

The Behavior of Blood Platelets in the Context of Inflammation

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“I am not great at everything, but I am great at one thing: surrounding myself with excellent people. And that tends to take care of the rest.”

- Sheldon Menery

Zusammenfassung

Blutplättchen gelten als zentrale Regulatoren der Hämostase und Thrombose. In jüngerer Zeit wurde gezeigt, dass sie zudem eine aktive Rolle bei Entzündungen und der Immunabwehr spielen. Über die biochemische Signalübertragung hinaus wird die Blutplättchenfunktion stark von biomechanischen Faktoren beeinflusst, darunter die Steifigkeit des Substrats, die Thrombusstruktur und die Organisation des Zytoskeletts. Wie diese biochemischen und biomechanischen Signale das Verhalten der Blutplättchen im Entzündungskontext beeinflussen, ist jedoch noch weitgehend ungeklärt.

In dieser Arbeit wurde das Verhalten von Blutplättchen im Entzündungskontext mithilfe von hochauflösender Rasterionenleitfähigkeitsmikroskopie (SICM), Fluoreszenzmikroskopie, Deep-Learning-gestütztem Tracking und biomechanischer Quantifizierung untersucht. Die Arbeit beleuchtet das Verhalten von Blutplättchen bei Entzündungen in drei unterschiedlichen Bereichen: den Einfluss von (oxidierten) Lipiden auf die zelluläre Steifigkeit und die Aktinarchitektur der Blutplättchen, die mechanosensitive Regulation der Blutplättchenmigration durch die Substratsteifigkeit sowie die durch zyklisches Guanosinmonophosphat (cGMP) regulierte dreidimensionale Struktur von Thromben. Eine kurze Zusammenfassung der Ergebnisse aus jedem Bereich findet sich in den folgenden Abschnitten.

Ein entzündetes Blutgefäß neigt zur Anreicherung von (oxidierten) Lipiden in der Gefäßwand, die mit Blutplättchen interagieren. Die Biomechanik und Morphologie der Blutplättchen wurde im Kontext der Aktivierung durch (oxidierte) Lipide und der Aktivierung des atypischen Chemokinrezeptors 3 (ACKR3) mittels SICM-Steifigkeitsabbildung untersucht. Die pharmakologische Aktivierung von ACKR3 reduzierte das Elastizitätsmodul der Blutplättchen signifikant. Darüber hinaus führte oxidiertes Low-Density-Lipoprotein (OxLDL), nicht jedoch natives LDL, zu einer Verringerung des Elastizitätsmoduls der Blutplättchen. Die ACKR3-Aktivierung kehrte die durch OxLDL induzierte Erweichung der Blutplättchen nicht um. Die Fluoreszenzmikroskopie zeigte eine deutliche Veränderung der Morphologie von anhaftenden Blutplättchen und der Aktin-Netzwerkstruktur nach Zugabe von (Ox)LDL, einschließlich der Bildung von Filopodien und Actin Nodules. Diese Ergebnisse zeigen, dass die Lipidaufnahme die Blutplättchen-Steifigkeit stark beeinflusst, und verdeutlichen, dass das Elastizitätsmodul der Blutplättchen ein biophysikalischer Marker für die Blutplättchenaktivierung unter hyperlipidämischen Bedingungen ist.

Entzündetes Gewebe verändert seine Steifigkeit. Da die Blutplättchenmigration ein wichtiger Bestandteil der Immunabwehr ist und eine wesentliche Rolle bei Entzündungen spielt, wurde der Einfluss dieser Veränderung der Substratsteifigkeit auf die haptotaktische Blutplättchenmigration untersucht. Die haptotaktische Migration der Blutplättchen wurde auf

drei mit Fibrinogen beschichteten Substraten auf Polydimethylsiloxan-(PDMS)-Basis mit definierten Elastizitätsmodulen mittels Lichtmikroskopie und Deep-Learning-basierter Bildanalyse verfolgt. Blutplättchen migrierten auf steifen Substraten effizient, doch die Migrationsgeschwindigkeit und die Anzahl der migrierenden Blutplättchen waren auf weicheren Substraten deutlich verringert. Die dreidimensionale Formanalyse der Blutplättchen zeigte, dass sich Blutplättchen auf weichen Substraten nicht polarisieren konnten. Die für migrierende Blutplättchen typische anisotropische Form war ebenfalls deutlich reduziert. Die Fluoreszenzmikroskopie von fluoreszierendem Fibrinogen zeigte, dass Blutplättchen während der Migration einen deutlichen Fibrinogengradienten erzeugen, wobei der von Blutplättchen auf weichen Substraten erzeugte Gradient deutlich reduziert war. Diese Ergebnisse belegen einen Zusammenhang zwischen der Substratsteifigkeit und der Fähigkeit der Blutplättchen zur haptotaktischen Migration.

Wenn die Entzündung der Gefäßwand ungehindert fortschreitet, sind ein Gefäßwandriss und eine daraus resultierende Thrombose unvermeidlich. Um die Bildung eines Thrombus nach einem Gefäßwandriss zu regulieren, nutzt der Körper eine auf Stickstoffmonoxid (NO) und cGMP basierende Signalkaskade, um die Blutplättchenaktivierung zu hemmen. Der Einfluss dieser Signalkaskade auf die Thrombusarchitektur wurde untersucht. Dreidimensionale SICM-Bildgebung zeigte, dass die pharmakologische Stimulation der NO-sensitiven löslichen Guanylatzyklase (NO-GC) mit Riociguat und NO-Donoren das Thrombusvolumen reduzierte, ohne die Hämostase vollständig zu stören. Zudem war die Oberflächenrauheit des Thrombus signifikant erhöht, was auf eine verminderte Kontraktionsfähigkeit des Gerinnsels hindeutet. Diese Ergebnisse unterstreichen die regulierende Wirkung der cGMP-basierten Signalkaskade auf die Thrombusstabilität bei gleichzeitiger Aufrechterhaltung der physiologischen Hämostase auf klinisch relevantem Niveau.

Summary

Platelets are known as central regulators of hemostasis and thrombosis. More recently, they have been revealed to play an active role in inflammation and immune defense. Beyond biochemical signaling, platelet function is strongly influenced by biomechanical cues, including substrate stiffness, thrombus structure and cytoskeletal organization. However, how these biochemical and biomechanical signals influence platelet behavior in an inflammatory context remains poorly understood.

In this thesis, platelet behavior in the context of inflammation was investigated using high-resolution scanning-ion conductance microscopy (SICM), fluorescence microscopy, deep-learning-supported tracking, and biomechanical quantification. The work illustrates platelet behavior in inflammation in three distinct areas: the influence of (oxidized) lipids on platelet cellular stiffness and actin architecture, the mechanosensitive regulation of platelet migration by substrate stiffness, and the three-dimensional structure of thrombi regulated by cyclic guanosine monophosphate (cGMP). A short summary of the results from each area is given in each of the following paragraphs.

An increased blood content of (oxidized) lipids is often a precursor of cardiovascular diseases, but the influence of those lipids on platelet biomechanics is poorly understood. Platelet biomechanics and morphology were investigated in the context of (oxidized) lipid exposure and atypical chemokine receptor 3 (ACKR3) signaling using SICM stiffness mapping. Pharmacological activation of ACKR3 significantly reduced platelet Young's modulus. In addition, oxidized low-density lipoprotein (OxLDL), but not native LDL, softened platelet Young's modulus. ACKR3 activation did not reverse platelet softening induced by OxLDL. Fluorescence microscopy revealed a distinct change in adhered platelet morphology and actin network structure upon (Ox)LDL addition, including filopodia formation and actin nodules. These results demonstrate that lipid uptake heavily influences platelet stiffness and illustrates platelet Young's modulus as a biophysical marker of platelet activation under hyperlipidemic conditions.

Inflamed tissue changes its stiffness. As platelet migration is an important part of the immune defense and plays a significant role in inflammation, the influence of this change in substrate stiffness on platelet haptotactic migration was investigated. Platelet haptotactic migration was tracked on three fibrinogen-coated polydimethylsiloxane (PDMS)-based substrates with defined Young's moduli using light microscopy and deep-learning-based image analysis. Platelets migrated efficiently on stiff substrates, but migration speed and number of migrating platelets was significantly diminished on softer substrates. Platelet three-dimensional shape analysis revealed that platelets on soft substrates failed to polarize. The shape anisotropy typical for migrating platelets was also significantly reduced. Fluorescence microscopy of

fluorescent fibrinogen revealed that platelets create a distinct fibrinogen gradient during migration, with the gradient generated by platelets migrating on soft substrate being significantly reduced. These findings demonstrate a dependency between the substrate stiffness and the ability of platelet to haptotactically migrate.

If inflammation of the blood vessel wall is unimpeded, a vessel wall rupture and resulting thrombosis is inevitable. To regulate this build-up of a thrombus after vessel wall rupture, the body uses a nitric oxide (NO) and cGMP-based signaling cascade to inhibit platelet activation. The influence of this signaling cascade on thrombus architecture was investigated. Three-dimensional SICM imaging revealed that pharmacological stimulation of NO-sensitive soluble guanylyl cyclase (NO-GC) with riociguat and NO-donors reduced thrombus volume, without fully disrupting hemostasis. In addition, thrombus surface roughness was significantly increased, hinting at a reduced capacity for clot contraction. These results highlight the regulatory effect of the cGMP-based signal cascade on thrombus stability while preserving physiological hemostasis at clinically relevant levels.

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1 Introduction

Cardiovascular disease (CVD) is the leading cause of death, causing nearly 20.5 million deaths in 2021 (Lindstrom et al., 2022; Di Cesare et al., 2024). The most common form of CVD is atherosclerosis (Libby et al., 2019; Libby, 2021a), the accumulation of fatty residues and inflammation in the large arteries, potentially leading to life-threatening diseases such as acute myocardial infarction (Reed et al., 2017), acute coronary syndrome (Bergmark et al., 2022) or ischemic stroke (Feske, 2021).

Our current understanding of the pathophysiology of atherosclerosis is based on a three-step process:

1. Damage to the blood vessel wall or endothelial dysfunction may cause a build-up of blood lipoproteins such as low-density lipoprotein (LDL) under the endothelium in the tunica media (Zhang et al., 2018; Björkegren & Lusis, 2022).
2. This increased lipoprotein accumulation is accompanied by lipoprotein modification, such as oxidation, leading to increased inflammation and foam cell formation, causing the build-up of plaques made from fat and cell debris in the blood vessel wall and reducing the flow of oxygenated blood to organs and limbs (Tabas et al., 2015; Gui et al., 2022).
3. In the final step, unstable plaques can burst, releasing their contents into the blood stream, which can cause platelets to form a blood clot or thrombus (a process called thrombosis), potentially leading to a full blockage of the blood vessel (Stark & Massberg, 2021).

This occlusion then can cause major thrombotic events such as myocardial infarction, stroke, or loss of limbs (Reed et al., 2017; Criqui et al., 2021; Feske, 2021). Effective intervention of these thrombotic events relies on disrupting platelet-induced thrombosis and treating underlying causes such as hyperlipidemia, a chronically increased level of lipids in the blood (Karr, 2017).

Current treatment options for cardiovascular diseases targeting platelets suffer from several drawbacks (Weber et al., 2017; Paraskevas et al., 2022; Shubietah et al., 2024). Using acetylsalicylic acid, more commonly known as aspirin, for prevention of cardiovascular diseases long-term might cause side effects such as (gastrointestinal) bleeding, kidney dysfunction or increased asthma severity, especially in elderly patients (Karsh, 1990). In addition, discontinuation of aspirin intake increases platelet aggregability and thus occurrence of thrombotic events (Biondi-Zoccai et al., 2006). Given these circumstances, there is still a need to understand the unique role of platelets in CVD and to find novel therapeutic targets (Esenwa & Gutierrez, 2015; Gawaz et al., 2023). For this endeavor, the interplay of platelets, thrombo-inflammation and atherosclerosis are of great importance (d'Alessandro et al., 2020; Rayes et al., 2020; Gawaz et al., 2023; Pan et al., 2024).

This thesis elucidates the roles of platelets in an inflammatory context from multiple aspects, such as platelet activation by oxidized lipoproteins and its regulation following treatment with a pharmacological atypical chemokine receptor 3 (ACKR3)-specific agonist that acts as an anti-thrombotic mediator (chapter 3), the influence of the substrate elastic modulus on platelet migration (chapter 4), and the thrombi structure investigated with scanning ion conductance microscopy (chapter 5).

1.1 Platelets and platelet hemostasis

Platelets are the smallest blood cells in our body. First described in 1882 by the pathologist Bizzozero (Bizzozero, 1882; Ribatti & Crivellato, 2007; Carlo, 2020), these anucleate cells derive from megakaryocytes and have a discoid shape, with about 2-3 μm in diameter and a volume of $7\text{-}12\cdot 10^{-15}$ L in their resting state (Demirin et al., 2011; Korniluk et al., 2019; Michelson, 2019).

A long-established function of platelets is the physiological response to stop bleeding and initiate wound repair, called hemostasis (Versteeg et al., 2013): If an injury destroys part of a blood vessel, extracellular matrix proteins such as collagen or von Willebrand factor (vWF) are released into the blood stream. Upon encountering such proteins, platelets bind quickly to the site of injury by the interaction of the receptor glycoprotein (GP)Ib-v-IX with vWF-collagen complexes as well as GPII with collagen (Savage et al., 1998; Nieswandt & Watson, 2003; Li et al., 2010). These interactions trigger an intracellular signal cascade, ultimately leading to platelet activation. Platelet activation is accompanied by the release of secondary platelet agonists such as thromboxane (TxA_2) and adenosine diphosphate (ADP) (José et al., 2009; Golebiewska & Poole, 2015; Patrono & Rocca, 2019). In addition, the ligand-affinity of other receptors is increased, e.g. the affinity of fibrinogen receptor GPIIb/IIIa/integrin $\alpha\text{IIb}\beta 3$ to fibrinogen (a process called inside-out signaling) (Payraastre et al., 2000; Shattil & Newman, 2004; Huang et al., 2019). Binding of soluble fibrinogen to high-affinity $\alpha\text{IIb}\beta 3$ then triggers a signal cascade inside the platelet to facilitate strong platelet adhesion and spreading (a process called outside-in signaling) (Durrant et al., 2017; Huang et al., 2019). At the same time, other platelets are recruited to the site of injury via coagulation factors and the production of thrombin (Agbani & Poole, 2017; Podoplelova et al., 2021; Denorme & Campbell, 2022). These procoagulant platelets show phosphatidylserine (PS) on their cell membrane, while the platelets initially binding to the site of injury show no such PS-expression, ultimately dividing the aggregating platelets into two subpopulations with distinct functions: Platelets with exposed PS control thrombin generation and fibrin crosslinking, while platelets with high-affinity $\alpha\text{IIb}\beta 3$ regulate clot retraction (Heemskerk et al., 2013). As a last step, thrombin produced by procoagulant platelets cleaves fibrinogen to form a cross-linked fibrin network, stabilizing the resulting blood clot (also called thrombus) (Hulshof et al., 2021; Larsen & Hvas, 2021).

Where hemostasis is the formation of a blood clot under normal physiological conditions (e.g. in response to a wound), thrombosis refers to the pathological formation of a blood clot (Guo & Rondina, 2019). Thrombosis is often the final result of chronic cardiovascular diseases, leading to blood vessel occlusion, myocardial infarction, or stroke (Koupenova et al., 2016; Buja et al., 2019). As these cardiovascular events are still the leading causes of death (Wendelboe & Raskob, 2016), understanding platelet behavior in inflammation and cardiovascular diseases remains a top priority for basic science and drug target discovery.

1.2 Blood vessel structure

The arterial blood vessel is formed by three distinctive layers (Pugsley & Tabrizchi, 2000; Björkegren & Lusi, 2022; Scipione et al., 2023): The innermost layer is a single layer of endothelial cells, forming a barrier between the blood and the rest of the arterial tissue. This endothelial layer is supported by a layer consisting of collagen and glycosaminoglycans, both layers forming the “tunica intima”. The next outer layer, the “tunica media”, is made from vascular smooth muscle cells. The outer coat of the arterial wall is a fibrous layer called the “tunica adventitia” (Figure 1.1).

