretics – no. (%)	41 (55.4 %)
can lead to PI3K PPI – no. (%)	32 (43.2 %)
Statins – no. (%)	41 (55.4 %)
Laboratory parameters	
Platelets	234 ± 82
Hemoglobin (g/dl)	12.59 ± 1.77
Hematocrit (%)	38.7 ± 4.9
Leucocytes	9.08 ± 6.08
GFR (ml/min)	62.96 ± 25.53
CRP (mg/l)	2.16 ± 5.80
LDL (mg/dl)	88.5 ± 32.2
HDL (mg/dl)	51.9 ± 15.4
TG (mg/dl)	105.33 ± 54.4
$HbA1_c$ (%)	6.2 ± 1.1

BMI = body mass index, PCI = percutaneous coronary intervention, OAC = oral anticoagulation, PPI = proton pump inhibitor, GFR = glomerular filtration rate, CRP = C-reactive protein, HDL = high-density lipoprotein, LDL = low-density lipoprotein, TG = triglycerides, $HbA1_c$ = glycated hemoglobin.

4 Discussion

As previously outlined, S1P is a sphingolipid that influences the cardiovascular system through its interactions with GPCRs [1]. In our study we focused on S1PR1, a GPCR expressed on ECs. We focused too on TM, an endothelial surface glycoprotein with anticoagulant properties, functioning by binding to thrombin and inhibit both coagulation and inflammation [72]. Given that S1P and TM are both endothelial receptors, we hypothesized a potential interaction between them although the underlying pathway has not yet been defined. The primary aim of this study was therefore to demonstrate such interaction and to elucidate the signaling cascade linking S1PR1 to TM. To achieve this, we employed a combination of *in vitro*, *ex vivo* and *in vivo* approaches, which collectively enabled us to identify and characterize the pathway connecting S1P-S1PR1 to TM.

4.1 S1P the regulator of endothelial TM *in vitro*

The central finding of this work is that S1P upregulates endothelial TM through S1PR1-PI3K signaling without inducing TM shedding, and that this endothelial program reduces the adhesive interactions that initiate thrombus growth. Across endothelial biology, this is conceptually consistent with a two-decade literature establishing S1PR1 as the barrier stabilizing S1P receptor on endothelium, which signals through Gi/PI3K/Rac to strengthen adherence junctions, increase eNOS activity, and promote cytoskeletal alignment [91]. Our data extends that paradigm beyond barrier function to coagulation control at the endothelial surface by showing that S1PR1 signaling increases the availability of membrane-bound TM, thereby reprogramming local thrombin from a procoagulant enzyme to anticoagulant anti-inflammatory signal via APC generation.

4.1.1 S1PR1 and PI3K dependency

Two mechanistic points deserve emphasis. First, the S1PR1 dependence. Pharmacologic antagonism with W146 abrogated the S1P effect on TM and on platelet adhesion, aligning with the notion that acute S1PR1 blockade destabilizes the endothelium and induces leak *in vivo* [92, 93]. Second, the PI3K node. PI3K inhibition eliminated S1P-driven TM upregulation, consistent with the Gi-PI3K axis downstream of S1PR1 seen in barrier enhancement and cytoskeletal remodeling [91]. Although the field has focused more on PI3K's role in junctional stability and eNOS activation, TM transcription of control is flow sensitive and integrates with PI3K linked endothelial programs. Shear stress, for example, modulates TM expression and promotes promoter activity in ECs [94, 95]. In addition, the flow-responsive transcription factors, Kruppel-like factors 2 and 4 (KLF2/KLF4) directly drive an antithrombotic gene set that includes TM and eNOS [96, 97]. Thus, our observation that S1P-PI3K tone increases surface TM is consistent with established endothelial quiescence

signaling. This information confirms our findings in section 3.1.1, where HUVECs incubation with S1P significantly increased TM surface expression in ECs in an S1PR1-dependent manner. Importantly, this effect was completely blunted by the selective S1PR1 antagonist W146, confirming the receptor-specific nature of this pathway (figure 12b). Moreover, in flow chamber experiment (section 3.1.2), S1P reduced platelet adhesion to HUVECs. Meanwhile W146 blunted this effect by blocking S1PR1 and TM-AB reversed it also, showing this interaction between S1PR1 and TM. However, when TM levels were examined in the supernatant (figure 12c), no differences were observed between conditions treated with S1P, W146 or the combination of both. This finding indicates that S1P-S1PR1 interaction promotes TM expression at the endothelial surface but does not trigger TM shedding into the extracellular environment. This distinction is critical, as proteolytic shedding of endothelial TM has been shown to reduce its anticoagulant activity up to 80-90 % while increasing sTM in the circulation [72]. Shedding typically occurs under pathological conditions characterized by inflammation, infection or ischemic stress [79]. Thus, in our setting, S1P enhances TM function by increasing its surface expression while simultaneously preventing its loss through shedding, thereby preserving its anticoagulant capacity.

The role of intracellular signaling in this process was also considered. As described earlier in section 1.1.3 “S1P receptors and functions”, S1P can activate PI3K, which contributes to endothelial glycocalyx restoration [22]. Activation of the S1P-S1PR1 axis leads to downstream PI3K/Akt phosphorylation, which promotes NO release, protection against oxidative stress, and reinforcement of endothelial barrier [98, 99]. Additionally, as noted in section 1.4.3 “Regulation of TM”, the PI3K/Akt pathway is directly implicated in TM regulation. Meanwhile, platelet derived growth factor has been shown to upregulate TM through PI3K/Akt activation [100], and other studies have reported that TM expression in endothelial contexts is modulated through PI3K/Akt signaling depending on the specific axis engaged [101]. Based on these findings, we hypothesized that PI3K could represent the central link between S1P-S1PR1 activation and TM expression. To test this, HUVECs were treated with the PI3K inhibitor LY294002 (figure 12d). The results were striking inhibition of PI3K not only prevented the S1P-mediated increase in TM but also reduced TM expression to levels lower than the untreated control cells. Moreover, co-treatment with S1P and LY294002 produced similar outcomes to LY294002 alone, further confirming that PI3K is indispensable for TM expression in this pathway. These findings strongly suggest that PI3K-Akt signaling functions as the principal downstream mediator, consistent with PI3K´s established role in vascular hemostasis, endothelial barrier protection, and glycocalyx restoration.

4.1.2 MMPs dependency

To further dissect the mechanism, we investigated whether MMPs are involved in the S1P-S1PR1-TM pathway. Using the broad-spectrum MMP inhibitor GM6001 (figure 12e), we found that blocking MMP activity completely abolished the S1P-induced increase in TM expression. Cells treated with GM6001 alone displayed only a minimal, non-significant rise in TM levels compared to untreated controls, and co-treatment with S1P and GM6001 yielded results indistinguishable from GM6001 alone. These findings indicate that MMP activity is essential for the S1P-mediated upregulation of TM. This requirement may reflect the role of MMPs in remodeling the extracellular matrix and regulating the turnover of membrane proteins, thereby influencing the presentation and stability of TM on the endothelial surface. This interpretation aligns with evidence presented in section 1.1.3 “S1P receptors and functions” showing that MMPs contribute to the regulation of membrane protein availability, including receptors and cofactors. Our results therefore support a model in which S1P stimulation via S1PR1 engages MMP activity to enable TM surface expression. One plausible mechanism is that S1P-S1PR1 signaling induces the activation of MMPs required either to process an extracellular ligand or to remove a membrane-tethered inhibitor, thereby permitting downstream signaling events, potentially through growth factor receptors, that enhance TM expression or localization [102]. Interestingly, S1P has also been shown to suppress MMP activity in certain endothelial contexts [21], underscoring the complexity of S1P-MMP interactions.