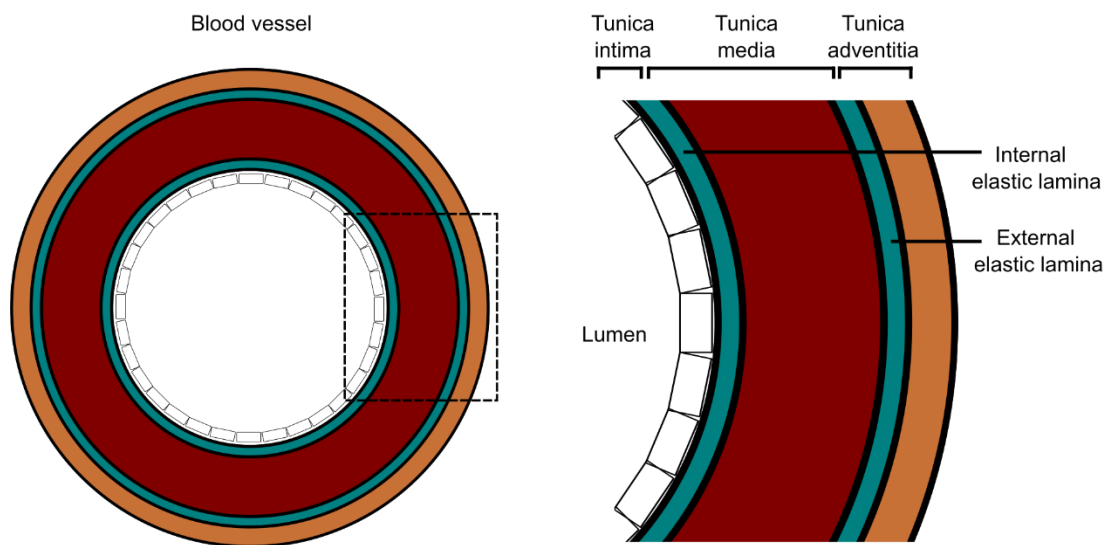


Figure 1.1: Schematic of the structure of a blood vessel. Blood vessels consist of three layers: The tunica intima (containing endothelial cells), the tunica media (containing vascular smooth muscle cells) and the tunica adventitia (containing fibroblasts).

These layers are also biomechanically distinct. Endothelial cells and endothelial tissue are softer (1-7 kPa) (Mathur et al., 2001; Lee, 2011; Guo et al., 2012) than the tissue of the arterial wall (10-40 kPa) (Asgari et al., 2022; Hooglugt et al., 2022; Petit et al., 2022).

1.3 Inflammation and atherosclerosis

Inflammation is the response of the human body to contact with unknown substances or to injury. Acute inflammation is accompanied by four key features of the tissue surrounding the contact or injury: redness, heat, swelling, and pain.

Local and systemic inflammation are highly associated with the onset and development of cardiovascular diseases (Ross, 1999; Libby, 2021a; Ministrini et al., 2021; Libby, 2024) such as coronary artery disease (Attiq et al., 2024) and stroke (Kelly et al., 2021). Several factors such as age, infection, lack of exercise, obesity or smoking lead to endothelial dysfunction and low-grade local and systemic inflammation (Mannarino & Pirro, 2008; Grover-Páez & Zavalza-Gómez, 2009; Gimbrone & García-Cardena, 2016; Koenen et al., 2021; Tyrrell & Goldstein, 2021; Kajikawa & Higashi, 2022; Vandercappellen et al., 2022; Mallah et al., 2023) (Figure 1.2 left). Inflammation in the arteries can lead to the build-up of plaques made from fat and cell debris in the sub-endothelium (Libby, 2021b) (Figure 1.2 middle). This process, called atherosclerosis, leads to the thickening and increased stiffness of arteries (Mattace-Raso et al., 2006; Hooglugt et al., 2022), which may restrict blood flow to vital organs and extremities (Sima et al., 2009; Gutierrez et al., 2022; Shamaki et al., 2022; Kwok et al., 2023). More severely, if these arterial plaques burst, their contents can leak into the circulation, causing platelets to activate, which in turn leads to thrombosis and vessel occlusion further down in the blood stream, causing critical clinical complications such as myocardial infarction or stroke (Holvoet & Collen, 1997; van der Wal & Becker, 1999; Bentzon et al., 2014; Wolberg, 2015; Kelly et al., 2021) (Figure 1.2 right). Endogenous anti-thrombotic mechanisms, such as endothelial nitric oxide can be employed to hinder excessive thrombosis (explored more in detail in chapter 5),

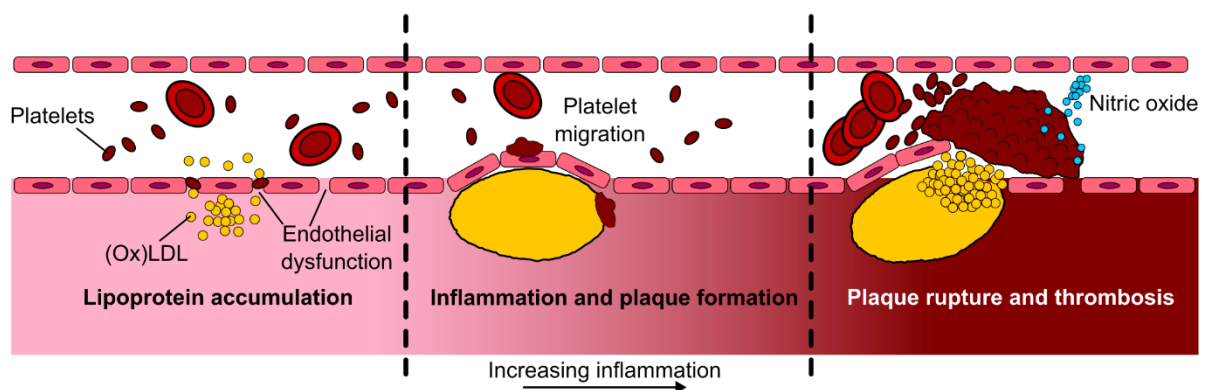


Figure 1.2: Pathogenesis of vascular inflammation and atherosclerosis. Onset of atherosclerosis can be divided into three steps. Local vascular inflammation leads to gaps in the endothelium layer, resulting in lipoprotein accumulation (left). This further increases inflammation triggering platelet and immune cell migration and the formation of foam cells, eventually resulting in plaques (middle). In the event of a plaque rupture, excessive thrombosis occurs, which triggers endogenous anti-thrombotic processes, such as the release of endothelial nitric oxide (right).

The pathophysiology of atherosclerosis is multifaceted and constantly being redefined (Libby & Hansson, 2019; Libby, 2021a; Björkegren & Lusis, 2022; Ajoobabady et al., 2024). Precise understanding of the environment of the atherosclerotic tissue is therefore needed for treatment of patients and the discovery of new therapeutical targets.

1.4 The role of platelets in inflammation and immunity

In recent years, it became increasingly clear that thrombosis and immune response cannot be easily separated, as platelets and immune cells show many paths of interaction (Nicolai et al., 2024; Escaig et al., 2026). Platelets guide innate immune cells (Zuchtriegel et al., 2016) and directly modulate the adaptive immune system (Elzey et al., 2003). Two terms can be used to describe the interaction between the thrombus and the immune system (Schrottmaier & Assinger, 2024): “Immunothrombosis”, first defined by Engelmann & Massberg in 2013 (Engelmann & Massberg, 2013), describes the effect of the immune system on thrombosis, while “thromboinflammation” refers to the influence of thrombosis on the immune system, defined by various researchers (Tanguay et al., 2004; Blair et al., 2009; Nieswandt et al., 2011). Another part of platelet immune activity is haptotactic migration.

1.5 Platelet haptotactic migration

For a long time, platelets were thought to be sessile after adhering to a substrate. In recent years, however, platelet migration has been revealed as another important function of platelets (Gaertner et al., 2017; Nicolai et al., 2020; Kaiser et al., 2023). Platelets can migrate using haptotaxis, a type of cell movement utilizing substrate-bound protein (Carter, 1965, 1967). By pulling on substrate-bound fibrinogen, platelets propel themselves forward, removing the fibrinogen from the substrate in the process (Carter, 1967; Gaertner et al., 2017; Sarkar et al., 2020). Indeed, it has been shown that platelet migration plays an important immune system function in inflamed tissue (Nicolai et al., 2020; Kaiser et al., 2023). Chapter 4 will explore the mechanosensing aspects of platelet migration in detail.

In contrast to other immune cells, which use phagocytosis as a defense mechanism, platelets can be classified as a “coverocytes” (White, 2005): Due to the increased surface area and substrate coverage in platelet migration, they bundle up bacteria, facilitating the phagocytosis process by primary immune cells such as neutrophils and macrophages (Gaertner et al., 2017). Actin related protein 2/3 complex (Arp2/3) is an important protein complex for building up the lamellipodia necessary for platelet migration. Consequently, mice with a platelet-specific Arp2/3 knockout had a wider bacteria spread after *S. aureus* infection, compared to control (Nicolai et al., 2020). These findings indicate that platelet actin and thus platelet biomechanics and morphology play an important role in their immune defense function.

1.6 Biomechanics

Biomechanics is the application of mechanical laws of physics to biological systems. The mechanical properties of biological cells, tissues and organs can change significantly during development, age or disease (Suresh, 2007; Guimarães et al., 2020; Taubenberger et al., 2020; von Eysmond & Schäffer, 2022; Wang et al., 2022). For example, it was found that cancer cells are typically softer than their healthy counterparts (Quan & Kim, 2016), supporting tissue invasion and metastasis (Xu et al., 2012; Rianna et al., 2020). Platelet biomechanics and its influence on platelet function has only emerged as a topic of interest in recent years (Sorrentino, Studt, et al., 2016; Sachs et al., 2020; Zaninetti et al., 2020; Bender & Palankar, 2021; Balmes et al., 2024; Gupta et al., 2025).

Since this thesis is partly concerned with platelet biomechanics and specifically the Young's modulus of platelets, chapter 1.7 provides an overview of Young's modulus and the cytoskeleton. Chapter 2.4 outlines the measurement techniques employed in this thesis to assess platelet biomechanics.

1.7 Cell stiffness and Young's modulus

One biomechanical property of cells is the Young's modulus E . E is a measure of the resistance of a material to deformation by an outside force and is defined as the ratio of stress (σ) and strain (ε) of a material and is usually expressed in pascal (Pa, force per unit area).

$$E = \frac{\sigma}{\varepsilon} \quad (1)$$

The mechanical stress σ is defined as a force F acting on the area A of an object (under the assumption that stress along the area A is evenly distributed):

$$\sigma = \frac{F}{A} \quad (2)$$

The strain ε is defined (for the one-dimensional deformation) as the ratio of the change of object length when a stress deforms it (Δl) and the original length l_0

$$\varepsilon = \frac{\Delta l}{l_0} = \frac{l - l_0}{l_0} \quad (3)$$

In biomechanical context, E is also commonly referred to as 'stiffness'. Under the same mechanical stress, a softer cell will deform more (i.e. has a higher strain) than a stiffer cell.

The cytoskeleton, consisting of filamentous actin (F-actin), microtubules (MTs) and intermediate filaments (IFs) (Fletcher & Mullins, 2010), is the main contributor to a cell's stiffness (Luo et al., 2016). F-actin is a filamentous gathering of globular actin (G-actin) molecules (Pollard, 2007; Oda et al., 2009; Merino et al., 2018). F-actin maintains the shape of the cell and is required to generate forces necessary for cell shape change and movement (Lappalainen et al., 2022; Goode et al., 2023; Rajan et al., 2023). F-actin is the main contributor to a cell's stiffness, as disrupting actin polymerization also softens the cell significantly (Hofmann et al., 1997; Rotsch & Radmacher, 2000). The cell membrane (Sanyour et al., 2019) as well as MTs and IFs only play a minor role in the determination of cell stiffness (Ketene et al., 2012).

The stiffness of cells and tissues varies greatly in the human body (Alonso & Goldmann, 2003; Kuznetsova et al., 2007; Levental et al., 2007; Arda et al., 2011; Garoffolo & Pesce, 2019; Guimarães et al., 2020), from multiple gigapascals (GPa) of bone tissue (Morgan et al., 2018), similar to glass or plastic, down to a few kilopascals (kPa) of single adherent platelets (Radmacher et al., 1996; Rheinlaender et al., 2015) and even to the 100 Pa range in human neural progenitor cells (Asnacios et al., 2026). The cell and tissue stiffness also varies greatly in the context of cancer (Suresh, 2007; Schächtele et al., 2021) and inflammation (Tracqui et al., 2011; Bufi et al., 2015; Rezvani-Sharif et al., 2019).

As a last note, cells and biological samples are often viscoelastic, meaning they combine both elastic and viscous features (Hoffman & Crocker, 2009; Liu et al., 2024). Other physical properties, such as surface tension or a larger indentation can influence the (apparent) stiffness of a sample (Ding et al., 2017; Rheinlaender & Schäffer, 2024). Furthermore, a 100- to 1000-fold difference in the obtained elastic and viscous moduli of MCF-7 breast cancer cells was observed, depending on the technique used to measure cell mechanics (P. H. Wu et al., 2018). Even when utilizing the same technique, using different probes might result in wider spreads of Young's modulus values (Kulkarni et al., 2023). Therefore, the results on cell stiffness reported in this thesis can only partially describe highly complex cell biomechanics.

1.8 Aims

The primary goal of this thesis is to elucidate platelet behavior beyond classical hemostasis. Special emphasis is played on understanding how biochemical and biomechanical modulation influence platelet activation, morphology, and migration on both the single platelet and the thrombus level.

At the single-platelet level, this thesis uses SICM to quantify platelet mechanical properties, such as stiffness in terms of Young's modulus. Fluorescence microscopy is used to relate these parameters to platelet activation state and cytoskeletal organization. The thesis further seeks to elucidate how lipid loading and lipid oxidation, including the effects of native and

oxidized low-density lipoproteins, alter platelet mechanics and morphology. The effects of an atypical chemokine receptor agonist are examined to assess its potential for inhibitory platelet regulation, especially in hyperlipidemic conditions.

Secondly, this thesis aims to investigate platelet migration and adaptive stiffness responses on substrates with defined mechanical properties using light microscopy and neural network-assisted tracking software.

Finally, large-range SICM was applied to characterize three-dimensional thrombus morphology and surface roughness, and to assess how these parameters are altered by modulation of cyclic guanosine monophosphate (cGMP) signaling. In particular, the effects of the NO-sensitive soluble guanylate cyclase (NO-GC) stimulator riociguat and nitric oxide donors on thrombus formation and structure are investigated.

Together, these aims try to give insights into how biochemical and biomechanical signals regulate platelet function in thrombo-inflammation and to identify potential avenues for future therapeutic intervention.

2 Experimental background

This chapter covers the most commonly used materials and methods used in this thesis. Additionally, chapters 3-5 also feature material and methods sections relevant to their respective chapters.

2.1 Materials

2.1.1 Commonly used reagents and chemicals

Compound	Manufacturer & Catalogue no.
Acid citrate dextrose (ACD)	Sigma Aldrich C3821
BSA	Sigma-Aldrich A7906
CaCl ₂	Sigma Aldrich 21115
DMSO	Sigma-Aldrich D8418
Formaldehyde	Sigma-Aldrich F1635
(D)-Glucose	Carl Roth HN06.1
Glutardialdehyde	Carl Roth 4157.1
HEPES	Merck H3537
KCl	Carl Roth HN02.1
NaCl	Carl Roth HN00.2
NaHCO ₃	Carl Roth HN01.1
NaH ₂ PO ₄	Carl Roth T879.1
PDMS Sylgard™ 184	DOW Chemical Company 4019862
PDMS Sylgard™ 527	DOW Chemical Company 2270030
Phosphate buffered saline (PBS)	Merck D8537
Prostaglandin I ₂ (Prostacyclin)	Cayman Chemicals 18220,
Triton X-100	Merck X100

2.1.2 Platelet activators and inhibitors

Compound	Manufacturer & Catalogue no.
ACKR3/CXCR7 agonist VUF11207	Sigma-Aldrich 239824
ADP	Sigma-Aldrich A2754
DEA-NO	Cayman Chemicals 82100
Collagen	Takeda 1130630
Fibrinogen	Sigma-Aldrich F3879
LDL	KalenBiomed 770200-4
OxLDL	KalenBiomed 770252-7
Riociguat	Biomol Cay9000554
Thrombin	Sigma-Aldrich T6884
Thromboxane (TxA ₂)	Biomol Cay16450

2.1.3 Fluorophor-bound proteins & stains

Compound	Manufacturer & Catalogue no.
Fibrinogen AlexaFluor594	ThermoFisher/Invitrogen F13193
Phalloidin	Abcam ab176753

2.2 Platelet isolation from human whole blood

Platelets have a short shelf life. After isolation, platelets can only be stored for 5 days at room temperature, before risks of bacterial contamination and high bioreactivity become too high (Aubron et al., 2018). Blood was therefore collected from healthy human volunteers and used directly after isolation. All procedures were approved by the institutional ethics committee (273/2018BO2) and comply with the declaration of Helsinki. Blood donors gave written informed consent.

Blood was collected into monovettes filled with 1.25 ml of anticoagulant acid-citrate-dextrose (ACD). Platelet isolation was done over two centrifugation steps: First, monovettes were centrifuged at 200 g for 20 min at room temperature (RT) to obtain platelet-rich plasma (PRP). PRP was resuspended with Tyrode-HEPES buffer (pH = 6.5, supplemented with 0.225 µg/ml prostacyclin; 136.89 mM NaCl, 2.81 mM KCl, 11.9 mM NaHCO₃, 1 g/L D-glucose, 10 mM HEPES) in a 1:3 ratio. Following a second centrifugation of the Tyrode-diluted PRP at 880 g for 10 min at RT, the resulting supernatant was removed, and the platelet pellet was carefully resuspended in 1 ml of Tyrode-HEPES buffer (pH = 7.4). Isolated platelets were allowed to rest at least 30 min before proceeding with other experiments.