Taken together, these *in vitro* experiments establish several key findings and align with the previous findings in many studies. First, S1P increases TM expression at the endothelial surface without inducing shedding, thereby maintaining its anticoagulant function. Second, the S1P-S1PR1 axis regulates TM through the PI3K-Akt pathway, highlighting a critical intracellular signaling mechanism. Third, MMP activity is required for this process, suggesting that extracellular matrix remodeling and protease-mediated regulation are integral to TM presentation. Collectively, these results provide strong evidence that S1P-S1PR1 signaling enhances endothelial anticoagulant function via a PI3K- and MMP-dependent mechanism, positioning this pathway as an important target in vascular protection against thrombosis.

4.2 Platelet activation and adhesion reduced by S1P-S1PR1-TM

4.2.1 Anti-thrombotic effect of S1P-TM

S1P has been known as a vascular mediator, which its role in thrombosis has been debated. Some studies have reported pro-thrombotic effects of S1P, particularly mediated by S1PR2 and S1PR3 in VSMCs and platelets. Many demonstrations showed that S1P triggers platelet shape change and aggregation and amplifies classic agonists, follow-ups tying aggregation and RhoA activation to S1PR2 [103, 104]. Newer mechanistic studies showed that S1PR1-PAR-1 crosstalk affect the platelet aggregation depending on S1P dose [105]. Exogenous S1P induces a concentration-dependent increase in intracellular calcium and platelet aggregation in human platelets [103]. These effects were sensitive to only S1PR2 but not to S1PR1. In other words, S1P triggers a translocation of RhoA to the platelet membrane after interacting with S1PR2 and induces platelet activation and aggregation through RhoA-Rho kinase pathway [103, 106].

In contrast, our data reveals a protective endothelial-dependent mechanism that is specifically mediated by S1PR1. Fewer studies test how endothelial S1P signaling alters platelet adhesion under flow. Endothelium literature is rich in S1PR1-driven barrier stabilization, anti-inflammation, eNOS signaling activation and glycocalyx protection, leading to less platelet adhesion [5, 107].

Importantly we provide evidence that S1P links to the anti-coagulant protein TM, shifting thrombin´s role from procoagulant to anti-coagulant and anti-inflammatory. To check S1P-S1PR1-TM effect on platelet adhesion, we conducted flow chamber experiments with HUVECs (3.1.2 Flow chamber). The platelets treated with S1P showed lower adhesion to HUVECs compared to the untreated platelets. Meanwhile the ones treated with S1P+W146 showed similar covered area to the control which is defined by higher platelet adhesion to HUVECs compared to the platelets treated only with S1P significantly (figure 13b). W146 abolished the interaction between S1P and S1PR1 leading to less TM expression and lower platelet adhesion. Likewise, the platelet adhesion effect was inhibited by using TM-AB (figure 13c). As previously shown, the platelets treated with S1P showed lower adhesion to HUVECs compared to the untreated platelets. Meanwhile the ones treated with S1P and TM-AB showed similar results to the untreated platelets explained by significantly higher platelet adhesion to HUVECs compared to the ones only treated with S1P. So, even by activating S1P-S1PR1-PI3K pathway, when TM is neutralized, the whole pathway is interrupted, and TM platelet anti-adhesive effect is abolished. The reduction in platelet adhesion may involve several parallel mechanisms. TM itself can interfere with thrombin-

mediated platelet activation by sequestering thrombin and redirecting its activity toward protein C activation.

Furthermore, we proceeded with *in vitro* tests to confirm that S1P-S1PR1-TM pathway downregulates the platelet reactivity and adhesion. Human P-selectin expression, measured by flow cytometry (figure 14b), was used as an indicator since P-selectin is released by activated platelets. Platelet groups were treated in different conditions then stimulated with Trap-6 each. The ones incubated with S1P showed no difference in P-selectin expression compared to platelets only stimulated with TRAP-6, whereas PRP treated with TM displayed reduced P-selectin significantly.

TRAP-6 is the used stimulant in our study because it is a thrombin agonist. Returning to figures 9 and 10, thrombin emerges as the central stimulant, converting fibrinogen to fibrin to drive the coagulation cascade. At the same time, thrombin is captured by TM, thereby inhibiting its activity and preventing further platelet activation. Thrombin-TM binding forms a complex that activates protein C. Then APC, in cooperation with its cofactor protein S, suppresses platelet-driven coagulation by inactivating factors Va and VIIIa and exerts anti-inflammatory effects through APC-EPCR complex via PAR signaling. This inhibitory pathway blocks FX activation and then prothrombin to thrombin conversion, ultimately suppressing platelet aggregation and coagulation (figure 10).

In summary, the findings of this section indicate that the S1P-S1PR1-TM axis exerts an antithrombotic effect, with S1P modulating platelet function via the whole pathway, while TM maintains direct contact with the platelets. Additionally, the absence of TM released into the supernatant suggests that S1P favors stabilization and upregulation of endothelial surface-bound TM, thereby maintaining its anticoagulant and anti-inflammatory functions rather than contributing to the pool of sTM that is typically considered a marker of endothelial injury.

4.2.2 Endothelial dependency

Our findings demonstrate that S1P exerts a clear endothelial-dependent anti-thrombotic effect that is mediated through activation of S1PR1 and subsequent induction of TM expression.

In the flow cytometry analysis (figure 14b), where human PRP was assessed in the absence of ECs, PRP treated with S1P did not exhibit any significant difference compared to the untreated control. In contrast, the TM-treated samples demonstrated a significantly reduced P-selectin expression relative to the control ($p=0.0443$). However, when following the same incubation and stimulation steps in the aggregation assay (figure 14c), no differences were observed between the samples.

This demonstrates that S1P does not influence platelet reactivity in absence of ECs. S1P-S1PR1-TM pathway impacts platelets activation but not the full aggregation process. In other words, it modulates the secondary hemostasis by reducing platelet activation without preventing complete aggregation. Platelet aggregation progresses to the stage in which thrombin converts fibrinogen to fibrin. However, in the presence of TM, thrombin becomes inactivated instead of driving this transformation. Therefore, the presence of ECs is essential.

4.2.3 Broader implications

The balance between different S1P receptor subtypes is key to understanding the dual roles of S1P in thrombosis and inflammation. While S1PR1 activation supports anticoagulant, anti-adhesive endothelial properties, signaling through S1PR2 and S1PR3 tend to promote pro-thrombotic responses such as platelet aggregation (S1PR2) and VWF release (S1PR3) [108]. Thus, the overall vascular outcome of S1P signaling depends on the receptor expression profile and cellular context. In healthy endothelium, S1PR1 predominates and promotes vascular quiescence, whereas in disease states characterized by endothelial dysfunction, loss of S1PR1 activity combined with upregulation of S1PR2 and S1PR3 may shift the balance toward thrombosis and inflammation.

In summary, our results identify TM as a novel downstream target of S1P-S1PR1-PI3K signaling in ECs, with functional consequences for limiting platelet adhesion under flow. By maintaining surface TM expression without inducing shedding, S1P shifts the endothelial phenotype toward an anticoagulant and anti-inflammatory state, complementing the known effects of S1PR1 on barrier stabilization, NO production and glycocalyx preservation. These findings extend the current understanding of S1P biology and emphasize the importance of endothelial signaling pathways in determining whether S1P functions as pro- or anti-thrombotic mediator.

4.3 Translation to *in vivo* thrombosis models

4.3.1 Rationale for *in vivo* translation

Our *in vitro* data established a clear endothelial S1PR1-PI3K-TM axis that suppresses platelet adhesion under physiological shear, without evidence on TM shedding. Because thrombosis is an emergent, multicellular process governed by the interplay of endothelium, platelets, leukocytes and plasma coagulation, it is essential to test whether the same protective signal translates to an organismal context. To address this, we pursued two complementary *in vivo* strategies: (i) pharmacological augmentation of S1P (systemic S1P administration) in wild-type mice, and (ii) genetic reduction of endogenous S1P (SphK1$^{-/-}$ mice), each linked to readouts of endothelial TM and arterial thrombosis (figures 16 to 19).

4.3.2 Pharmacologic S1P augments aortic TM and limits arterial thrombosis

Systemic S1P administration (38 ng/g, i.v.) showed higher aortic endothelial TM expression (ELISA) compared with vehicle (figure 16), consistent with our S1PR1-dependent TM upregulation.

Functionally, S1P-treated C57BL/6J mice exhibited attenuated carotid artery thrombus formation in the injury model (figure 17b) verified under IVM with an infinite time of occlusion (figure 17a). This explains that S1P reduces the thrombus size and abolishes the occlusion process representing the aggregation concept. Meanwhile the blood loss did not show any difference between the control and mice treated with S1P (figure 18). The latter point is critical: antithrombotic interventions often trade efficacy for bleeding liability. Thus, preservation of normal hemostasis alongside reduced arterial thrombosis is consistent with a surface-confined endothelial mechanism rather than coagulation function.

Mechanistically, several features align across *in vitro* and *in vivo* readouts:

1. Endothelial specificity: our flow chamber experiments show that S1P lowers platelet adhesion to endothelium, without directly decreasing platelet intrinsic reactivity; *in vivo*, the unchanged bleeding time supports this endothelium-first mechanism.
2. TM dependence: TM neutralization abolishes S1P´s anti-adhesive effect *in vitro*; *in vivo*, increased aortic TM (figure 16) and reduced platelet deposition/occlusion (figure 17) are coherent outcomes of the same axis.
3. No TM shedding: *in vitro*, S1P raises surface TM without increasing sTM; *in vivo*, the anti-thrombotic phenotype without bleeding fits retention of functional, endothelial-bound TM.

These findings extend classical endothelial-protective actions of S1P-barrier stabilization and eNOS activation into the hemostatic domain by identifying TM as a downstream effector

that functionally biases thrombin away from procoagulant substrates toward protein C and TAFI activation. Prior *in vivo* work often attributed the vascular benefits of S1P/S1PR1 to leak protection and glycocalyx preservation [91, 109]. Our data add anti-coagulant reprogramming of the endothelial surface as a parallel protective arm.

4.3.3 Genetic reduction of endogenous S1P

SphK1$^{-/-}$ mice, which exhibit 70 % lower circulating plasma S1P, showed reduced aortic TM expression (figures 19a). This genetic model strengthens causality in two ways. First, it demonstrates that physiological S1P is required to maintain endothelial TM *in vivo*. Second, the prothrombotic phenotype aligns with the blood loss of an endothelial anticoagulant program rather than gain of a procoagulant platelet program. Again, tail bleeding was not pathologically increased, pointed away from global hemostatic defects and coagulation.

Rescue experiments further support this interpretation: exogenous TM corrected the heightened thrombosis in the low-S1P setting (figure 19b). TM inhibited carotid artery thrombus formation in C57BL/6J mice. Meanwhile, in SphK1$^{-/-}$ mice, with reduced S1P production, displayed diminished TM expression and a pro-thrombotic phenotype. Supplementation of rTM reversed this effect, directly linking S1P deficiency to TM loss and increased thrombosis risk. This experiment not only validates TM as the key effector but also underscores its therapeutic potential in S1P-deficient states.

Together, the pharmacological and genetic data set a coherent narrative: S1P sustains endothelial TM, and TM is necessary and sufficient to oppose arterial thrombosis under arterial shear stress.

4.3.4 Reconciling divergent *in vivo* literature on S1P and thrombosis

The S1P field has long grappled with apparently conflicting *in vivo* findings. Some studies report pro-thrombotic actions (often attributed to S1PR2 and S1PR3 on platelets on VSMCs), whereas others describe vasculoprotective effects (typically via S1PR1 on the endothelium). Several variables explain this heterogeneity and contextualize our results:

4.3.4.1 Receptor subtype balance and cellular compartment

1. Endothelium (S1PR1-high): promotes barrier integrity, eNOS/NO, glycocalyx preservation, and as our study indicates, TM upregulation, anti-adhesive and anticoagulant surface [21, 91].
2. Platelets/VSMCs (S1PR2 and S1PR3-high): foster RhoA/ROCK signaling, calcium mobilization, VWF release, and contractile tone, which can favor thrombosis [106, 108].

Our models emphasize endothelial S1PR1 dominance in the carotid injury context, shifting the balance toward anticoagulation.

4.3.4.2 Injury model and shear environment

Laser-induced, photochemical, and carotid artery injury (IVM) differ in endothelial damage, tissue factor exposure, and VWF mobilization, which can differently weight S1PR1 and S1PR3 effects. Carotid artery injury (used in our study for IVM) includes endothelial denudation and strong platelet/VWF dependence, precisely where raising endothelial TM would be expected to blunt growth of the platelet-rich thrombus without disabling systemic hemostasis.

4.3.4.3 Ligand formulation and carrier biology

S1P circulates largely bound to albumin and HDL-apoM. HDL-bound S1P has been linked to S1PR1-biased endothelial signaling and vascular protection *in vivo*, whereas free albumin bound pools may more readily engage S1PR2 and S1PR3 in other compartments. Our i.v. dosing likely elevated both pools, but the dominant phenotype, enhanced endothelial TM and reduced arterial thrombosis, indicate that, under these conditions, endothelial S1PR1 signaling prevailed. This aligns with the established cardioprotective effect of HDL/S1P *in vivo* [89] and provides a plausible carrier-context explanation for the disparate outcomes reported across studies.

4.3.4.4 Dose, timing and receptor regulation

S1P responses are dose- and time-dependent. High local concentrations or chronic exposure can internalize S1PR1 pathways. Our regimen (single S1P dose, 16h pre-injury) appears to prime endothelium, raising TM by the time of arterial injury, rather than provoking acute off-target effects. This “vascular preconditioning” window is consistent with prior barrier-enhancement observations after S1P/S1PR1 agonism.