2.3 Microscopy techniques

Microscopy is used to image objects that are not visible to the naked eye. Biological research has been significantly enhanced by the inventions of diverse microscopy techniques, each with their own advantages and disadvantages (Lemon & McDole, 2020). Microscopy techniques can be separated by their method of visualization of the object of interest (Apollon et al., 2022). The following section focuses on the several light and scanning probe microscopy techniques used in this thesis.

2.3.1 Light and fluorescence microscopy

Light or optical microscopy uses visible light, lenses and cameras to illuminate, enhance and image a sample, allowing for non-invasive visualization of live biological samples (such as platelets) and their inner workings with relative ease (Montague et al., 2020).

Fluorescence is the phenomenon of a material to emit light after being excited by a certain wavelength of light. Fluorescence microscopy uses fluorescent molecules called fluorochromes or fluorophores to label specific molecules of interest. After being excited by light, these fluorophores emit light themselves, typically of a lower wavelength. This so-called

Stokes shift allows for the separation of excitation and emission light using filters, thus enabling the observer to only view the fluorescently labelled parts of an object of interest.

This technique thus allows visualization and tracking of a single molecule species, its location and interaction with other molecules (Lichtman & Conchello, 2005; Hickey et al., 2022). For example, phalloidin allows the labeling of F-actin in a fixed and permeabilized cell (Adams & Pringle, 1991). The efficacy of fluorescence microscopy has been increased further with new techniques: Using lasers and a pinhole, confocal laser scanning microscopy allows imaging of fluorescent samples with high signal-to-noise ratio in both two and three spatial dimensions due to the reduction of scattered and out-of-focus illumination (Sandison et al., 1995).

The overall fluorescence intensity of a sample can be quantified numerous ways, chief among them is the calculation of the mean fluorescence intensity (MFI) or the corrected total cell fluorescence (CTCF) (Gavet & Pines, 2010). MFI can be calculated by calculating the sum intensity of all pixels of a sample and dividing by the number of pixels:

$$MFI = \frac{\text{Sum of the intensity of all pixels of the sample}}{\text{number of all pixels of the sample}} \quad (4)$$

MFI does not take the sample area and background fluorescence into account. Therefore, it is often more instructive to use CTCF to compare fluorescence of differently large objects, using the following equation:

$$CTCF = \frac{\text{Sum of the intensity of all pixels of the sample} - (\text{number of all pixels in the sample area} \cdot \text{background intensity})}{\text{number of all pixels in the sample area}} \quad (5)$$

However, optical microscopy is limited to a resolution of roughly half the wavelength of the employed light. For visible light, this so-called diffraction limit, which was first described by Ernst Abbe in 1873 (Abbe, 1873), amounts to roughly 200 nm, below which two features cannot be distinguished from each other. Since a lot of biological features such as proteins or cell organelles are often in the nanometer-size range, significant efforts have been made to overcome or bypass the diffraction limit (Erickson, 2009; Guo et al., 2018). Super-resolution fluorescence microscopy uses fluorescence and specific techniques to overcome the diffraction limit (Hell & Wichmann, 1994; Schermelleh et al., 2019). Other microscopy techniques avoid using light as a probe in general and employ a different probe to visualize the sample.

2.3.2 Scanning Probe Microscopy

Techniques using a probe to measure physical interaction with a sample are loosely gathered under the name of scanning probe microscopy. Two techniques used in this thesis, atomic force microscopy and scanning ion conductance microscopy, are described in detail in the following sections. A detailed analysis of the capabilities of these techniques beyond the scope of this thesis can be found in elsewhere (von Eysmond & Schäffer, 2022).

2.3.2.1 Atomic force microscopy

Atomic force microscopy (AFM) is a microscopy technique that uses a sharp tip attached to a flexible cantilever as a scanning probe to obtain three-dimensional information about a sample. Invented in 1986, it is possible to achieve a resolution on the scale of single atoms, hence the name of the technique (Binnig et al., 1986; Binnig et al., 1987). AFM can be used to measure conductive and non-conductive samples in vacuum (Meyer & Amer, 1990), air (Binnig et al., 1986) and liquid environments (Drake et al., 1989). The geometry and spring constant of the cantilever as well as the shape and size of the cantilever tip (e.g. pyramid, cone, sphere, tipless etc.) can be chosen by the operator to best fit the parameters of interest (Dzedzickis et al., 2023; Kulkarni et al., 2023). AFM allows the measurement of biological samples under physiological conditions and without the need to label them. Moreover, AFM can be used to measure and employ forces on cells. These properties allowed the use of AFM to investigate a plethora of biological samples, such as eukaryotic cells (Braet et al., 1998; Amarouch et al., 2018), lipids (Hansma et al., 1991; Singh & Keller, 1991), DNA and proteins (Weisenhorn et al., 1990; Hansma et al., 1993; Beckwitt et al., 2018; Kasas & Dietler, 2018), bacteria (Dufrêne et al., 2021), and viruses (Ray et al., 2025). Platelets have also been previously investigated with AFM (Radmacher et al., 1996).

In a typical AFM setup, piezoelectric actuators move a flexible cantilever with a sharp tip over the surface of a sample (Figure 2.1). A laser beam is reflected off the backside of the cantilever onto a segmented photodiode. As the cantilever tip touches the sample, the bending of the cantilever causes the position of the laser beam on the photodiode to change, which can be used as a measure of cantilever deflection and therefore the topography of the sample. This technique is also called ‘optical lever’ (Meyer & Amer, 1988; Alexander et al., 1989).

In addition, the deflection and the spring constant of the cantilever can be used to ascertain the Young’s modulus of the sample. Hooke’s law describes the dependence of the cantilever deflection d with the force F and the cantilever spring constant k :

$$F = k \cdot d$$

(6)

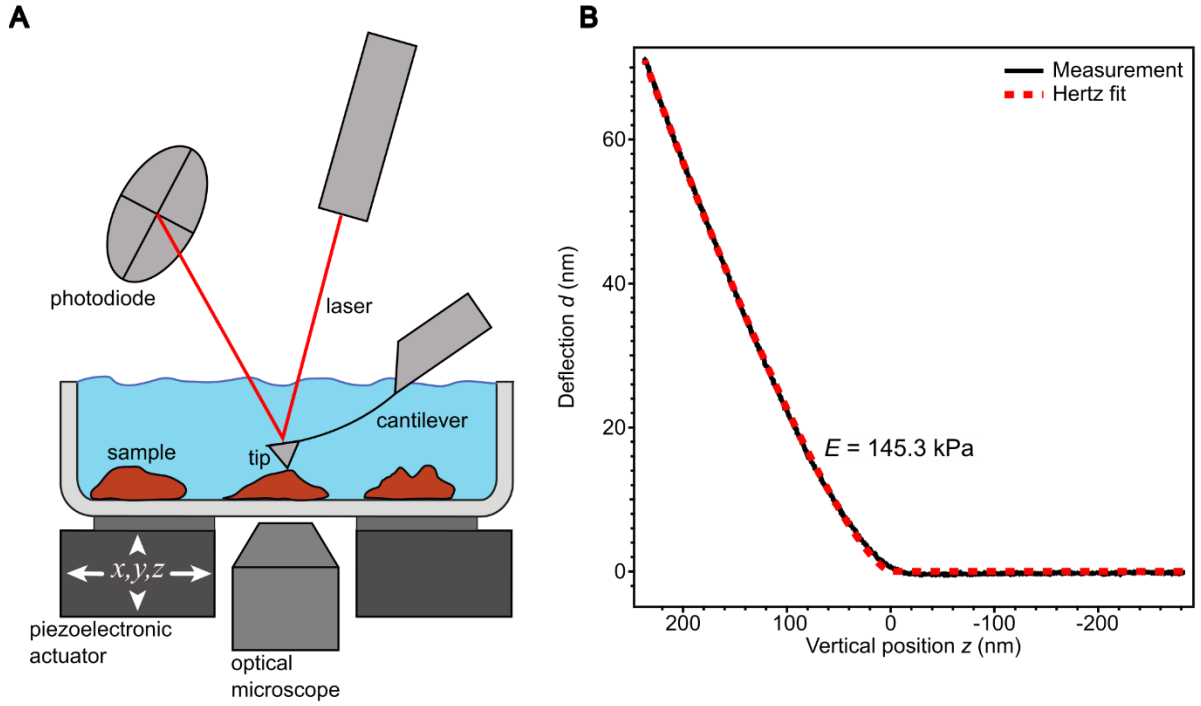


Figure 2.1: AFM measurement principle. (A) Schematic of a typical AFM setup. In AFM, the tip of a flexible cantilever is moved across a sample, while a laser is reflected from the cantilever tip onto a photodiode. The cantilever is deflected by the interaction of the sample with the tip, changing the laser position on the photodiode. This allows for the measurement of cantilever deflection (and therefore force). (B) Exemplary measured d - z -curve. The measurement was performed on a Sylgard™ 184 polydimethylsiloxane substrate (mixed in 1:25 ratio of curing agent and silicone base) using a cantilever with a spherical tip (tip radius $R = 2 \mu\text{m}$, spring constant $k = 0.348 \text{ N/m}$).

The cantilever deflection d can be mapped against the vertical cantilever position z (also called the d - z -curve, Figure 2.1B). As the cantilever tip approaches a sample surface (non-contact area), the vertical cantilever position z increases, and cantilever deflection d is near 0. Upon reaching contact with the sample (contact point z_0), the cantilever tip begins to indent the sample, causing the cantilever deflection to slowly increase (contact area). As the cantilever indents the sample, the degree of cantilever deflection is dependent on the sample stiffness. At a given indentation, a soft sample deflects the cantilever less than a stiffer one. This indentation δ is defined as the difference between the vertical cantilever position z and the contact point z_0 and the deflection d :

$$\delta = (z - z_0) - d$$

(7)

The relationship between the indentation δ and the force F can be described with a contact mechanics model of a spherical cantilever tip with radius R (also called Hertz-Model) (Hertz, 1882):

$$F(\delta) = \frac{4}{3} \sqrt{R} \frac{E}{1-\nu^2} \delta^{\frac{2}{3}} \quad (8)$$

with the Poisson's ratio ν (usually assumed to be ≈ 0.5 for cells, although this is often an oversimplification (Mokbel et al., 2020; Javanmardi et al., 2021)).

Assuming a constant R and ν , the applied force F and indentation depth δ can be calculated using Equation 6 and Equation 8, so the model can be fitted to the d - z -curve. This allows the extraction of the sample Young's modulus E .

Platelets have been previously investigated using AFM, revealing a Young's modulus of 1-50 kPa for adherent platelets (Radmacher et al., 1996) depending on the platelet region, confirmed by a recent study using AFM and cryo-electron tomography (Sorrentino, Jan-Dirk, et al., 2016). However, as platelets are mechanosensitive, direct contact of the cantilever tip could result in platelet activation (Fritz et al., 1993), signifying the need for a contactless SPM technique explained in the next chapter.

2.3.2.2 Scanning ion conductance microscopy

Scanning ion conductance microscopy (SICM) was invented by Hansma et al. in 1989 (Hansma et al., 1989). Similar to AFM, SICM is a SPM technique. In contrast to AFM, however, an electrolyte-filled pipette with a tip diameter of tens to hundreds of nanometers is scanned over a sample submerged in electrolyte (Figure 2.2A). An ionic current I is passed through the nanopipette using the voltage V between two electrodes, one inside the nanopipette and one in the electrolyte above the sample. The nanopipette can be moved horizontally in x - and y - and vertically in z -direction. If the nanopipette tip comes in close proximity of the sample, the ion current I rapidly decreases (Hansma et al., 1989; Zhu et al., 2020)(Figure 2.2B). Using a feedback-loop between I and Δz , the ion current I can be held constant (e.g. at 99% of the ion current at a far distance (I_0)), resulting in a constant nanopipette-sample distance Δz from the sample while rapidly scanning the sample topography in the horizontal x - and y -directions (this is called constant current mode (Hansma et al., 1989)). Recording the z -position for each x - and y -position then results in a three-dimensional image of sample topography. However, abrupt changes in sample height (such as the outer edges of adherent cells) might result in nanopipette-sample contact or even damage (Zhu et al., 2020; von Eysmond & Schäffer, 2022).

Imaging using SICM in this thesis was done using a mode called “backstep” (Mann et al., 2002) or “hopping” mode (Novak et al., 2009), which significantly reduces the risk of nanopipette-sample contact even with abrupt changes in sample height. In this mode, the vertical and horizontal movements of the nanopipette are separated: First, the nanopipette is vertically approached until a predefined current threshold (a trigger event, e.g. 99% of I_0) is reached. Then z is recorded and the nanopipette is retracted from the sample before it is moved laterally and approached towards the sample again.

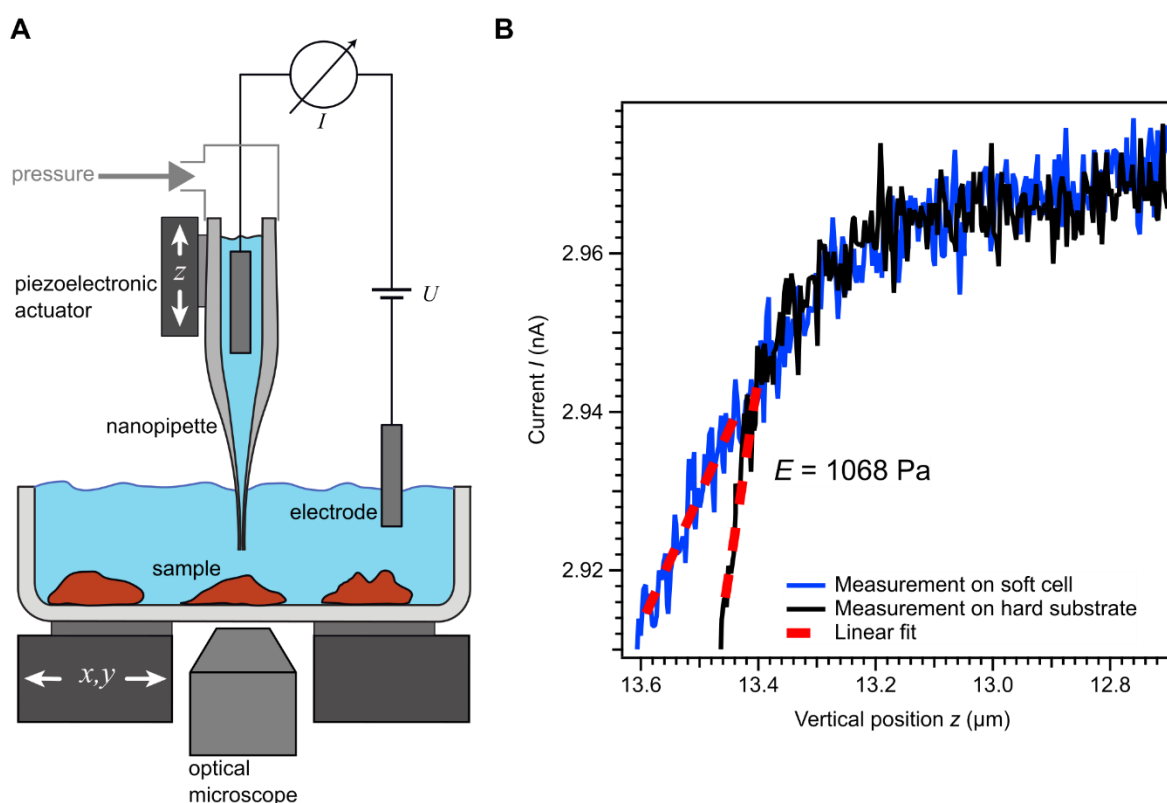


Figure 2.2: SICM measurement principle. (A) Schematic of a typical SICM setup. In SICM, a voltage is applied via two electrodes resulting in an ion current through a nanopipette. The ion current decreases rapidly in close proximity of the nanopipette tip to the sample. This decrease can be used to quantify the topography of a sample without mechanical contact. In addition, pressure can be applied to the end of the nanopipette to measure the sample Young's modulus. (B). Exemplary measured ion current-vertical position curve. The measurement was performed on an adherent platelet (blue line) and on an infinitely stiff substrate made of polystyrene (black line). The linear fits (red dashed lines) show the difference in slope of the ion current depending on the Young's modulus of the sample. The Young's modulus E of the adherent platelet was calculated using Equation 9.

It is also possible to measure the Young's modulus of a sample using SICM. The method for Young's modulus measurement using SICM used in this thesis was established by Rheinlaender and Schäffer (Rheinlaender & Schaffer, 2013). A constant pressure of tens to hundreds of kilopascals is applied to the back of the nanopipette, inducing a microfluidic outflow at the nanopipette tip. Soft samples are deformed by the water pressure, widening the nanopipette-sample distance and thus slowing the current decrease when the nanopipette approaches the sample, resulting in a shallower slope. On a stiff sample, there is no such

deformation, meaning the nanopipette-sample distance is not altered and that the current still decreases sharply, resulting in a steeper slope. The sample Young's modulus can then be determined using the slope s of a linear fit of the I - z -curve between 99 and 98 % of I_0 and the following equation:

$$E(s) = p_0 A \left(\frac{s_\infty}{s} - 1 \right)^{-1}, \quad (9)$$

where p_0 is the applied pressure in Pa, A is a constant depending on the nanopipette tip geometry and s_∞ is the slope on an infinitely stiff sample (Rheinlaender & Schaffer, 2013).