4.3.5 Mechanistic deepening: how might TM reduce thrombus growth *in vivo*?

TM-thrombin coupling reprograms thrombin specificity from procoagulant to anticoagulant and anti-inflammatory, generating APC (with protein S) and activating TAFI. In the arterial setting, this dual action yields several reinforcing effects:

1. Less platelet activation: by attaching thrombin to TM complexes, PAR-dependent platelet activation is reduced. Fewer platelets transition to pro-coagulant phenotype, decreasing phosphatidylserine exposure and prothrombinase assembly.
2. Weaker fibrin reinforcement: lower free thrombin reduces fibrin generation and cross-linking, giving the nascent thrombus less structural stability.

3. Endothelial quiescence and anti-adhesion: APC-PAR-1 signaling stabilizes junctions, increases NO, and transactivates S1PR1, further enhancing the anti-adhesive endothelial phenotype.
4. Fibrinolysis tuning: TAFI activation reduces inflammatory signals and modulates fibrinolysis at the thrombus-endothelium interface, which may reduce thrombus propagation without precipitating bleeding.

These mechanisms fit the observed antithrombotic effect, without bleeding signature in our mice models and offer a coherent bridge between molecular signaling and macroscopic clot behavior.

4.4 Relevance to human cardiovascular disease

4.4.1 Endothelial dysfunction as the clinical backdrop

Endothelial dysfunction is a common dominator across cardiovascular diseases and a mechanistic bridge between inflammation, thrombosis and atherogenesis. Clinically, it manifests as loss of barrier integrity, reduced NO bioavailability, heightened leukocyte and platelet adhesion, and a shift from anticoagulant to procoagulant endothelial surface properties. These alterations can be captured by circulating endothelial biomarkers and by functional tests of relevance. Here, sTM in plasma is widely interpreted as an indicator of endothelial damage or shedding. In many studies, higher sTM has been investigated as a potential predictor of developing coronary heart disease and subclinical atherosclerosis, consistent with the concept that TM loss from the endothelial surface and appearance in plasma tracks endothelial damage and prothrombotic milieu [85].

4.4.2 TM in the clinic: biomarker and therapeutic target

From a clinical perspective, TM occupies a dual role. First, as a biomarker, its soluble form increases when the endothelium is inflamed, oxidatively stressed, or proteolytically injured. A study linked sTM to coronary heart disease and carotid atherosclerosis, supporting its use as a readout of vascular risk and subclinical endothelial damage. The mechanistic interpretation remains consistent: higher sTM reflects loss of protective surface-bound TM, not a gain of anticoagulant function [85, 110].

Second, as a therapeutic, human rTM has reached clinical testing, based on its ability to neutralize thrombin and augment protein C activation. Meta-analytical data indicate a tendency toward lower mortality rates and enhanced resolution of disseminated intravascular coagulation without an associated increase in bleeding risk. However, large trials have produced mixed primary endpoints, illustrating both the promise and the complexity of translating TM biology to heterogenous clinical syndromes [111, 112]. These

experiences underscore the clinical importance of preserving endothelial TM at the vessel wall, where it reprograms thrombin and maintains anti-adhesive endothelial tone, precisely the phenotype our data show is reinforced by S1P-S1PR1-PI3K signaling.

4.4.3 Circulating S1P in human: vascular protection and risk association

Human S1P circulates predominantly bind to lipoproteins and albumin, with a major protective fraction carried by HDL via apoM. This carrier biology matters: apoM-bound S1P preferentially activates endothelial S1PR1, stabilizes barrier function and supports vascular quiescence. In mice and human, apoM determines HDL-S1P content. Loss of apoM reduces the S1P cargo and weakens endothelial protection, whereas apoM enrichment increases HDL-S1P and augments S1PR1 signaling. These observations provide a clinically relevant framework in which higher functional S1P signaling (especially via HDL/apoM) correlates with more antithrombotic and anti-inflammatory endothelium [113].

Clinical studies relating plasma S1P to cardiovascular phenotypes are accumulating. Several reports link lower S1P with worse cardiovascular status (e.g. heart failure severity in ischemic disease), although associations can be modulated by comorbidities and cardiac remodeling indices [114]. Others describe nuanced relationships between S1P and outcomes, reminding us that absolute S1P levels, carrier partitioning (apoM-HDL and albumin), and receptor balance jointly shape net vascular effects [115]. Together these human data are consistent with our mechanistic model: when endothelial S1PR1 signaling predominates, S1P promotes an anticoagulant anti-adhesive endothelial phenotype.

4.4.4 Integrating our human cohort data

In our patient cohort with established cardiovascular diseases, we quantified plasma S1P (LC-MS/MS) and thrombin activity markers (TATC). A negative correlation was identified between plasma S1P concentrations and thrombin levels (figure 20), concordant with the hypothesis that stronger S1P signaling tracks with less systemic thrombin generation.

Mechanistically, this aligns with our experimental findings: S1P-S1PR1 upregulates endothelial TM, which binds and reprograms thrombin away from procoagulant substrates toward protein C activation. Thus, individuals with lower circulating S1P might have less TM-supported endothelial anticoagulant tone, fostering higher thrombin activity at baseline. In the aggregate, our clinical observations are consistent with the directionality of the endothelial pathway demonstrated *in vitro* and *in vivo*.

Importantly, this relationship was detected in a real-world cardiovascular disease population with substantial comorbidity and diverse medications (e.g. frequent β-blocker, statin, OAC use) (table 7). The inverse S1P-thrombin association emerges despite these confounders

and increases confidence in a biologically meaningful link between S1P tone and thrombin burden at the system level.

4.4.5 How this framework explains clinical patterns of endothelial biomarkers

The mechanistic picture that emerges S1P-S1PR1 raises surface TM without shedding, thereby reducing thrombin activity and platelet adhesion, helps reconcile clinical biomarker patterns.

When endothelial health is preserved, one would expect lower thrombin generation and low sTM, mirroring our cohort´s inverse S1P-thrombin relationship. When endothelial injury dominates, sTM rises due to proteolytic shedding while the surface TM pool and its anticoagulant capacity decrease is associated with higher thrombin activity and clinical events. This is consistent with the Atherosclerosis Risk in Communities (ARIC) study and related observations that sTM tracks vascular risk and damage [85].

In this context, sTM and S1P offer complementary information: sTM reflects endothelial damage, whereas S1P (particularly its HDL/apoM-bound fraction) reflects endothelial protective signaling capacity. Their combined assessment may therefore improve risk stratification: low S1P + high sTM would indicate both insufficient protective signaling and active endothelial injury.

In summary, in human cardiovascular disease, endothelial dysfunction and thrombo-inflammation are intertwined. Our data show that S1P-S1PR1 activation restores an anticoagulant endothelial phenotype via TM, and in our cohort, higher S1P associates with lower thrombin activity. Placed alongside population evidence that sTM reflects endothelial damage and alongside mechanistic insights into apoM-HDL-mediated endothelial S1PR1 signaling, these findings support a clinically coherent model: preserving surface TM through endothelial S1PR1-PI3K signaling is a plausible route to reduce thrombin generation and platelet adhesion in patients. Future clinical studies that co-measure S1P, sTM and thrombin markers, and that test S1PR1-biased interventions, will be decisive in determining how this axis can be leveraged to reduce cardiovascular risk.

4.5 Mechanistic and therapeutic implications

These findings help explain discrepancies in previous S1P studies. Some report prothrombotic and others anti-thrombotic effects. Our data indicates that in intact vascular systems, S1P acts protectively via TM, but in endothelial-free or damaged systems, this pathway cannot operate, and direct platelet effects are negligible.