This technique enabled the investigation of the cell mechanics of mouse embryonic fibroblasts (Rheinlaender & Schaffer, 2013), cancer cells (Schächtele et al., 2021), adult rat cardiomyocytes (Simeonov & Schaffer, 2019) as well as mouse (Baumann et al., 2022) and human blood platelets (Rheinlaender et al., 2015; Seifert et al., 2022). The contact-free measurement of cell Young's modulus using SICM is especially advantageous for mechanosensitive platelets, as contact with the cantilever in AFM can activate platelets on its own (Fritz et al., 1993).

In conclusion, AFM and SICM are both excellent techniques to measure and quantify the shape and Young's modulus of cells and are therefore used throughout this thesis.

2.4 Quantification of two- and three-dimensional shape

The shape of a cell or tissue often changes significantly during its life cycle. Platelets change their shape rapidly upon their activation (Loftus et al., 1984; Shin et al., 2017; Bender & Palankar, 2021). This process can be started by a platelet adhering and spreading onto a surface (Kuwahara et al., 2002; Watson, 2009; Lee et al., 2012) or via endogenous agonist such as thrombin (Harmon & Jamieson, 1986), TxA_2 (FitzGerald, 1991) or ADP (Gaarder et al., 1961; Daniel et al., 1998).

Abnormal platelet shape change, adhesion or spreading as well as abnormal thrombus shape can be a sign of platelet dysfunction (Parr et al., 2011; Seifert et al., 2021; Dziedzic et al., 2024; Swieringa et al., 2025). Quantification of cell and tissue morphology using "shape parameters" can be used to characterize these deviations (Figure 2.3). The shape parameters used in this thesis are the maximum height *max h* (defined as the 99th percentile of all height values of an object), the mean height *mean h*, the area A , the volume V , the circularity C , as well as the aspect ratio AR .

The circularity C is a unitless parameter to measure similarity of an object of interest to a circle of the same area A . It ranges between 0 and 1, with 1 being a perfect circle:

$$C = \frac{4\pi A}{P^2}, \quad (10)$$

where P is the perimeter of the object of interest. Similarly, the aspect ratio AR of an object of interest is obtained by fitting an ellipse to the the object of interest and calculating the ratio of the long axis a and the short axis b :

$$AR = \frac{a}{b} \quad (11)$$

AR is in the range from 1- ∞ , with the fitted ellipse forming a perfect circle when AR is 1.

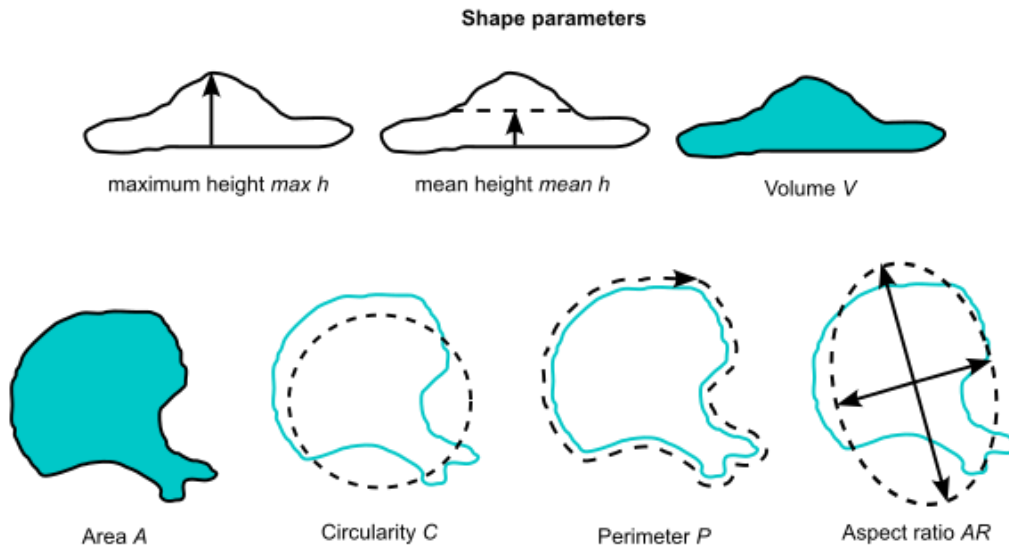


Figure 2.3: Shape parameters. Overview of parameters used for sample morphology quantification: Maximum height $max\ h$, mean height $mean\ h$, volume V , area A , circularity C , perimeter P , aspect ratio AR .

2.5 Cell Young's modulus measurement with scanning ion conductance microscopy

As mentioned in chapter 1.7, each material and therefore each cell has a certain Young's modulus, a measure of its ability to withstand deformation. The cytoskeleton, consisting of filamentous actin, microtubules and intermediate filaments, is the major contributor to a cell's Young's modulus (Luo et al., 2016).

Platelets undergo a significant shape change upon adhesion and activation, which is essential for their function (R. D. Allen et al., 1979; Moskalensky & Litvinenko, 2019; Bender & Palankar, 2021). Upon encountering immobilized adhesive proteins such as fibrinogen and collagen, platelets start to firmly adhere and thereafter spread (Savage et al., 1996; Ruggeri & Mendolicchio, 2007; Bender & Palankar, 2021). Platelet spreading can be distinguished into four phases: Platelet adhesion (1st phase), filopodia formation (2nd phase), filopodia + lamellipodia formation (3rd phase) and completely spread lamellipodia (4th phase) (Bender & Palankar, 2021). Thread-like protrusions of parallel actin filaments extending from the platelet center are called filopodia ("filum"= latin for "thread"), while the cell protrusion forming between the filopodia are called lamellipodia ("lamellum = latin for "sheet") (R D Allen et al., 1979; Hartwig, 1992; Mattila & Lappalainen, 2008; Aslan et al., 2012; Bender & Palankar, 2021; Lee et al., 2023).

This shape change is also accompanied by a significant change in the platelet actin network and thus platelet Young's modulus (Hartwig, 1992; Rheinlaender et al., 2015). When measured with SICM, Non-activated platelets had a Young's modulus between 1-10 kPa (3 kPa on average) (Rheinlaender et al., 2015). During platelet adhesion and spreading, the platelet actin network forms actin nodules (Calaminus et al., 2008, Poulter, 2015 #608) in the early phase of the spreading, which then later turn into lamellipodia as well as stress fibers, which are actin filaments interlaced with the motorprotein myosin (Tanaka & Itoh, 1998). The platelets use these stress fibers to build up the forces needed to spread (Tojkander et al., 2012). Upon the built-up of the actin filament network, the local platelet Young's modulus increases, with fully spread platelets having a Young's modulus of 10-50 kPa (15 kPa on average) (Radmacher et al., 1996; Rheinlaender et al., 2015). Thrombin activation of spread platelets is associated with a decrease in Young's modulus, differing from the rapid decrease in platelet Young's modulus caused by F-actin depolymerization with the addition of cytochalasin D (CytoD) (Rheinlaender et al., 2015).

Platelet biomechanics has been investigated using a plethora of methods (Sachs et al., 2020). One of those methods is the measurement of cell Young's modulus using SICM (Rheinlaender & Schaffer, 2013; Rheinlaender et al., 2015; Seifert et al., 2022). The main advantage of SICM for the measurement of platelet Young's modulus is its non-contact probe, since platelets can be activated or damaged by indentation during AFM measurements or analysis (Fritz et al.,

1993; Seifert et al., 2015). The measurement of the platelet topography and Young's modulus is simultaneous (Figure 2.4A+B). In addition, since platelets are very thin when spread (roughly 500-1000 nm), an underlying hard substrate such as glass or plastic can falsify the measurement of spread platelets, overestimating the apparent Young's modulus of a platelet. Therefore, there are correctional models that were developed to rectify this “thickness effect” (Rheinlaender & Schaffer, 2020) (Figure 2.4C).

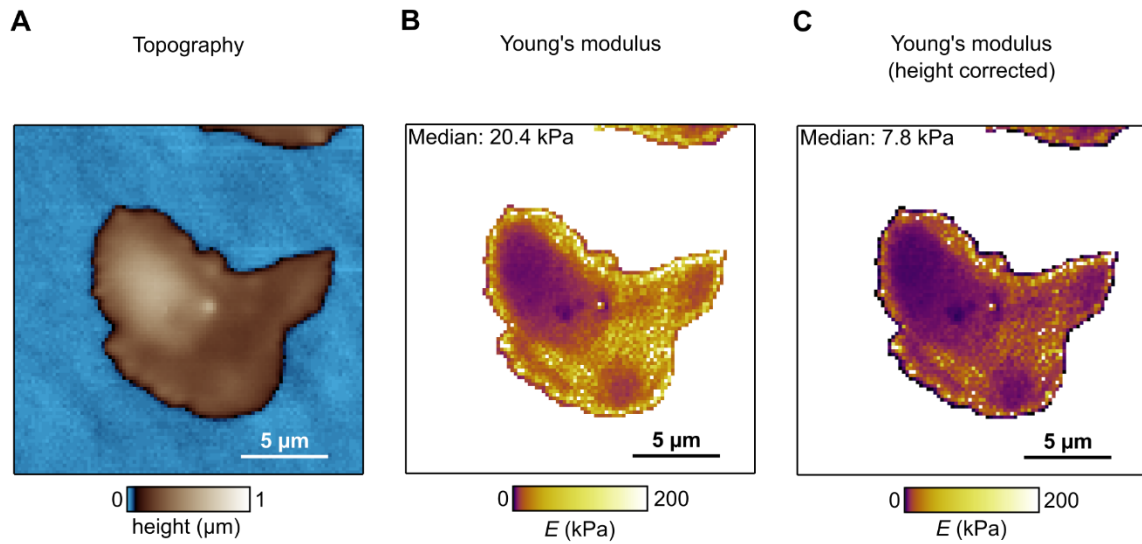


Figure 2.4: Platelet Young's modulus measurement with SICM. (A) Topography image of a spread platelet imaged with SICM. (B) Platelet Young's modulus image of the platelet in (A). (C) height-corrected Young's modulus of spread platelet from (B) to avoid measuring the underlying substrate's Young's modulus through thin platelet areas. Correction was done using the procedure of (Rheinlaender & Schaffer, 2020). Scale bar: 5 μm , 20 x 20 μm^2 scan size, 100 x 100 pixels resolution, pixel size 0.2 x 0.2 μm^2 .

3 Investigation of the influence of lipid oxidation and ACKR3 agonist on platelets

3.1 Introduction

3.1.1 (Oxidized) Low-density lipoprotein

Low-density lipoprotein (LDL) is a key mediator of extracellular lipid transport in the body. It facilitates the cholesterol influx or uptake into cells, where they can be further metabolized (Brown & Goldstein, 1976; Pfisterer et al., 2016) by subsequent oxidation or peroxidation.

LDL and its oxidized form (oxidized low-density lipoprotein, OxLDL) have been recognized as major contributors to atherosclerosis and cardiovascular diseases (Aviram & Gerald Brook, 1987). LDL in its native state is known to make platelets more sensitive to activation by other platelet agonists (also called hyperactivity) (S. J. A. Korpelaar & J.-W. N. Akkerman, 2006; S. J. A. Korpelaar & J. W. N. Akkerman, 2006). In addition, LDL exerts a synergistic effect on platelet activation induced by other platelet agonists like ADP and thrombin, which ensures that hyperlipidemia and atherosclerosis contribute to a pro-thrombotic environment (Akkerman, 2008; Qiao et al., 2022). During the onset of atherosclerosis, LDL accumulates in the sub-endothelium (tunica intima) as plaques (Zhang et al., 2018). As inflamed tissue is typically rich in reactive oxygen species (Mittal et al., 2014), the trapped LDL is oxidized to OxLDL (Matsuura et al., 2008; Förstermann et al., 2017; Kattoor et al., 2017)(**Figure 3.1**). (Ox)LDL- and (ox)LDL-activated platelets are phagocytized by macrophages, leading to the formation of lipid-rich foam cells in the sub-endothelium and increased atherosclerotic plaque size (Chandler & Hand, 1961; Siegel-Axel et al., 2008; Tabas et al., 2015). Björkegren, 2022 #512}.

Depending on the degree of oxidation (Chou et al., 2006; Miyazaki et al., 2020), OxLDL is also known to activate and aggregate platelets, leading to thrombus formation (Korpelaar et al., 2005; Wraith et al., 2013; Magwenzi et al., 2015; Chatterjee et al., 2017)(**Figure 3.1**). Both LDL and OxLDL enhance platelet activation, however LDL does so only mildly, while OxLDL does so more intensely (Korpelaar et al., 2005; S. J. A. Korpelaar & J.-W. N. Akkerman, 2006; S. J. A. Korpelaar & J. W. N. Akkerman, 2006; Akkerman, 2008). LDL binds to the platelet specific LDL-receptor ApoER2 (Riddell et al., 1999), while OxLDL binds to scavenger receptor CD36 (Volf et al., 1999; Podrez et al., 2007), lectin-type OxLDL receptor 1 (LOX-1), and scavenger receptor class A 1 (SR-A1) (Korpelaar et al., 2007). Notably, binding of OxLDL to CD36 and SR-A1 leads to platelet activation (Magwenzi et al., 2015), while binding of OxLDL to LOX-1 synergistically enhances platelet activation triggered by other agonists, rather than activate platelets on its own (Berger & Naseem, 2022). Binding of OxLDL to platelet CD36 counteracts the platelet inhibitory NO-GC-cGMP-PKG signaling cascade, leading to increased generation of reactive oxygen species via NOX-2 (Magwenzi et al., 2015). Furthermore, platelets themselves uptake and oxidize LDL intracellularly upon exposure with LDL

(Chatterjee et al., 2017) or oxidize intraplatelet LDL following activation with collagen related peptide, making platelets an important intracellular reservoir for oxidized lipids secluded from the blood plasma.

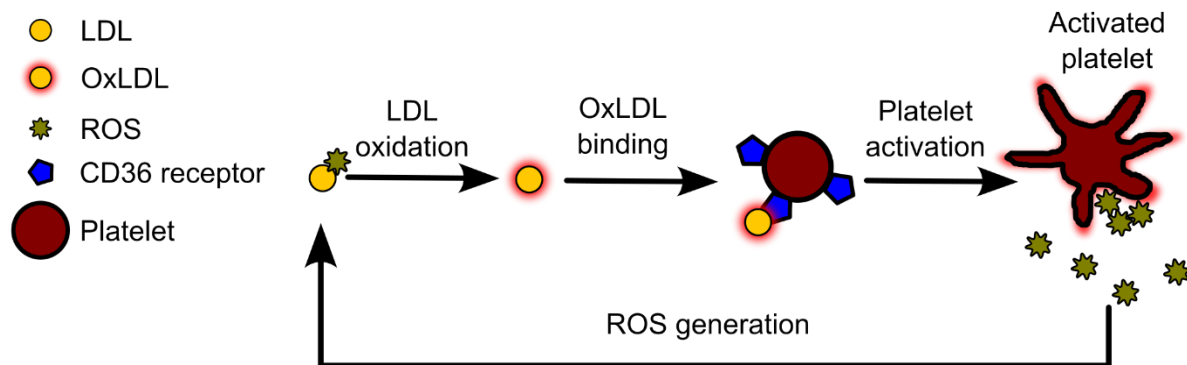


Figure 3.1: Lipoprotein oxidation and platelet activation. Low-density lipoprotein (LDL) can be oxidized by reactive oxygen species (ROS), which are abundant in inflamed tissue, to oxidized LDL (OxLDL). OxLDL can bind to the platelet CD36 scavenger receptor, triggering platelet activation and further ROS generation, creating a feedback loop, potentially resulting in excessive thrombosis. Adapted from (Gąsecka et al., 2021).

These circumstances make the interaction of platelets and (Ox)LDL a unique target of research in the context of inflammation, atherosclerosis and atherothrombosis. To reduce (Ox)LDL-induced platelet activation leading to atherothrombosis, hyperlipidemia is managed with statins and proprotein convertase subtilisin/kexin type 9 inhibitors (Karr, 2017; Wright & Subramanian, 2024). Nevertheless, intraplatelet oxidized LDL was significantly enhanced in acute coronary syndrome patients as compared to matched healthy control subjects (Chatterjee et al., 2017), hinting to the fact that the intraplatelet lipidome is not altered by statin treatment. Moreover, platelets act as vehicles for OxLDL in circulation (Chatterjee et al., 2017). These results point towards the unmet need of better and improved therapeutic strategies that could regulate the platelet lipid uptake and lipid induced platelet activation in atherosclerotic cardiovascular diseases.

3.1.2 ACKR3 is a promising target for regulatory platelet inhibition

A promising target for these purposes is the platelet atypical chemokine receptor 3, formally known as C-X-C chemokine receptor type 7 (ACKR3 or CXCR7), a noncanonical G-protein coupled receptor (Bachelierie et al., 2014; Chatterjee et al., 2014; Chatterjee, 2022; Duval et al., 2022; Rohlfing et al., 2022). To avoid confusion between the two nomenclatures, the receptor will hereafter be referred to as ACKR3.