From a therapeutic standpoint, the S1P-TM axis offers an attractive target for preventing arterial thrombosis without bleeding complications. This is particularly relevant in settings where current anti-platelet or anti-coagulant therapy is contraindicated, such as in primary prevention high risk patients with elevated bleeding risk. Furthermore, in acute MI, where S1P is already known to confer cardio-protection, the added benefit of TM-mediated thrombosis suppression could offer dual protection for the myocardium and vasculature.

4.6 Limitations and future directions

Nevertheless, several limitations must be acknowledged. Our murine models cannot fully recapitulate human vascular pathology. The patient cohort, through translationally informative, was relatively small and cross-sectional, preventing causal inference. Moreover, the complex interplay between S1PR subtypes warrants further exploration, as activation S1PR2 and S1PR3 may counteract the protective effects mediated by S1PR1. Future studies should investigate whether pharmacological S1P analogues or TM-targeted therapies can reproducibly enhance vascular protection in larger patient populations, and whether these effects extend to inflammatory and atherosclerosis disease models.

4.7 Conclusion

This study identifies a novel anti-thrombotic mechanism in which S1P enhances endothelial TM expression via S1PR1 and PI3K signaling. This upregulation strengthens the endothelium's natural anti-coagulant capacity, reducing platelet adhesion and arterial thrombus formation without impairing normal hemostasis.

Our *in vitro* findings show that S1P´s effects are strictly endothelium-dependent, as platelet activation and aggregation remain unaffected in endothelial-free systems. *In vivo*, S1P injection in wild-type mice increased TM expression and conferred robust protection against carotid artery thrombosis, while SphK1 deficiency produced the opposite phenotype. The TM supplementation effectively reversed the prothrombotic state in SphK1$^{-/-}$ mice, underscoring TM´s pivotal role in this pathway.

In patients with cardiovascular disease, elevated plasma S1P levels are correlated with lower circulating thrombin, supporting the translational relevance of this mechanism.

Together, these results position the S1P-TM axis as a promising therapeutic target for preventing arterial thrombosis without the bleeding risk inherent to current anti-thrombotic agents. By preserving endothelial integrity and exploiting endogenous anti-coagulant pathways, S1P modulation could offer a safer, dual-action strategy in both primary prevention and acute vascular protection.

In summary, the key outcomes of this study demonstrate that S1P upregulates endothelial TM through S1PR1 and PI3K dependent signaling, which in turn diminishes platelet adhesion and limits arterial thrombus formation, all without increasing the bleeding risk. Thus, targeting S1PR1 signaling could represent a promising therapeutic approach for thrombotic diseases.

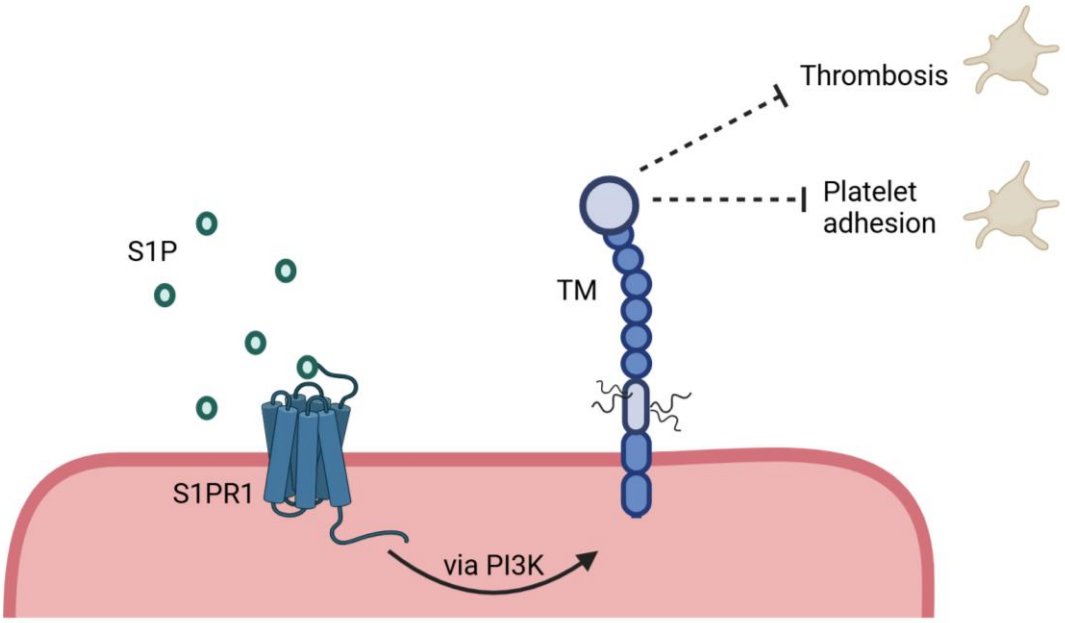

Figure 21: S1PR1 and endothelial TM signaling pathway.

S1PR1 interacts with TM through PI3K, leading to thrombosis and platelet adhesion inhibition without TM shedding or affecting the bleeding risk.

5 References

1. Jozefczuk, E., T.J. Guzik, and M. Siedlinski, *Significance of sphingosine-1-phosphate in cardiovascular physiology and pathology.* Pharmacol Res, 2020. **156**: p. 104793.
2. Spiegel, S. and S. Milstien, *Sphingosine-1-phosphate: an enigmatic signalling lipid.* Nature Reviews Molecular Cell Biology, 2003. **4**(5): p. 397-407.
3. Hla, T., et al., *S1P and the birth of platelets.* J Exp Med, 2012. **209**(12): p. 2137-40.
4. Thuy, A.V., et al., *Sphingosine 1-phosphate in blood: function, metabolism, and fate.* Cell Physiol Biochem, 2014. **34**(1): p. 158-71.
5. Weigel, C., J. Bellaci, and S. Spiegel, *Sphingosine-1-phosphate and its receptors in vascular endothelial and lymphatic barrier function.* J Biol Chem, 2023. **299**(6): p. 104775.
6. Igarashi, J. and T. Michel, *Sphingosine-1-phosphate and modulation of vascular tone.* Cardiovasc Res, 2009. **82**(2): p. 212-20.
7. Raza, Z., U. Saleem, and Z. Naureen, *Sphingosine 1-phosphate signaling in ischemia and reperfusion injury.* Prostaglandins Other Lipid Mediat, 2020. **149**: p. 106436.
8. Ouyang, J., et al., *The role of sphingosine 1-phosphate and its receptors in cardiovascular diseases.* J Cell Mol Med, 2020. **24**(18): p. 10290-10301.
9. Fyrst, H. and J.D. Saba, *An update on sphingosine-1-phosphate and other sphingolipid mediators.* Nat Chem Biol, 2010. **6**(7): p. 489-97.
10. Książek, M., et al., *Sources, metabolism, and regulation of circulating sphingosine-1-phosphate.* J Lipid Res, 2015. **56**(7): p. 1271-81.
11. Adams, D.R., S. Pyne, and N.J. Pyne, *Sphingosine Kinases: Emerging Structure-Function Insights.* Trends Biochem Sci, 2016. **41**(5): p. 395-409.
12. Cannavo, A., et al., *Sphingosine Kinases and Sphingosine 1-Phosphate Receptors: Signaling and Actions in the Cardiovascular System.* Front Pharmacol, 2017. **8**: p. 556.
13. Zhang, J., et al., *Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia.* Am J Physiol Heart Circ Physiol, 2007. **293**(5): p. H3150-8.
14. Landeen, L.K., et al., *Mechanisms of the negative inotropic effects of sphingosine-1-phosphate on adult mouse ventricular myocytes.* Am J Physiol Heart Circ Physiol, 2008. **294**(2): p. H736-49.

15. Thompson, B., et al., *Protein kinase Calpha and sphingosine 1-phosphate-dependent signaling in endothelial cell.* Prostaglandins Other Lipid Mediat, 2006. **80**(1-2): p. 15-27.
16. Alewijnse, A.E., S.L. Peters, and M.C. Michel, *Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites.* Br J Pharmacol, 2004. **143**(6): p. 666-84.
17. Bonnaud, S., et al., *Sphingosine-1-phosphate activates the AKT pathway to protect small intestines from radiation-induced endothelial apoptosis.* Cancer Res, 2010. **70**(23): p. 9905-15.
18. Wang, B., et al., *Sphingosine 1-phosphate receptor 1 governs endothelial barrier function and angiogenesis by upregulating endoglin signaling.* Ann Transl Med, 2022. **10**(3): p. 136.
19. Wang, H., H. Huang, and S.F. Ding, *Sphingosine-1-phosphate promotes the proliferation and attenuates apoptosis of Endothelial progenitor cells via S1PR1/S1PR3/PI3K/Akt pathway.* Cell Biol Int, 2018. **42**(11): p. 1492-1502.
20. Okamoto, Y., et al., *Sphingosine-1-Phosphate-Specific G Protein-Coupled Receptors as Novel Therapeutic Targets for Atherosclerosis*. Pharmaceuticals (Basel). 2011 Jan 4;4(1):117-37. doi: 10.3390/ph4010117. eCollection 2011 Jan.
21. Zeng, Y., et al., *Sphingosine-1-phosphate protects endothelial glycocalyx by inhibiting syndecan-1 shedding.* Am J Physiol Heart Circ Physiol, 2014. **306**(3): p. H363-72.
22. Zeng, Y., et al., *Sphingosine 1-phosphate induced synthesis of glycocalyx on endothelial cells.* Exp Cell Res, 2015. **339**(1): p. 90-5.
23. Spiegel, S. and S. Milstien, *Sphingosine-1-phosphate: an enigmatic signalling lipid.* Nat Rev Mol Cell Biol, 2003. **4**(5): p. 397-407.
24. Sanchez, T. and T. Hla, *Structural and functional characteristics of S1P receptors.* J Cell Biochem, 2004. **92**(5): p. 913-22.
25. Sanna, M.G., et al., *Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P1 antagonist in vivo.* Nature Chemical Biology, 2006. **2**(8): p. 434-441.
26. Liu, Y., et al., *Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation.* J Clin Invest, 2000. **106**(8): p. 951-61.
27. Vestri, A., et al., *Sphingosine 1-Phosphate Receptors: Do They Have a Therapeutic Potential in Cardiac Fibrosis?* Front Pharmacol, 2017. **8**: p. 296.
28. Liu, X., et al., *Endothelial S1pr1 regulates pressure overload-induced cardiac remodelling through AKT-eNOS pathway.* J Cell Mol Med, 2020. **24**(2): p. 2013-2026.

29. Yung, B.S., et al., *Selective coupling of the S1P(3) receptor subtype to S1P-mediated RhoA activation and cardioprotection.* J Mol Cell Cardiol, 2017. **103**: p. 1-10.
30. Liu, X., et al., *Cardiomyocyte S1PR1 promotes cardiac regeneration via AKT/mTORC1 signaling pathway.* Theranostics, 2025. **15**(4): p. 1524-1551.
31. Tang, L., et al., *Autophagy: a double-edged sword in ischemia-reperfusion injury.* Cell Mol Biol Lett, 2025. **30**(1): p. 42.
32. Sun, G., et al., *How do sphingosine-1-phosphate affect immune cells to resolve inflammation?* Front Immunol, 2024. **15**: p. 1362459.
33. Spampinato, S.F., M.A. Sortino, and S. Salomone, *Sphingosine-1-phosphate and Sphingosine-1-phosphate receptors in the cardiovascular system: pharmacology and clinical implications.* Adv Pharmacol, 2022. **94**: p. 95-139.
34. Zhang, F., et al., *Sphingosine 1-phosphate signaling contributes to cardiac inflammation, dysfunction, and remodeling following myocardial infarction.* Am J Physiol Heart Circ Physiol, 2016. **310**(2): p. H250-61.
35. Polzin, A., et al., *Sphingosine-1-phosphate improves outcome of no-reflow acute myocardial infarction via sphingosine-1-phosphate receptor 1.* ESC Heart Fail, 2023. **10**(1): p. 334-341.
36. Jin, Z.Q., et al., *A sphingosine kinase 1 mutation sensitizes the myocardium to ischemia/reperfusion injury.* Cardiovasc Res, 2007. **76**(1): p. 41-50.
37. Behrangi, N., F. Fischbach, and M. Kipp, *Mechanism of Siponimod: Anti-Inflammatory and Neuroprotective Mode of Action.* Cells, 2019. **8**(1).
38. Coyle, P.K., et al., *Sphingosine 1-phosphate receptor modulators in multiple sclerosis treatment: A practical review.* Ann Clin Transl Neurol, 2024. **11**(4): p. 842-855.
39. in *Mechanisms of Vascular Disease: A Reference Book for Vascular Specialists*, R. Fitridge and M. Thompson, Editors. 2011, University of Adelaide Press

© The Contributors 2011.: Adelaide (AU).

40. LaPelusa, A. and H.D. Dave, *Physiology, Hemostasis*, in *StatPearls*. 2024, StatPearls Publishing

Disclosure: Heeransh Dave declares no relevant financial relationships with ineligible companies.

41. van der Meijden, P.E.J. and J.W.M. Heemskerk, *Platelet biology and functions: new concepts and clinical perspectives.* Nat Rev Cardiol, 2019. **16**(3): p. 166-179.