Circulating platelets contain a plethora of diverse molecular mediators stored in their granules (Gawaz et al., 2005; Yao & Kahr, 2025). Following activation, platelets release these small molecules, cytokines and chemokines, into the extracellular environment (Weber, 2005; Chen et al., 2020). These cytokines and chemokines, such as macrophage migration inhibitor factor (MIF) or C-X-C motif chemokines (e.g. CXCL4/Platelet-factor4, CXCL8/Interleukin-8,

CXCL12/SDF-1, and CXCL14), are mainly concerned with inflammatory and immune response regulation via monocyte and neutrophil recruitment (Kowalska et al., 2010; Chatterjee & Gawaz, 2013; Futosi et al., 2013), phagocytosis, and reactive oxygen species (ROS) generation (Woller et al., 2008). This makes platelet activation a significant contributor to thrombo-inflammation and atherosclerosis. Interestingly, the atherogenic prothrombotic chemokine CXCL12/SDF-1 changes LDL and OxLDL uptake in platelets. This boosts the prothrombotic actions of LDL and OxLDL, leading to an enhanced thrombotic response (Chatterjee et al., 2017). Use of anti-platelet therapy such as the administration of aspirin and/or ADP/P2Y₁₂-receptor inhibitors offers significant reduction in thrombus formation and inflammation (Müller et al., 2015; Capodanno et al., 2023; Iglesias et al., 2025). However, anti-platelet therapy severely increases the risk of bleeding due to the limited hemostasis by platelets (Lewis et al., 2018). A therapy that attenuates platelet activation and regulates platelet lipid uptake without compromising hemostasis would thus be an ideal candidate for the treatment of cardiovascular diseases.

For this premise, ACKR3 has revealed itself to be a promising target (Duval et al., 2022). ACKR3 activation enhances cholesterol uptake by adipose tissue, reducing hyperlipidemia in a mouse model of atherosclerosis (Li et al., 2014). In coronary artery diseases patients, ACKR3 platelet surface expression is significantly increased, particularly in patients which suffered an acute myocardial infarction (Rath et al., 2013). Surface expression of ACKR3 correlates with the level of intraplatelet OxLDL (Chatterjee et al., 2017). On the other hand, patients with relatively higher levels of plasma LDL show enhanced surface expression of ACKR3 on their platelet surface (Cebo, Dittrich et al., Blood 2022), suggesting the availability of ACKR3 as a potential anti-thrombotic target in hyperlipidemia. Upon treatment of platelets with the pharmacological ACKR3 agonist VUF11207, platelet activation by ADP, thrombin, collagen, and other platelet activators is attenuated (Cebo et al., 2022; Rohlfing et al., 2022). In addition, ACKR3 activation also changes the lipidome to be more anti-thrombotic, reduces thrombo-inflammation, and lowers platelet Young's modulus (Cebo et al., 2022).

Conventional chemokine receptors belong to the class of G-protein-coupled receptors, a class of receptors with 7 transmembrane units, that typically activate intracellular processes by activating a coupled G-protein (Offermanns, 2006; Gurbel et al., 2015; Chaudhary et al., 2024). As an atypical chemokine receptor, ACKR3 activates intracellular pathways independent from g-coupled protein (Ulvmar et al., 2011; Bachelierie et al., 2014). In other cells, ACKR3 activation prolongs cell survival by β -arrestin activation (Smith & Rajagopal, 2016), second messenger degradation and receptor internalization (Torossian et al., 2014; Nguyen et al., 2020), proposing a similar function for platelets. In addition, ACKR3 activation has shown to trigger the formation of heterodimers of CXCR4 and ACKR3 receptors, inhibiting CXCR4-mediated

platelet activation (Levoye et al., 2009; Dicenta-Baunach et al., 2025). Another proposed mode of platelet inhibition involves the platelet lipidome. ACKR3 activation by VUF11207 turned the platelet lipidome anti-thrombotic, increasing the level of 12-hydroxyeicosatrienoic acid, which in turn increases cyclic adenosine monophosphate levels by prostaglandin I₂- receptor, reducing platelet activation (Cebo et al., 2022; Chatterjee, 2022). The specific pathways through which ACKR3 exerts its anti-thrombotic action in platelets are yet to be fully elucidated, highlighting the need for future studies (Duval et al., 2022).

Platelets do change their shape upon treatment with ACKR3 agonist (Cebo et al., 2022), however the effects of (Ox)LDL and ACKR3 activation on platelet morphology and actin cytoskeleton as well as platelet Young's modulus are still poorly understood. In this coming chapter, potential effects of the genetic deletion of ACKR3 on murine platelet morphology are investigated. In addition, the change in platelet shape, actin content and Young's modulus in presence of (Ox)LDL and its modulation by the pharmacological ACKR3 agonist VUF11207 are quantified.

3.2 Material & Methods

3.2.1 Shape parameter quantification of *Ackr3*^{-/-} mice platelets using SICM

Murine platelets from *Ackr3*^{fl/fl} and *Ackr3*^{-/-} mice were isolated as described previously (Borst et al., 2012). Washed platelets were stimulated with thrombin (0.1 U/ml) and allowed to adhere to collagen-coated petri dishes (100 µg/ml for 1 h at 37°C) for 30 min. Platelets were fixed with 4% formaldehyde (*This part was done by Dr. rer. nat. Anne-Katrin Rohlfing from Medizinische Klinik, Innere Medizin III, Kardiologie und Angiologie, University Hospital Tübingen*).

Platelets were then imaged using a custom-built scanning ion conductance microscope operated in backstep/hopping mode with borosilicate nanopipettes (typically 50 nm radius). Topography images were acquired over a 10 × 10 µm² area at 100 nm/pixel resolution. Morphological analysis was performed using custom Igor Pro software, with cell boundaries defined by a 50 nm height threshold within manually selected regions of interest. Parameters including area, volume, mean and maximum height, circularity, and perimeter were quantified (for details, see chapter 2.4) (Rohlfing et al., 2022).

3.2.2 SICM imaging of ACKR3-activated human platelets

Human platelets were isolated as previously described (see chapter 2.2), however no prostacyclin was used during the first centrifugation step. Isolated platelets were added to a fibrinogen-coated (100 µg/ml for 1 h at 37°C) cell culture dish and allowed to adhere for 10 min in the presence of 0.1 U/ml thrombin and 1 mM CaCl₂. The cell culture dish was rinsed with Tyrode's buffer twice to wash away non-adherent platelets, then Tyrode's buffer containing ACKR3 agonist or dimethyl sulfoxide (DMSO) as well as 0.1 U/ml thrombin and 1 mM CaCl₂ were added. The adherent platelets were then visualized with SICM.

Platelet topography and Young's modulus were recorded with SICM using a nanopipette with $r_i \approx 150$ nm and 32×32 pixels in a square scan area of $15 \times 15 \mu\text{m}^2$ (higher resolutions of 64×64 pixels were used to record images for publication). For Young's modulus imaging, pressure of 20 kPa was applied.

Young's modulus E of an individual pixel was calculated via the method provided in chapter 2.3.2.2 using IGOR PRO (Wavemetrics, Inc., Lake Oswego, USA). Only regions of cell height above 50 nm were included in the analysis. The geometric mean of all pixels of one individual platelet was taken as the Young's modulus E of that platelet. Statistical analysis was done using Student's t-test on logarithmized geometric mean data.

3.2.3 SICM imaging of lipid-loaded platelets

Platelets were isolated as described in the previous chapter (see chapter 3.2.2). Isolated platelets were again preincubated for 30 min with 50 $\mu\text{g}/\text{ml}$ ACKR3 agonist or 0.1 % DMSO (control), and then further incubated for 15 min with either 20 $\mu\text{g}/\text{ml}$ LDL or OxLDL, before their adhesion on fibrinogen-coated surfaces and Young's modulus measurement was conducted using SICM. Control platelets not activated with (Ox)LDL were activated with 0.1 U/ml thrombin.

Platelet topography and Young's modulus were recorded with SICM using a nanopipette with $r_i \approx 150$ nm and 32×32 pixels in a square scan area of $15 \times 15 \mu\text{m}^2$ (higher resolutions of 64×64 pixels were used to record images for publication). For Young's modulus imaging, pressure of 20 kPa was applied. Johannes Rheinlaender assisted in the measurement of this data.

Young's modulus E of an individual pixel was calculated via the method provided in chapter 2.3.2.2 using IGOR PRO (Wavemetrics, Inc., Lake Oswego, USA). Only regions of cell height above 50 nm were included in the analysis. The median of all pixels of one individual platelet was taken as the Young's modulus E of that platelet. Statistical analysis was done using Dunn-Holland-Wolfe test for non-parametric data.

3.2.4 Actin staining of lipid-loaded platelets

Coating of surfaces, preparation of platelets, preincubation with DMSO, ACKR3 agonist or lipids, and adhesion followed the same protocol as described in the previous chapter (see chapter 3.2.3).

After 15 min of adhesion, non-adherent platelets were removed by washing. Afterwards, platelets underwent fixation and permeabilization involving a 10-minute treatment with 2% formaldehyde, followed by 10 minutes in 0.1% Triton X-100, washing with PBS three times between each step. F-actin was then stained for 30 minutes using FITC-Phalloidin (1:1000 dilution in PBS). Imaging was performed using an Eclipse Ti inverted microscope (Nikon) with

a monochrome digital camera (DS-Qi2, Nikon) and a 100× objective. Phase contrast images were acquired with 300 ms exposure at 10.0 V, and FITC channel images were captured with a 300 ms exposure at 5% lamp power for all samples.

F-actin fluorescence was quantified by calculating the corrected total cell fluorescence of adhered platelets (Gavet & Pines, 2010) using a built-in analysis in image analysis software ImageJ/fiji. Area and circularity were analyzed with ImageJ using a custom-built function utilizing built-in shape analysis (for full code, see chapter 11.1). The number of actin nodules and filopodia were counted. (For details on calculating CTCF, see chapter 2.3.1)

3.2.5 Image analysis and statistics

Image analysis & statistical evaluation was done in Igor Pro 9 (WaveMetrics, Lake Oswego, OR, USA) using built-in functions. Unless noted otherwise, all data are tested using either the two-tailed Mann-Whitney-U test for two samples or the Dunn-Holland-Wolfe test for non-parametric multiple comparisons for three or more samples. *P*-values below 0.05 were considered significantly different.

3.3 Results

3.3.1 Shape parameter quantification of *Ackr3*^{-/-} mice platelets using SICM

To test, if the genetic deletion of ACKR3 changed platelet morphology, SICM was used to image murine platelet spread on collagen. Spread platelet morphology of the ACKR3-deletion variant (*Ackr3*^{-/-}, n=28) was identical to the wildtype (*Ackr3*^{fl/fl}, n=17) as confirmed by SICM (Figure 3.2):

Platelet mean height (*Ackr3*^{fl/fl}: 452 ± 85 nm vs. *Ackr3*^{-/-}: 574 ± 500 nm, means ± standard deviation, *P*=0.22, t-test), maximum height (1.09 ± 0.28 μm vs. 1.45 ± 0.95 μm, *P*=0.06), perimeter (23.1 ± 5.4 μm vs. 26.7 ± 9.9 μm, *P*=0.22), area (16.7 ± 5.1 μm² vs. 18.2 ± 8.1 μm², *P*=0.46), circularity (0.44 ± 0.20 vs. 0.36 ± 0.16, *P*=0.19) and volume (7.50 ± 2.48 fL vs. 8.80 ± 4.41 fL, *P*=0.21) were not significantly different between the two phenotypes (Figure 3.2B), meaning a ACKR3 deletion does not affect platelet shape per se (Rohlfing et al., 2022).

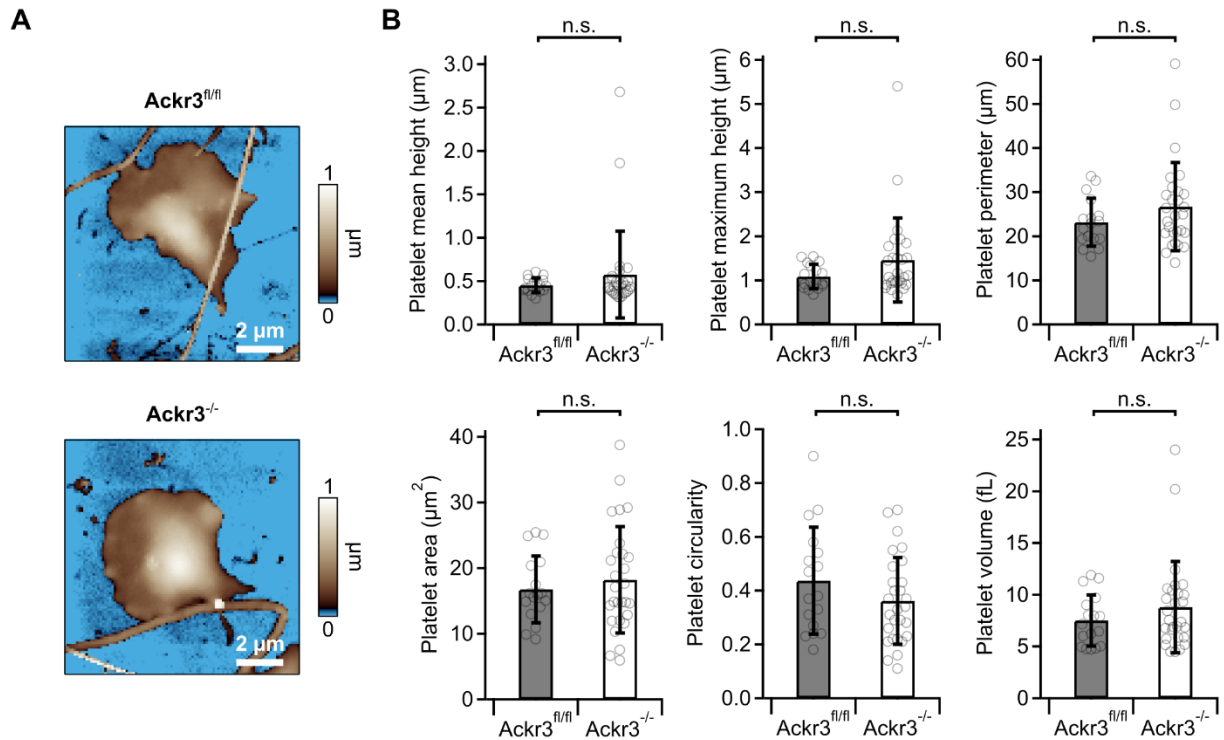


Figure 3.2: Platelet-specific ACKR3 deletion does not change platelet morphology and spreading behavior. (A) Representative topography images of fixed spread platelets, that were activated with thrombin and spread on collagen-coated petri dishes. Image size $10 \times 10 \mu\text{m}^2$ (100 x 100 pixels), pixel size $100 \times 100 \text{ nm}^2$. Scale bar: $2 \mu\text{m}$ (B) Barplots + error bar show means + standard deviations of platelet morphology parameters, with data points corresponding to individual platelets ($n=17$ for Ackr3^{fl/fl}, $n=28$ for Ackr3^{-/-}). Student t-test, n.s.= $P \geq 0.05$. Own data, also published in (Rohlfing et al., 2022). Murine blood collection, adhesion and fixation were performed by Anne-Katrin Rohlfing, SICM imaging, analysis and data visualization was done by Hendrik von Eysmond.

3.3.2 ACKR3 agonist reduces platelet Young's modulus

Since ACKR3 agonist VUF11207 reduces platelet hyperreactivity (Cebo et al., 2022), and increased adhesion and spreading is associated with an increase in platelet Young's modulus (Rheinlaender et al., 2015), it can be hypothesized that platelets preincubated with VUF11207 are reduced in their Young's modulus.

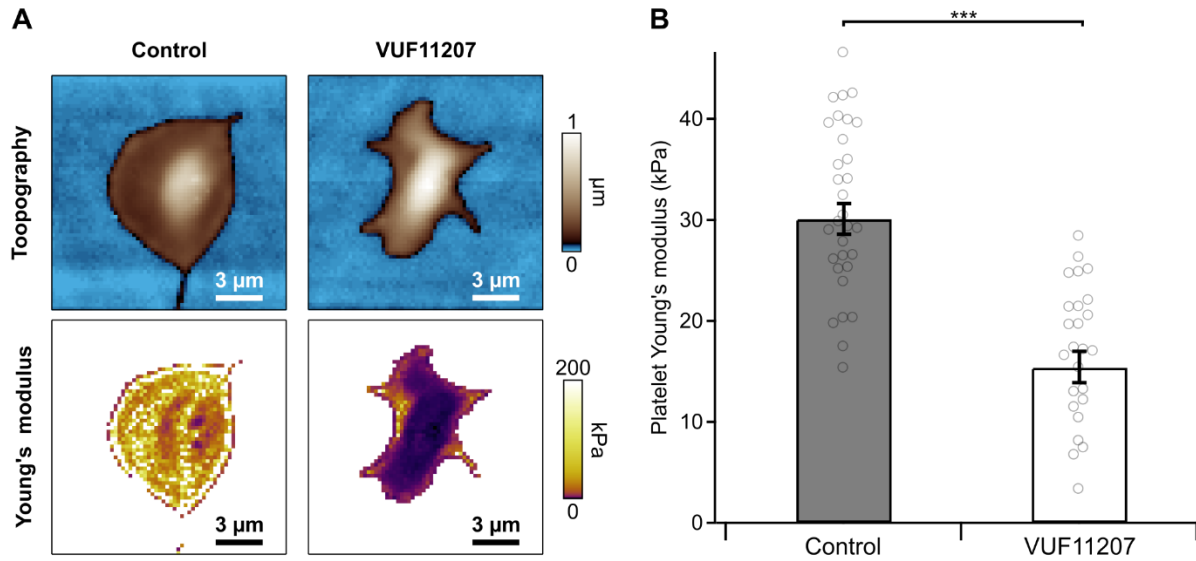


Figure 3.3: Influence of ACKR3 activation on platelet Young's modulus. (A) Representative topography (top row) and Young's modulus in terms of Young's modulus (bottom row) images of spread platelets pre-incubated with either 0.1 % DMSO (vehicle control) or 50 $\mu\text{g}/\text{ml}$ ACKR3 agonist VUF11207, before spreading on fibrinogen-coated surfaces. Image size $15 \times 15 \mu\text{m}^2$ (64×64 pixels), pixel size $234 \times 234 \text{ nm}^2$. Scale bar: $3 \mu\text{m}$ (B) Barplots of platelet Young's modulus showing geometric mean $\pm$ SEM from 3 donors, with data point corresponding to Young's modulus of individual platelets ($n=31$ for Control, $n=25$ for ACKR3 agonist). Student t-test on logarithmized data, *** $P < 0.001$. Own data, also published in (Cebo et al., 2022).

Indeed, Young's modulus of platelets spreading on fibrinogen was significantly reduced, when platelets were incubated with ACKR3 agonist VUF11207 (Figure 3.3, $P = 9.2 \cdot 10^{-7}$, t-test of logarithmized data). Control platelets showed an average Young's modulus of $30 \pm 1.05 \text{ kPa}$ (geometric mean $\pm$ geometric SEM factor), while ACKR3 agonist treated platelets showed an average Young's modulus of only $15.3 \pm 1.10 \text{ kPa}$.

3.3.3 OxLDL-activated platelets are softer than thrombin- and LDL-activated platelets

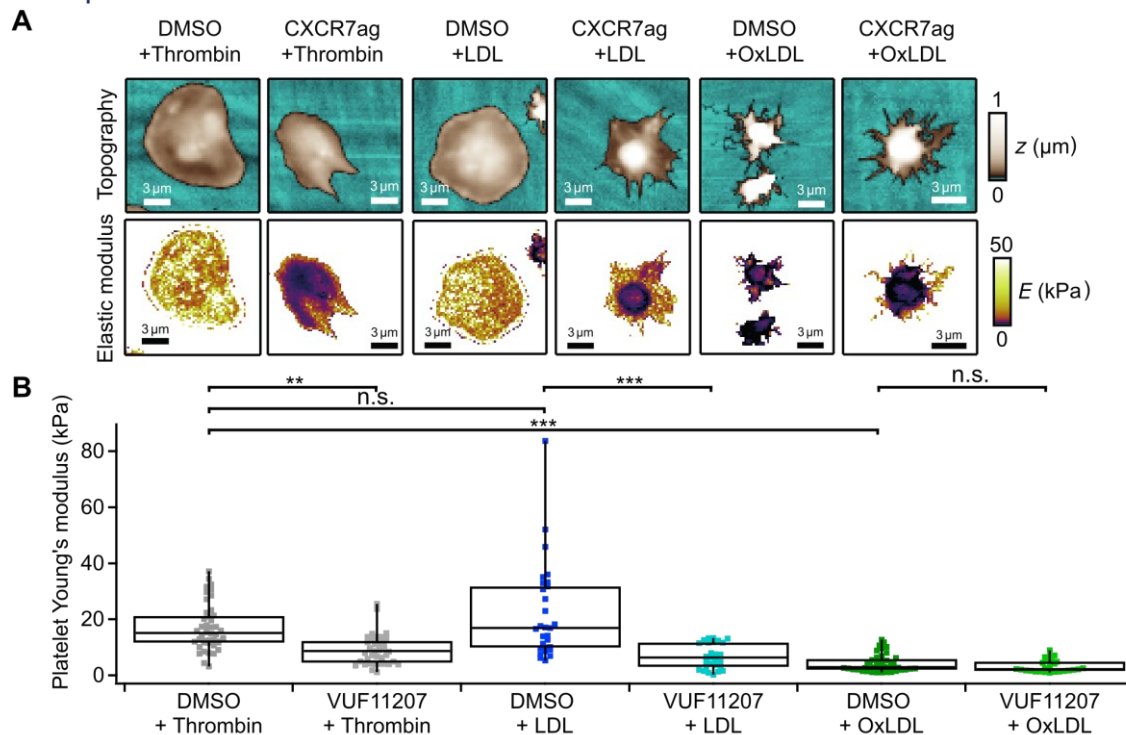


Figure 3.4: OxLDL and ACKR3 agonist, but not LDL, lower platelet Young's modulus. (A) Representative SICM topography and Young's modulus images of human platelets spread on fibrinogen-coated surfaces after preincubation with ACKR3 agonist or vehicle control DMSO and activation with OxLDL or LDL. Image size $15 \times 15 \mu\text{m}^2$ (64×64 pixel), pixel size $234 \times 234 \text{ nm}^2$. Scale bar: $3 \mu\text{m}$ (B) Platelet Young's modulus for each condition. Boxplots showing pooled data from 3 donors, with data points corresponding to individual platelets. Number of platelets: 44 (DMSO+Thr), 39 (VUF11207 +Thr), 29 (DMSO+LDL), 27 (VUF11207+LDL), 40 (DMSO+OxLDL) and 26 (VUF11207+OxLDL). Dunn-Holland-Wolfe test for non-parametric data, n.s. $P \geq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Next, the influence of (Ox)LDL and ACKR stimulation of platelet Young's modulus was investigated. Surprisingly, platelet Young's modulus was lowered only by OxLDL, not by LDL, compared to control (Figure 3.4A+B). ACKR3 agonist VUF 12207 lowered Young's modulus in both control and LDL-treated platelet but did not further decrease platelet Young's modulus when used with OxLDL. As the actin cytoskeleton significantly influences Young's modulus of a cell (Hofmann et al., 1997; Rotsch & Radmacher, 2000), it was investigated if there were significant changes in the platelet actin cytoskeleton following the use of ACKR3 agonist and/or lipids.

3.3.4 ACKR3 agonist and lipids change platelet shape and actin cytoskeleton

Platelet actin cytoskeleton composition differed greatly between samples (Figure 3.5A). While platelets activated with thrombin were often fully spread and circular, platelets activated with (Ox)LDL showed more spike-like protrusion, mostly likely filopodia (Bender & Palankar, 2021). Interestingly, platelets activated with lipids often showed small, actin-enriched areas, presumably so-called actin nodules (Calaminus et al., 2008; Poulter et al., 2015).

The area of platelets treated with LDL ($43.44 \pm 12.26 \mu\text{m}^2$, median $\pm$ MAD) was similar to that of platelets activated with thrombin ($43.79 \pm 12.47 \mu\text{m}^2$, $P = 1$, Dunn-Holland-Wolfe test for non-parametric data, Figure 3.5B). Also, the area of platelets activated with OxLDL ($46.72 \pm 13.77 \mu\text{m}^2$) was not significantly different to platelets activated with thrombin or LDL ($P = 0.99$ and $P = 0.99$, respectively). ACKR3 agonist significantly lowered the spreading area of thrombin- and LDL-activated platelets ($37.92 \pm 12.67 \mu\text{m}^2$, $P = 7.93 \cdot 10^{-5}$ and $29.99 \pm 10.26 \mu\text{m}^2$, $P = 1.63 \cdot 10^{-14}$, respectively), however not for OxLDL-activated platelets ($38.92 \pm 12.90 \mu\text{m}^2$, $P = 0.0985$).

Due to the spiky appearance of platelets activated with LDL/OxLDL, platelet circularity was significantly lower for platelets activated with LDL (Figure 3.5C, 0.51 ± 0.1 , $P = 1.63 \cdot 10^{-14}$) and even lower for platelets activated with OxLDL (0.43 ± 0.08 , $P = 1.63 \cdot 10^{-14}$), compared to platelets activated with thrombin (0.72 ± 0.12). ACKR3 agonist significantly increased platelet circularity by reducing spikiness for thrombin-activated platelet (0.80 ± 0.08 , $P = 0.0324$) but did not change platelet circularity for LDL- and OxLDL-activated platelets (0.52 ± 0.12 , $P = 0.99$ and 0.45 ± 0.1 , $P = 0.99$, respectively) which already had reduced circularity.

No actin nodules were visible within thrombin-activated platelets (Figure 3.5D). An increased number of actin nodules were visible within LDL- ($P = 4.87 \cdot 10^{-6}$ vs. DMSO + thrombin) and even more within OxLDL-activated platelets ($P = 1.63 \cdot 10^{-14}$ vs. DMSO + Thrombin). ACKR3 agonist increased the number of actin nodules for all conditions (DMSO: $P = 0.0224$; LDL: $P = 1.63 \cdot 10^{-14}$; OxLDL: $P = 2.53 \cdot 10^{-7}$).

Thrombin-treated platelets only sporadically showed filopodia, regardless of ACKR3 agonist treatment ($P = 1$, Figure 3.5E). The number of filopodia per platelet increased significantly with platelet activation with lipids (DMSO + LDL vs. DMSO + Thrombin: $P = 1.63 \cdot 10^{-14}$; DMSO + OxLDL vs. DMSO + Thrombin: $P = 1.63 \cdot 10^{-14}$).

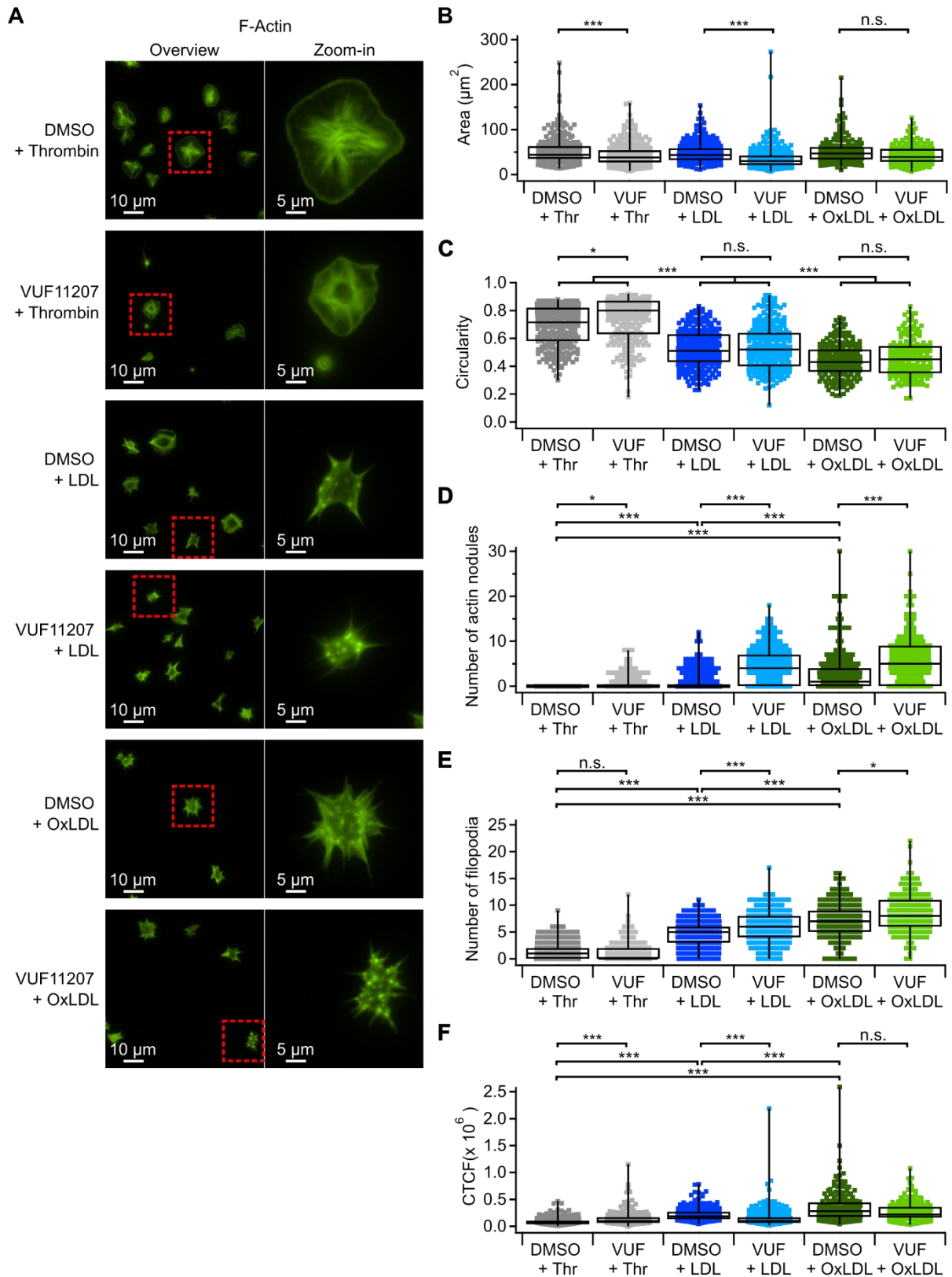


Figure 3.5: ACKR3 agonist and lipids change platelet actin cytoskeleton. (A) Representative fluorescence microscopy images of phalloidin-stained F-actin of platelets treated with either DMSO (vehicle control) or ACKR3 agonist VUF11207 and activated with either thrombin (Thr) or lipoproteins (LDL or OxLDL) and spreading on fibrinogen-coated surfaces. Left column: Overview, right column: zoom-in, indicated by red-dashed box in left column. Scale bar: 10 μ m and 5 μ m, respectively. (B)-(F) Shape parameter analysis of the actin cytoskeleton of lipid-loaded platelets. Boxplots show difference in (B) area, (C) circularity, and (D) number of actin nodules and (E) filopodia (E). (F) Corrected total cell fluorescence (CTCF) of platelet F-actin. Boxplots showing median $\pm$ IQR from

pooled data of 2 donors, with data points corresponding to individual platelets. Number of platelets: 270 (DMSO+Thr), 262 (VUF11207+Thr), 267 (DMSO+LDL), 276(VUF11207+LDL), 207 (DMSO+OxLDL) and 184 (VUF11207+OxLDL). Dunn-Holland-Wolfe test for non-parametric data, n.s. $P \geq 0.05$, * $P < 0.05$, *** $P < 0.001$.

The number of filopodia per platelet increased even further, when ACKR3 agonist VUF11207 was added (ACKR3 + LDL: $P = 0.00076$ vs. DMSO + LDL, ACKR3 + OxLDL: $P = 0.0412$ vs. DMSO + OxLDL), which potentially contributed to reduced platelet spreading and circularity.

F-actin CTCF was highest in OxLDL-treated platelets (Figure 3.5F, DMSO + OxLDL: 281948 ± 117263 , median $\pm$ MAD), and less in thrombin- (DMSO + Thrombin: 64114 ± 29866 ; $P = 1.63 \cdot 10^{-14}$ vs. DMSO + OxLDL, Dunn-Holland-Wolfe nonparametric test) and LDL-treated platelets (186802 ± 67224 , $P = 1.63 \cdot 10^{-14}$ vs. DMSO + Thrombin and $P=0.00022$ vs. DMSO + OxLDL). ACKR3 agonist treatment significantly changed the CTCF for both thrombin- (VUF11207 + Thrombin: 98377 ± 46206 , $P = 1 \cdot 10^{-7}$ vs. DMSO + Thrombin) and LDL-treated (VUF11207 + LDL: 100880 ± 46357 , $P = 1.63 \cdot 10^{-14}$ vs. DMSO + LDL), but not for OxLDL-treated platelets (VUF11207 + OxLDL: 218766 ± 81226 , $P = 0.99$ vs. DMSO + OxLDL).

3.4 Discussion

The major findings of this section are the following: 1) Genetic ACKR3 deletion does not significantly change platelet morphology, 2) ACKR3 agonist VUF11207 reduces platelet Young's modulus, 3) OxLDL, but not LDL significantly lowers platelet Young's modulus and 4) Stimulation of ACKR3 and platelet activation by (oxidized) lipids severely change platelet shape and actin network composition to a filopodia-and actin-nodule dominated shape. As platelet Young's modulus is directly correlated to its activation status (Rheinlaender et al., 2015), these findings might be relevant for the potential platelet inhibitory effects of ACKR3 agonist in a hyperlipidemic microenvironment.