42. Tyagi, T., et al., *A guide to molecular and functional investigations of platelets to bridge basic and clinical sciences.* Nat Cardiovasc Res, 2022. **1**(3): p. 223-237.
43. Thomas, M.R. and R.F. Storey, *The role of platelets in inflammation*. Thromb Haemost, 2015. **114**(3): p. 449-58.
44. Ware, J.A. and D.D. Heistad, *Seminars in medicine of the Beth Israel Hospital, Boston. Platelet-endothelium interactions.* N Engl J Med, 1993. **328**(9): p. 628-35.
45. McRae, S., *Physiological Haemostasis*, in *Mechanisms of Vascular Disease: A Reference Book for Vascular Specialists*, R. Fitridge and M. Thompson, Editors. 2011: Adelaide (AU).
46. Yun, S.H., et al., *Platelet Activation: The Mechanisms and Potential Biomarkers.* Biomed Res Int, 2016. **2016**: p. 9060143.
47. Moroi, M., *[Platelet receptors for collagen].* Seikagaku, 2000. **72**(7): p. 543-6.
48. Monnet, E., et al., *Different role of platelet glycoprotein GP Ia/IIa in platelet contact and activation induced by type I and type III collagens.* Thromb Res, 2000. **98**(5): p. 423-33.
49. Benkhoff, M., *Lipide und Lipoproteine in der Kardioprotektion*. 2022, Heinrich-Heine-University.
50. Kahn, M.L., et al., *Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin.* J Clin Invest, 1999. **103**(6): p. 879-87.
51. Aslan, J.E., et al., *Platelet shape change and spreading.* Methods Mol Biol, 2012. **788**: p. 91-100.
52. Shin, E.K., et al., *Platelet Shape Changes and Cytoskeleton Dynamics as Novel Therapeutic Targets for Anti-Thrombotic Drugs.* Biomol Ther (Seoul), 2017. **25**(3): p. 223-230.
53. Reed, G.L., *Platelet secretory mechanisms.* Semin Thromb Hemost, 2004. **30**(4): p. 441-50.
54. Lemons, P.P., D. Chen, and S.W. Whiteheart, *Molecular mechanisms of platelet exocytosis: requirements for alpha-granule release.* Biochem Biophys Res Commun, 2000. **267**(3): p. 875-80.
55. Lai, K.C. and R. Flaumenhaft, *SNARE protein degradation upon platelet activation: calpain cleaves SNAP-23.* J Cell Physiol, 2003. **194**(2): p. 206-14.
56. Frojmovic, M.M. and J.G. Milton, *Human platelet size, shape, and related functions in health and disease.* Physiol Rev, 1982. **62**(1): p. 185-261.
57. Piersma, S.R., et al., *Proteomics of the TRAP-induced platelet releasate.* J Proteomics, 2009. **72**(1): p. 91-109.

58. Maynard, D.M., et al., *Proteomic analysis of platelet alpha-granules using mass spectrometry.* J Thromb Haemost, 2007. **5**(9): p. 1945-55.
59. Kaplan, K., et al., *Platelet alpha-granule proteins: studies on release and subcellular localization.* Blood, 1979. **53**: p. 604-618.
60. Orkin, S.H., et al., *Nathan and Oski's hematology of infancy and childhood.* Elsevier Health Sciences, 2009: p. 1386.
61. Ruggeri, Z., *Perspective series: cell adhesion in vascular biology. von Willebrand factor.* J Clin Invest, 1999. **99**: p. 559-564.
62. Jurk, K. and B.E. Kehrel, *Platelets: physiology and biochemistry.* Semin Thromb Hemost, 2005. **31**(4): p. 381-92.
63. Sharda, A. and R. Flaumenhaft, *The life cycle of platelet granules.* F1000Res, 2018. **7**: p. 236.
64. White, J.G., *Use of the electron microscope for diagnosis of platelet disorders.* Semin Thromb Hemost, 1998. **24**(2): p. 163-8.
65. Sang, Y., et al., *Interplay between platelets and coagulation.* Blood Reviews, 2021. **46**.
66. Sierra, C., M. Moreno, and J.C. Garcia-Ruiz, *The physiology of hemostasis.* Blood Coagul Fibrinolysis, 2022. **33**(Suppl 1): p. S1-S2.
67. Chaudhry, R., S.M. Usama, and H.M. Babiker, *Physiology, Coagulation Pathways*, in *StatPearls*. 2023: Treasure Island (FL).
68. van Herrewegen, F., et al., *Clinical practice: the bleeding child. Part II: disorders of secondary hemostasis and fibrinolysis.* Eur J Pediatr, 2012. **171**(2): p. 207-14.
69. Davie, E.W. and O.D. Ratnoff, *Waterfall Sequence for Intrinsic Blood Clotting.* Science, 1964. **145**(3638): p. 1310-2.
70. Hoffman, M. and D.M. Monroe, 3rd, *A cell-based model of hemostasis.* Thromb Haemost, 2001. **85**(6): p. 958-65.
71. Giannitsi, S., et al., *Endothelial dysfunction and heart failure: A review of the existing bibliography with emphasis on flow mediated dilation.* JRSM Cardiovasc Dis, 2019. **8**: p. 2048004019843047.
72. Martin, F.A., R.P. Murphy, and P.M. Cummins, *Thrombomodulin and the vascular endothelium: insights into functional, regulatory, and therapeutic aspects.* Am J Physiol Heart Circ Physiol, 2013. **304**(12): p. H1585-97.
73. Koupenova, M., et al., *Circulating Platelets as Mediators of Immunity, Inflammation, and Thrombosis.* Circ Res, 2018. **122**(2): p. 337-351.

74. Wilkins, G.C., et al., *Dissecting the Therapeutic Mechanisms of Sphingosine-1-Phosphate Receptor Agonism during Ischaemia and Reperfusion.* Int J Mol Sci, 2023. **24**(13).
75. Nussbaum, C., et al., *Sphingosine-1-phosphate receptor 3 promotes leukocyte rolling by mobilizing endothelial P-selectin.* Nat Commun, 2015. **6**: p. 6416.
76. Dittman, W.A. and P.W. Majerus, *Structure and function of thrombomodulin: a natural anticoagulant.* Blood, 1990. **75**(2): p. 329-36.
77. Okamoto, T., et al., *Thrombomodulin: a bifunctional modulator of inflammation and coagulation in sepsis.* Crit Care Res Pract, 2012. **2012**: p. 614545.
78. Suzuki, K., et al., *A domain composed of epidermal growth factor-like structures of human thrombomodulin is essential for thrombin binding and for protein C activation.* J Biol Chem, 1989. **264**(9): p. 4872-6.
79. Ito, T., et al., *Thrombomodulin in disseminated intravascular coagulation and other critical conditions-a multi-faceted anticoagulant protein with therapeutic potential.* Crit Care, 2019. **23**(1): p. 280.
80. Esmon, C.T., *The protein C pathway.* Chest, 2003. **124**(3 Suppl): p. 26s-32s.
81. Conway, E.M., *Thrombomodulin and its role in inflammation.* Semin Immunopathol, 2012. **34**(1): p. 107-25.
82. Glaser, C.B., et al., *Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A potential rapid mechanism for modulation of coagulation.* J Clin Invest, 1992. **90**(6): p. 2565-73.
83. Loghmani, H. and E.M. Conway, *Exploring traditional and nontraditional roles for thrombomodulin.* Blood, 2018. **132**(2): p. 148-158.
84. Giri, H., I. Biswas, and A.R. Rezaie, *Thrombomodulin Regulates PTEN/AKT Signaling Axis in Endothelial Cells.* Arterioscler Thromb Vasc Biol, 2024. **44**(2): p. 352-365.
85. Salomaa, V., et al., *Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study.* Lancet, 1999. **353**(9166): p. 1729-34.
86. Corporation, A.K., *Thrombomodulin alfa (ART-123, Recomodulin®) – Approval and Use in Japan.* 2025.
87. Peng, B., et al., *Identification of key lipids critical for platelet activation by comprehensive analysis of the platelet lipidome.* Blood, 2018. **132**(5): p. e1-e12.
88. Adams, K.J., et al., *Skyline for Small Molecules: A Unifying Software Package for Quantitative Metabolomics.* J Proteome Res, 2020. **19**(4): p. 1447-1458.