Platelet shape analysis by SICM showed that platelet spreading and morphology of *Ackr3*^{-/-} mice was not changed compared to those of *Ackr3*^{fl/fl} mice, meaning both have the same baseline of platelet activity and can be used to compare the influence of ACKR3, as was done in (Rohlfing et al., 2022).

Hyperlipidemia is associated with platelet hyperreactivity (Aviram & Gerald Brook, 1987) and increases the risk of severe thrombosis and cardiovascular diseases (Akkerman, 2008). Targeting ACKR3 for the treatment of hyperlipidemic patients offers a two-point advantage: 1) A reduction of pro-thrombotic lipids and platelet hyperreactivity while 2) maintaining hemostasis (Cebo et al., 2022). As an increased platelet Young's modulus is associated with increased platelet adhesion and spreading (Rheinlaender et al., 2015), a lower Young's modulus points to a lowered activity of ACKR3 agonist-treated platelets. However, platelet adhesion or spreading is not fully abolished, allowing for hemostasis, like reported previously (Cebo et al., 2022).

Simulating a hyperlipidemic environment using LDL and OxLDL, the given results show that platelets activated by LDL show no decrease in Young's modulus, while OxLDL significantly reduces platelet Young's modulus (Figure 3.5B). In previous studies with other cells of the blood system, the effect was the opposite: The Young's modulus of endothelial cells (Byfield et al., 2006; Chouinard et al., 2008) and erythrocytes (Wang et al., 2009) increased after exposure to OxLDL. However, these increases were only observed after hours of OxLDL exposure (Byfield et al., 2006; Chouinard et al., 2008; Wang et al., 2009). In fact, the endothelial cell Young's modulus seems to be slightly decreased after 30 min of OxLDL exposure (Chouinard et al., 2008). Endothelial stiffening is mediated via the CD36/RhoA/ROCK/MLCP/MLC2 pathway, where the activation of the scavenger receptor CD36 by OxLDL leads to the activation of small GTPase RhoA and its Rho-associated kinase (ROCK), which dephosphorylates myosin light chain 2 (MLC2) and myosin light-chain phosphatase (MLCP) (Oh et al., 2016). OxLDL induces platelet adhesion and spreading using the same pathway (Wraith et al., 2013), so platelet stiffening in response to OxLDL might be expected. However, further activation of already spread platelets with platelet activator thrombin decreased platelet Young's modulus (Rheinlaender et al., 2015). Thrombin also activates the RhoA pathway in platelets (Bodie et al., 2001). There might be a time-dependent component, that exposure of (resting) platelets to OxLDL leads to a short-term softening, but a long-term stiffening. There might potentially be a difference between adhered and non-adhered platelet. Substrate-bound fibrinogen, as was used in this thesis, might also create its own effects (Qiu et al., 2014).

As platelet adhesion and spreading is accompanied by an increase in cellular Young's modulus (Rheinlaender et al., 2015), platelet spreading is seemingly impaired by OxLDL, but not by LDL, a conclusion which is supported by the results of the actin staining (Figure 3.5). Interestingly, while ACKR3 agonist counteracts OxLDL-induced platelet activation in many facets, such as generating antithrombotic lipids (Cebo et al., 2022) and lowering platelet activation (Rohlfing et al., 2022), addition of ACKR3 agonist VUF11207 did not revert the reduction of platelet Young's modulus by OxLDL activation. This is not very surprising as application of ACKR3 agonist VUF11207 itself lowered platelet Young's modulus (see Figure 3.3). In conclusion, the exact effects of (Ox)LDL and ACKR3 activation on platelet Young's modulus need to be investigated further, ideally via protein-protein interaction detection methods (Cho & Park, 2026) to detect the direct interaction partners of (Ox)LDL and VUF11207 in the platelets.

Similar to a previous report (Seifert et al., 2021), exposure of human platelets to LDL lead to no significant change in platelet spreading area in comparison to platelets activated with thrombin. In contrast to that report, however, LDL significantly reduced platelet circularity and

increased number of visible filopodia formations. OxLDL only reduced platelet circularity and increased the number of visible platelet filopodia, whereupon there was no change in platelet surface coverage or area, unlike the previous report (Seifert et al., 2021). However, the incubation time of LDL & OxLDL in that report was only 10 min, here the incubation time was 15 min, in addition to the 30 min of incubation in 0.1% DMSO vehicle control, which could have an influence on spreading behavior. An additional influence could be the number of data points measured. For example, the decrease in platelet circularity and the increase in filopodia with LDL is also visible in the data of the previous report (Seifert et al., 2021). Additional research is necessary to validate these findings.

Filamentous actin (F-actin) is a major contributor to the overall Young's modulus of a cell, since disruption of the F-actin network reduces the Young's modulus of cells by a significant amount (Hofmann et al., 1997; Rotsch & Radmacher, 2000; Ujihara et al., 2012). A natural assumption would then be that a platelet with a lower Young's modulus (such as a platelet activated with OxLDL) shows a lower F-actin content than a stiffer platelet. However, the study showed the opposite: F-actin content as measured by CTCF was highest in OxLDL-activated platelets. The reasons for this could be manifold: First, OxLDL-activated platelets showed a high number of actin nodules. These structures have a high fluorescence intensity, potentially skewing the CTCF analysis towards higher values. Second, the quantification of F-actin content via CTCF is only an indirect measurement. A direct quantification of the F-actin itself using an absorbance assay, Western Blot, enzyme-linked immunosorbent assay, or mass spectrometry could result in a more accurate representation of F-actin content in these platelets. Third, the F-actin content is not the lone contributing factor to a cell's Young's modulus. The F-actin organization also plays a significant role. If F-actin is organized in stress fibers (Tojkander et al., 2012), then the Young's modulus is significantly increased compared to unorganized F-actin (Calzado-Martín et al., 2016). Stress fibers are easily visible in the thrombin-activated platelets (long, straight lines with high fluorescence; Figure 3.5), while they are fewer in the LDL- and OxLDL-activated platelets. Areas with no stress fibers are softer, so the median Young's modulus of the cell is lowered. Fourth, other proteins of the cytoskeleton such as microtubules and intermediate filaments contribute to a cell's Young's modulus (Vakhrusheva et al., 2022), which could effectively contribute to a reduced Young's modulus not accounted for by a reduced F-actin content.

Actin nodules are actin-rich structures formed early in the platelet spreading process (Calaminus et al., 2008; Poulter et al., 2015; Joubert et al., 2024), before actin stress fibers are formed during the last stages of platelet spreading (Paknikar et al., 2019). Interestingly, this process is reversible, as actin nodules also seem to reappear after platelets are spread for around 2 h (Joubert et al., 2024). This process is possibly linked to clot retraction and based

on a decreased cytosolic calcium concentration, a reversal of the calcium mobilization in platelets that ensures platelet activation (Varga-Szabo et al., 2009). In addition, platelets with actin nodules seemingly appear more often when spreading on fibrinogen (as compared to vWF) (Mollica et al., 2023).

As evident from the results (see Figure 3.5), platelets activated with lipoprotein LDL or OxLDL were more likely to show actin nodules than those activated with thrombin. Since actin nodules can appear in the early spreading process as well as afterwards, the question remains whether platelets are inhibited or accelerated in their spreading. A direct comparison of spreading behavior of thrombin- and lipid-activated platelets showed that OxLDL-activated platelets had a significantly lower initial spreading rate (Seifert et al., 2021), hinting more towards the former being true. For mouse platelets, the number of nodules per platelet during spreading averages between four and six (Poulter et al., 2015) before being reduced to zero, similar to the human (Ox)LDL-activated platelets treated with ACKR3 agonist here, hinting at an inhibited spreading rate as well. A dynamic measurement of actin content during platelet spreading might clarify these findings.

The formation of actin nodules in platelets is dependent on the Arp2/3 complex and its activator Wiskott Aldrich syndrome protein (WASp), as the platelets of patients afflicted with Wiskott-Aldrich syndrome show no actin nodules (Poulter et al., 2015). Arp2/3-rich actin nodules (Calaminus et al., 2008) are also necessary for the generation of lamellipodia (Bender & Palankar, 2021; Campellone et al., 2023), whereas filopodia can be formed independently of Arp2/3 (Steffen et al., 2006; Wu et al., 2012; Romero et al., 2020). Since platelets treated with OxLDL display a high number of actin nodules and a filopodia-dominated shape lacking lamellipodia (shown in the results here and also in (Seifert et al., 2021)), OxLDL might be interfering with Arp2/3 or one of its activators, such as WASp, WASp-family verprolin-homologous protein (WAVE) (Campellone & Welch, 2010; Campellone et al., 2023) or the small GTPase Rac1 (McCarty et al., 2005).

Supporting this hypothesis is the filopodia-dominated shape of Arp2/3-inhibited platelets. Murine platelets with a Rac1-knockout showed a filopodia-dominate shape with only partial lamellipodia (McCarty et al., 2005; Pleines et al., 2009), similar to the platelets derived from mice having Arp2/3-depleted megakaryocytes (Paul et al., 2017). Interestingly, the F-actin content of these platelets was significantly lowered compared to platelets of wildtype mice, opposite to the results shown in this thesis. However, in that study, F-actin of resting or activated platelets was quantified on using flow cytometry, meaning the platelets did not adhere or spread. Since actin nodules are a significant part of the spreading process, this could explain this discrepancy in F-actin content change.

These findings with murine platelets are similar to human platelets. In a study using platelets from patients with a Arp2/3 deficiency or loss, platelets still adhered, but failed to form lamellipodia (Kahr et al., 2017). However, the Arp2/3 deficient-platelets in that study lacked actin nodules, which is opposite to the effect induced by (Ox)LDL as revealed in current results. In other cell types, Arp2/3 deletion leads to a similar phenotype as shown here. In a Arp2/3-knockout mouse embryonic fibroblast (MEF) cell line, cells showed no lamellipodium and a spiky shape (Wu et al., 2012). Lamellipodial extrusions were restored 20 min after administering bovine Arp2/3 to the MEFs. In a reversal of this phenomenon, human platelets spread on fibrinogen retracted their lamellipodium 30 min after administering OxLDL (Seifert et al., 2021). A similar retraction was seen in OxLDL-treated macrophages, caused by a CD36- and Rac1-mediated inhibition of non-muscle myosin II (Park et al., 2012). In addition, OxLDL-induced CD36 activation increased actin polymerization in macrophages (Park et al., 2009). Taking these reports and the results shown here together, there is an urgent need for further investigation on the influence of OxLDL on Arp2/3, the cytoskeleton and lamellipodial spreading to clarify these phenomena.

In conclusion, ACKR3 is a promising target to lower platelet activity. Platelet Young's modulus is an important biomarker that should not be ignored. More intensive research focusing on platelet responsiveness to hyperlipidemia and the possible treatment with ACKR3 agonist to alleviate prothrombotic events is needed.

4 Mechanosensing alters platelet migration

The following chapter contains the scientific publication titled:

“Mechanosensing alters platelet migration”

Authors: Hendrik von Eysmond, Jan Seifert, Johannes Rheinlaender, Tilman E. Schäffer

Author position: 1

Status in publication process: Published (von Eysmond et al., 2025)

Detailed breakdown of contributions:

The percentages in parentheses indicate the author's (Hendrik von Eysmond's) contribution as a percentage. This applies to the entirety of this chapter.

- Scientific ideas (50 %)
- Data generation (100 %)
 - Isolation of human platelets & optimization of platelet migration
 - Creation of PDMS substrates with different stiffnesses
 - Light microscopy and SICM measurements
 - Any data collection related to the figures shown
- Analysis & Interpretation (65 %)
 - Code writing in IGOR Pro (Analysis)
 - Composition and design of figures
- Paper writing (50 %)

Platelets have long been established as a safeguard of our vascular system. Recently, haptotactic platelet migration has been discovered as a part of the immune response. In addition, platelets exhibit mechanosensing properties, changing their behavior in response to the stiffness of the underlying substrate. However, the influence of substrate stiffness on platelet migration behavior remains elusive. Here, we investigated the migration of platelets on fibrinogen-coated polydimethylsiloxane (PDMS) substrates with different stiffnesses.

Using phase-contrast and fluorescence microscopy as well as a deep-learning neural network, we tracked single migrating platelets and measured their migration distance and velocity. We found that platelets migrated on stiff PDMS substrates ($E = 2$ MPa), while they did not migrate on soft PDMS substrates ($E = 5$ kPa). Platelets migrated also on PDMS substrates with intermediate stiffness ($E = 100$ kPa), but their velocity and the fraction of migrating platelets were diminished compared to platelets on stiff PDMS substrates. The straightness of platelet migration, however, was not significantly influenced by substrate stiffness. We used scanning ion conductance microscopy (SICM) to image the three-dimensional shape of migrating platelets, finding that platelets on soft substrates did not show the polarization and shape change associated with migration. Furthermore, the fibrinogen density gradient, which is generated by migrating platelets, was reduced for platelets on soft substrates.

Our work demonstrates that substrate stiffness, and thus platelet mechanosensing, influences platelet migration. Substrate stiffness for optimal platelet migration is quite high (>100 kPa) in comparison to other cell types, with possible implications on platelet behavior in inflammatory and injured tissue.

4.1 Introduction

Platelets are small, anucleate cells, which are mainly involved in hemostasis and wound closure. Platelets contribute to vascular integrity by adhering to a site of injury, spreading, and forming a blood clot (R. D. Allen et al., 1979; Hartwig, 1992; Sorrentino et al., 2015). For decades, it has been known that platelets use chemotaxis, migrating towards structure proteins such as collagen, chemokines such as stromal-derived factor 1 (SDF-1), or bacteria-derived proteins such as N-formyl peptide to help with host defense in injury or inflammation (Valone et al., 1974; Lowenhaupt, 1978; Czapiga et al., 2005; Kraemer et al., 2010; Schmidt et al., 2011; Schmidt et al., 2012). In addition, platelets show mechanosensing properties, scanning their environment and changing their spreading behavior according to the mechanical properties of this environment (Qiu et al., 2014; Kee et al., 2015).

In recent years, multiple reports have shown that platelets have the ability to migrate independently of chemical attractants (Gaertner et al., 2017; Nicolai et al., 2020; Sarkar et al., 2020; Kaiser et al., 2023; Schoen et al., 2024). This process was revealed to be part of the early immune response, including the migration of platelets to collect bacteria (Gaertner et al.,

2017). Platelets migrate by depleting substrate-bound ligands such as fibrinogen and self-generate a ligand gradient on the underlying substrate, a process called ligand-depleting migration (Sarkar et al., 2020) or haptotaxis (Carter, 1967; Nicolai et al., 2020; Schoen et al., 2024). For platelet migration to occur, platelets need to polarize (Lauffenburger & Horwitz, 1996) and assume a “half-moon”-like shape (Gaertner et al., 2017; Nicolai et al., 2020; Kaiser et al., 2023).

Recently, more details of the platelet migration process such as the necessity of Arp2/3-dependent lamellipodia formation (Nicolai et al., 2020) or the integrin-GPIIb-dependent outside-in signaling cascade (Kaiser et al., 2023) have been revealed.

Substrate stiffness has been known to influence migration behavior for other cells including fibroblasts (Yeung et al., 2005), cancer cells (McKenzie et al., 2018), vascular smooth muscles cells (VSMCs) (Isenberg et al., 2009), retinal ganglion cells (Koser et al., 2016), and mast cells (Yu et al., 2021). In addition, for some cells, there seems to be an “optimal substrate stiffness” where the cell migration speed is highest (Bangasser et al., 2017). For mast cells, a substrate stiffness of ~50 kPa gave the highest migration speed (Yu et al., 2021), while the “optimal” substrate stiffness for neutrophils was 4-7 kPa (Stroka & Aranda-Espinoza, 2009). For platelets, however, a possible connection between mechanosensing and substrate stiffness in migration behavior has not yet been shown.

We therefore investigated the migration of platelets on fibrinogen-coated polydimethylsiloxane (PDMS) substrates with different stiffnesses. Using optical phase-contrast microscopy combined with a previously established convolutional neural network (CNN) (Seifert et al., 2021), we show that platelets migrate slower and less frequently on softer PDMS substrates. Using high-resolution scanning ion conductance microscopy (SICM), we then demonstrate that platelets migrating on softer PDMS substrates lack the distinct polarized shape associated with migration (Gaertner et al., 2017; Seifert et al., 2022). In addition, the fibrinogen density gradient of platelets on soft PDMS was diminished compared to platelets on stiff or intermediate PDMS. We thereby demonstrate that substrate stiffness affects platelet migration behavior.

4.2 Material and methods

4.2.1 Preparation of polydimethylsiloxane (PDMS) substrates

To prepare substrates with different stiffnesses, PDMS silicone elastomers Sylgard™ 184 (S184) and Sylgard™ 527 (S527) (both from Dow Inc., Midland, USA) were used (Prager-Khoutorsky et al., 2011; Palchesko et al., 2012). S184 was mixed 10:1 (base : cross-linker, weight ratio) to create the stiff PDMS substrate and S527 was mixed 1:1 (Part A : Part B, weight ratio) to create the soft PDMS substrate. For intermediate PDMS, premixed S184 and S527 were subsequently mixed in a 1:10 weight ratio. Mixing was done for 10 min at room temperature (RT) using a magnetic stirrer. The PDMS was then degassed using a desiccator

for 10 min. Depending on the experiment, 80 mg of uncured PDMS was poured into the center of a glass-bottom dish (ibidi, 81218) or a glass-bottom dish with a 500 μm grid (ibidi, 81168), followed by another 60 min of degassing. PDMS substrates were then cured for 12 h at 80°C and stored for at least 5 days before use (a shorter storage duration sometimes gave a reduced platelet adhesion). By optical microscopy it was ensured that the cured PDMS substrates were flat and had a thickness of about 30-40 μm to avoid an influence of the stiff underlying glass surface on the stiffness sensed by the platelets.