89. Theilmeier, G., et al., *High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor.* Circulation, 2006. **114**(13): p. 1403-9.
90. Mourikis, P., et al., *Icosapent ethyl reduces arterial thrombosis by inhibition of cyclooxygenase-1-induced platelet reactivity.* Sci Transl Med, 2025. **17**(799): p. eado0610.
91. Garcia, J.G., et al., *Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement.* J Clin Invest, 2001. **108**(5): p. 689-701.
92. Tarrasón, G., et al., *The sphingosine-1-phosphate receptor-1 antagonist, W146, causes early and short-lasting peripheral blood lymphopenia in mice.* Int Immunopharmacol, 2011. **11**(11): p. 1773-9.
93. Oo, M.L., et al., *Engagement of $S1P_1$-degradative mechanisms leads to vascular leak in mice.* J Clin Invest, 2011. **121**(6): p. 2290-300.
94. Malek, A.M., et al., *Endothelial expression of thrombomodulin is reversibly regulated by fluid shear stress.* Circ Res, 1994. **74**(5): p. 852-60.
95. Takada, Y., et al., *Fluid shear stress increases the expression of thrombomodulin by cultured human endothelial cells.* Biochem Biophys Res Commun, 1994. **205**(2): p. 1345-52.
96. Lin, Z., et al., *Kruppel-like factor 2 (KLF2) regulates endothelial thrombotic function.* Circ Res, 2005. **96**(5): p. e48-57.
97. Sangwung, P., et al., *KLF2 and KLF4 control endothelial identity and vascular integrity.* JCI Insight, 2017. **2**(4): p. e91700.
98. Langeslag, M., et al., *Sphingosine 1-phosphate to p38 signaling via S1P1 receptor and Gαi/o evokes augmentation of capsaicin-induced ionic currents in mouse sensory neurons.* Mol Pain, 2014. **10**: p. 74.
99. Safarian, F., et al., *Activation of $S1P_1$ receptor regulates PI3K/Akt/FoxO3a pathway in response to oxidative stress in PC12 cells.* J Mol Neurosci, 2015. **56**(1): p. 177-87.
100. Ramachandran, A., et al., *An Akt- and Fra-1-dependent pathway mediates platelet-derived growth factor-induced expression of thrombomodulin, a novel regulator of smooth muscle cell migration.* Am J Pathol, 2010. **177**(1): p. 119-31.
101. Hsu, Y.Y., et al., *Thrombomodulin promotes focal adhesion kinase activation and contributes to angiogenesis by binding to fibronectin.* Oncotarget, 2016. **7**(42): p. 68122-68139.

102. Yang, W., Q. Li, and Z. Pan, *Sphingosine-1-phosphate promotes extravillous trophoblast cell invasion by activating MEK/ERK/MMP-2 signaling pathways via S1P/S1PR1 axis activation.* PLoS One, 2014. **9**(9): p. e106725.
103. Urtz, N., et al., *Sphingosine 1-Phosphate Produced by Sphingosine Kinase 2 Intrinsically Controls Platelet Aggregation In Vitro and In Vivo.* Circ Res, 2015. **117**(4): p. 376-87.
104. Yatomi, Y., et al., *Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets.* Blood, 1995. **86**(1): p. 193-202.
105. Liu, H., et al., *Sphingosine-1-phosphate modulates PAR1-mediated human platelet activation in a concentration-dependent biphasic manner.* Scientific Reports, 2021. **11**(1): p. 15308.
106. Randriamboavonjy, V., et al., *The S1P(2) receptor expressed in human platelets is linked to the RhoA-Rho kinase pathway and is down regulated in type 2 diabetes.* Basic Res Cardiol, 2009. **104**(3): p. 333-40.
107. Zheng, H., et al., *S1PR1-biased activation drives the resolution of endothelial dysfunction-associated inflammatory diseases by maintaining endothelial integrity.* Nat Commun, 2025. **16**(1): p. 1826.
108. van Hooren, K.W., et al., *Sphingosine-1-phosphate receptor 3 mediates sphingosine-1-phosphate induced release of weibel-palade bodies from endothelial cells.* PLoS One, 2014. **9**(3): p. e91346.
109. Wang, L., et al., *FTY720-induced human pulmonary endothelial barrier enhancement is mediated by c-Abl.* Eur Respir J, 2011. **38**(1): p. 78-88.
110. Cella, G. and M.L. Randi, *Soluble thrombomodulin as predictor of incident coronary heart disease.* Lancet, 1999. **354**(9176): p. 425.
111. Kato, H., et al., *Efficacy and safety of recombinant human soluble thrombomodulin in patients with sepsis-induced disseminated intravascular coagulation - A meta-analysis.* Thromb Res, 2023. **226**: p. 165-172.
112. Valeriani, E., et al., *Efficacy and safety of recombinant human soluble thrombomodulin in patients with sepsis-associated coagulopathy: A systematic review and meta-analysis.* J Thromb Haemost, 2020. **18**(7): p. 1618-1625.
113. Christoffersen, C., et al., *Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M.* Proc Natl Acad Sci U S A, 2011. **108**(23): p. 9613-8.

114. Polzin, A., et al., *Plasma sphingosine-1-phosphate concentrations are associated with systolic heart failure in patients with ischemic heart disease.* J Mol Cell Cardiol, 2017. **110**: p. 35-37.
115. Xue, Y., et al., *U-shaped association between plasma sphingosine-1-phosphate levels and mortality in patients with chronic systolic heart failure: a prospective cohort study.* Lipids in Health and Disease, 2020. **19**(1): p. 125.

Acknowledgements

I would like to express my deepest gratitude to Prof.Dr. Malte Kelm for the opportunity to conduct my research in the Cardiovascular Research Laboratory (CVRL). I would also like to thank all the cooperation partners who contributed to the overall picture of this dissertation with their work. Further, I would like to sincerely thank my doctor advisor, Prof.Dr.med. Amin Polzin, for providing an interesting topic and for supervising me throughout the entire duration of the work. Over the last five years in his research group, I was able to grow professionally and personally. I would like also to thank PD Dr.med. Christian Buchbender for taking on the role of second supervisor.

I would like to thank Polzin´s research group members. I am grateful to the entire CVRL lab team and Prof. Norbert Gerdes, the lab director. Without all of you and your support, it would not have been possible to accomplish all this work. Thank you for the great friendship, wonderful memories, laughter and celebration times. Thank you for the time we shared conversations, professional and moral support. I faced many challenges and hard times during these five years, and I am deeply grateful to each of you for your support, helping me get back on my feet and continue moving forward.

I would also like to extend my sincere thanks to Polzin´s group leaders for their valuable feedback, guidance and support all this time, especially Dr. Marcel Benkhoff.

I would like to express my heartfelt appreciation to the Medical Research School for their continuous support and guidance through my PhD journey. Their assistance in providing academic resources, training opportunities and stimulating research environment has been remarkable to the successful completion of this work.

On a more personal note, I would like to warmly thank my family and my friends for the invaluable encouragement. My greatest thanks to my parents Gaby and Rima, my uncle Youssef, my brother Georgio and my sister Maria, who supported me all the time and were always by my side. Your belief in me has been the greatest source of strength. Thank you all for being by my side in the ups and downs, for your endless patience and understanding during this process.

Finally, I dedicate this work to everyone who believed in me and helped me see it through to the end.

Thank you all,

Gabrielle Al Kassis