To produce substrates with a stiffness gradient, first, stiff S184 was mixed and degassed as described above, poured in the form of a wedge into a glass-bottom dish, and cured for 12 h. Second, soft S527 was mixed and degassed, poured in the form of a wedge on top of the cured S184, and cured for 12 h to produce a sharp transition with stiffness gradient (see Figure 4.7 for details).

4.2.2 Characterization of PDMS substrates

The Young's modulus of PDMS substrates was measured using a commercial material testing machine (Zwicki Line 500, Zwick & Roell, Ulm, Germany), equipped with a flat-ended cylindrical indenter with a radius of 1 mm and a force sensor with spring constant 667 kN/m. Force-displacement curves were obtained at three different positions on each sample with a velocity of 0.5 mm/s and a maximum indentation depth of 1 mm (Figure 4.1A-C). Each PDMS sample was at least 2 cm high and 2 cm wide to avoid any effect of sample size on the measurement. The Young's modulus of each PDMS sample was obtained by fitting the contact model for a flat-ended cylindrical indenter to the force-displacement curves assuming a Poisson ratio of 0.5 for PDMS (Müller et al., 2019) (Figure 4.1D).

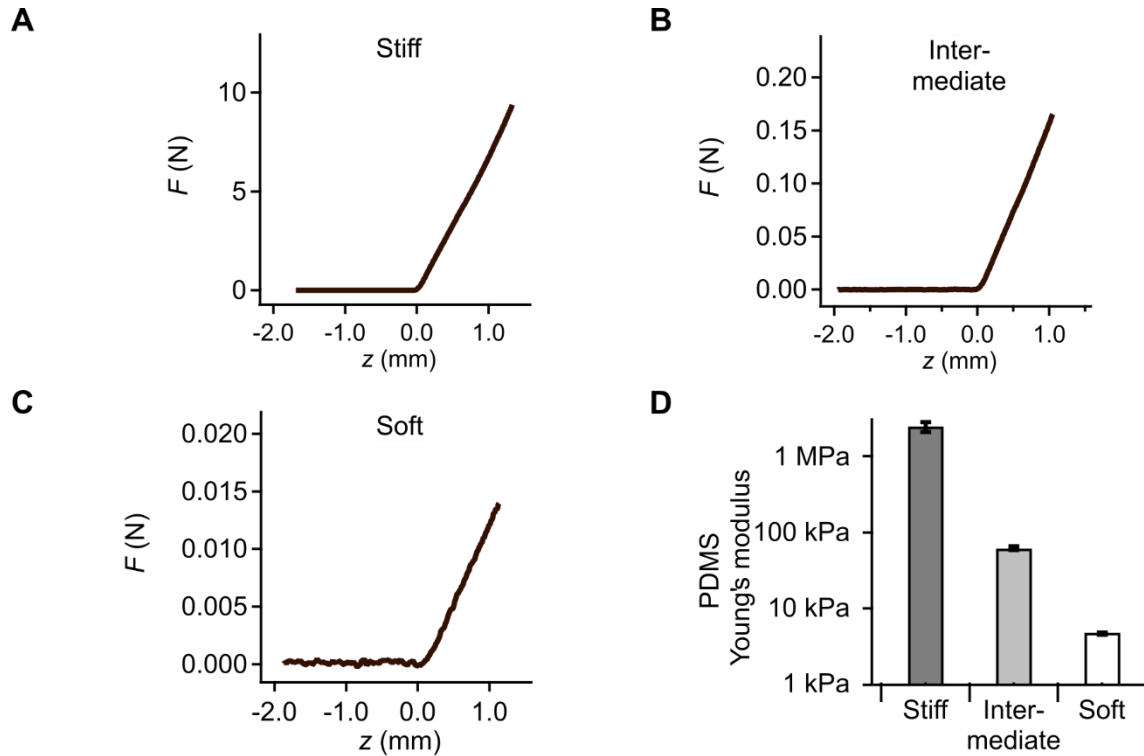


Figure 4.1: Indentation measurements on PDMS substrate using cylindrical indenter. Representative force-displacement curves on (A) stiff, (B) intermediate, and (C) soft PDMS substrates. The contact model for a flat-ended cylindrical indenter was fit to force-displacement curves to obtain the Young's modulus. (D) Average Young's modulus of the PDMS substrates. Bar plots show mean $\pm$ standard deviation from 3 independent experiments each.

4.2.3 Platelet isolation

All procedures were approved by the institutional ethics committee (273/2018BO2) and comply with the declaration of Helsinki. Blood donors gave written informed consent. Blood was drawn from healthy donors into monovettes (04.1926.001, Sarstedt) containing 1/4 volume of acid-citrate-dextrose (ACD, C3821, Sigma Aldrich). Monovettes were centrifuged at 200 g for 20 min at RT to obtain platelet-rich plasma (PRP). PRP was resuspended at a 1:3 ratio in Tyrode-HEPES buffer (pH = 6.5) (136.89 mM NaCl, 2.81 mM KCl, 11.9 mM NaHCO₃, 1 g/L D-glucose, 10 mM HEPES) supplemented with 0.225 μ g/ml prostacyclin (Prostaglandin I₂, Cat. No. 18220, Cayman Chemical) and centrifuged again at 880 g for 10 min at RT. Afterwards, the supernatant was discarded, and the platelet pellet was carefully resuspended in 1 ml of Tyrode-HEPES buffer (pH = 7.4). Washed platelets were allowed to rest for at least 30 min before proceeding with the experiments.

4.2.4 Serum preparation

Human serum contains free calcium ions and human serum albumin (HSA), which are both necessary for platelet migration (Gaertner et al., 2017). Therefore, we additionally prepared serum from each donor for each day of experiments. Blood was collected from healthy donors into serum monovettes (01.1601.001, Sarstedt, Nümbrecht, Germany). After collection, the

monovettes were left at rest for 30 min to allow blood coagulation. The monovettes were then centrifuged at 2000 g for 15 min at RT. The supernatant was extracted and centrifuged again at 2000 g for 15 min at RT. The resulting supernatant, i.e., the serum, was split into 500 μ l aliquots and stored at -80°C until further use.

4.2.5 Platelet migration assay

The platelet migration assay was done similarly as previously described (Seifert et al., 2021). In short, migration buffer (Tyrode-HEPES buffer, pH 7.4, including 5% human serum of the same donor, 3 μ M U-46619 thromboxane (TxA_2 , Cay16450, biomol, Hamburg, Germany) and 20 μ M adenosine diphosphate (ADP, A2754, Sigma Aldrich)) was supplemented with 20 μ g/ml fibrinogen Alexa Fluor™ 594 Conjugate (F13193, ThermoFisher) to make the coating buffer. The PDMS substrates were coated with 2 ml of coating buffer and were incubated for 10 min at 37°C. Afterwards, the PDMS substrates were carefully washed 3 $\times$ with migration buffer without fibrinogen and imaged with fluorescence microscopy to evaluate fibrinogen density. Afterwards, 5 μ l of washed platelets were activated for 1 min in 500 μ l of migration buffer without fibrinogen, pipetted onto the PDMS substrates, and incubated for 10 min at 37°C to allow platelet adhesion. Then, the samples were carefully washed with migration buffer to remove non-adhered platelets. Samples were then transferred to the microscope for platelet migration imaging. Non-migrating control samples (Figure 4.2) were prepared by using migration buffer without serum, thus without free calcium ions and HSA necessary for migration (Gaertner et al., 2017).

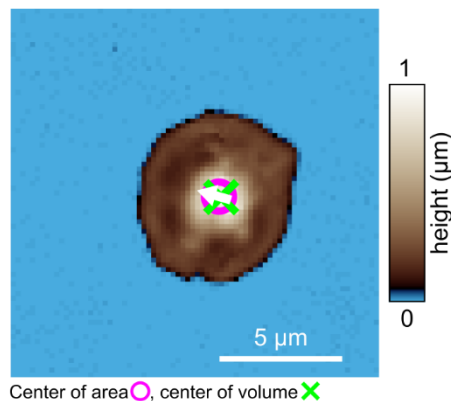


Figure 4.2: SICM topography of a non-migrating platelet on a stiff PDMS substrate. The platelet was fixed during a migration assay on a stiff PDMS substrate while inhibiting migration by using migration buffer without serum. The distance between the center of area (marked with a purple circle O) and the center of volume (marked with a green cross X) defines the platelet shape anisotropy, illustrated by the white arrow (enlarged 10x for visibility). Scale bar: 5 μ m

4.2.6 Light and fluorescence microscopy setup and imaging

Light and fluorescence microscopy images were taken using an Eclipse Ti inverted microscope (Nikon) with a monochrome digital camera (DS-Qi2, Nikon) and a 40x objective (Plan Fluor ELWD 40x Ph2 DM, Nikon). All phase contrast images were taken at 7.0 V lamp voltage (DIA, Nikon) with 50 ms of exposure time. All epifluorescence images were taken at 30% lamp

voltage (Sola, Lumencor) with 1 s of exposure time using Texas-Red filter. For fibrinogen density gradient measurements, images of PDMS substrates were taken with phase contrast and Texas-Red epifluorescence before and after coating, as well as before and after migration. For platelet migration assays, phase contrast images were taken with intervals of 90 s for 90 min duration. Live cell imaging of migrating platelets was carried out at a RT of 25-30 °C. For subsequent SICM measurements, the samples were fixed with 2 % formaldehyde and 0.05 % glutardialdehyde for 10 min. Aldehydes were removed by washing 3× with phosphate buffered saline (PBS, D8537, Sigma-Aldrich), then the samples were mounted into the SICM setup.

4.2.7 SICM setup and imaging

A custom-built SICM setup described previously (Seifert et al., 2022) was used. Images were acquired in the hopping mode using a pipette with an inner radius of ≈ 90 nm. The pipette was moved with a vertical approach and retract speed of 150 $\mu\text{m/s}$ with a hopping distance of 2 μm and a trigger value of 1% current drop. Images were recorded in a scan area of $20 \times 20 \mu\text{m}^2$ at a pixel resolution of 100×100 , resulting in a pixel size of $0.2 \times 0.2 \mu\text{m}^2$. Sample tilt and offset were corrected by first order plane flattening. Pixels with height values above 50 nm were considered as pixels belonging to platelets.

The relative shape anisotropy was determined as the distance from the center of volume to the center of area of the platelets (for details, see ref. (Seifert et al., 2022)), divided by the effective radius $r = \sqrt{\frac{A}{\pi}}$ of the platelet area A (to be independent of platelet size). The platelet volume was calculated as the sum of all single pixel volumes of the platelet.

4.2.8 Data analysis and convolutional neural network

Data analysis was performed in Igor Pro 9 (WaveMetrics, Lake Oswego, OR, USA). For migration assays, the positions of single platelets in platelet migration assays were tracked as previously described (Seifert et al., 2021). In short, binarized images of platelets were predicted from phase contrast images using a convolutional neural network (CNN). Platelet center position and area over time were tracked using a custom-built tracking function. Obtained tracking data were manually corrected for drift and smoothed over five time steps.

The straightness of the platelet migration movement was defined as the ratio of the distance between the platelet positions at the start and the end (after 90 min) of the measurement (“start-to-end-distance”) and the sum of the distances between the platelet positions in consecutive images (“accumulated distance”). A perfectly straight migration movement corresponds to a straightness of 1. The mean migration velocity was defined as the ratio of the start-to-end distance over 90 min.

For fibrinogen density gradient analysis, normalized fibrinogen fluorescence images were calculated as $I' = \frac{(I - I_{bg})}{(I_{fib} - I_{bg})}$, which sets the mean intensity of the background (I_{bg}) to 0 and the mean intensity of the fibrinogen (I_{fib}) to 1. This was done to reduce the influence of the varying fibrinogen layer intensity, which slightly varied locally and between experiments (not statistically significant, see Figure 4.3). In the normalized images, the platelet areas were automatically identified using the prediction images produced by the CNN. For calculating the fibrinogen gradient, the areas of the substrate around the platelets and of the high intensity at the platelet pseudo-nucleus were excluded (see dashed lines in Figure 4.11B). Using a two-dimensional plane fit, the absolute value of the gradient of the normalized fluorescent intensity below the remaining platelet area was quantified, as shown in Figure 4.11. The detailed code can be found in the appendix (chapter 11.2).

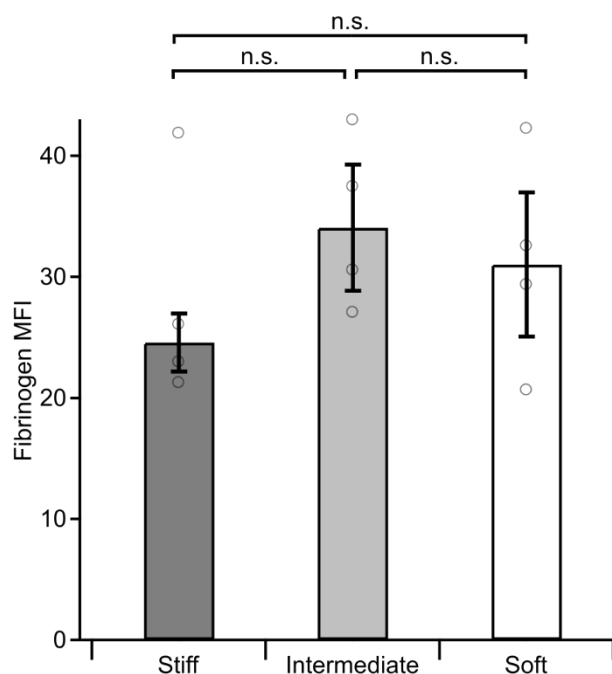


Figure 4.3: Fibrinogen coverage of PDMS does not depend on PDMS stiffness. Bar plots show median $\pm$ MAD, data points show mean fluorescence intensity (MFI) of 4 independent experiments. n.s. for $P > 0.05$ for Dunn-Holland-Wolfe test.

4.2.9 Statistics

Statistical evaluation was done in IGOR Pro 9 using built-in functions. Unless noted otherwise, all data are presented as median $\pm$ median absolute deviation (MAD) and tested using either the two-tailed Mann-Whitney-U test or the Dunn-Holland-Wolfe test for non-parametric multiple comparisons. P -values below 0.05 were considered significantly different.

4.3 Results

4.3.1 PDMS substrate stiffness influences platelet migration velocity

To investigate the influence of substrate stiffness on platelet migration, we prepared platelets on fibrinogen-coated PDMS substrates with different stiffnesses: pure S184 (Young's modulus approx. $E = 2$ MPa, "stiff"), pure S527 (approx. $E = 5$ kPa, "soft"), and a 1:10 mixture of S184:S527 (approx. $E = 100$ kPa, "intermediate"). First, we imaged migrating platelets for 90 min using phase-contrast microscopy. Platelets on stiff PDMS (Figure 4.4, top row; a donor-wise break-down is provide in Figure 4.5) migrated over a distance of several tens of micrometers (red trace), similar as in previous studies on stiff glass surfaces (Gaertner et al., 2017; Sarkar et al., 2020; Seifert et al., 2022; Kaiser et al., 2023). In comparison, platelets on intermediate PDMS (Figure 4.4A, middle row) migrated over a smaller distance than on stiff PDMS (red trace). Platelets on soft PDMS (Figure 4.4A, bottom row) spread, but did not migrate. We used fluorescent fibrinogen to ensure that the fibrinogen coverage of the PDMS was similar for all PDMS stiffnesses (Figure 4.3).

To quantify platelet migration, platelet positions were automatically tracked using a CNN and a custom-made tracking analysis (Seifert et al., 2021). The tracking analysis revealed that platelet velocity and likewise migration distance is strongly influenced by the PDMS substrate stiffness (Figure 1B): Platelets migrated significantly faster and farther on stiff PDMS with a mean velocity of 119 ± 28 nm/min and a start-to-end distance of 10.7 ± 2.5 μ m (median $\pm$ median absolute deviation, MAD) than on intermediate PDMS with 48 ± 23 nm/min and 4.3 ± 2.1 μ m, respectively. For platelets on soft PDMS, which did not migrate, we nevertheless measured a non-zero velocity of 9 ± 4 nm/min due to the tracking noise (stiff vs. intermediate $P = 3.25 \cdot 10^{-10}$, intermediate vs. soft $P = 3.25 \cdot 10^{-12}$, and stiff vs. soft $P = 3.25 \cdot 10^{-15}$).

We then investigated the fraction of migrating platelets for the different substrate stiffnesses (Figure 4.4C). A platelet was considered migrating if its start-to-end distance was larger than its effective diameter (defined as the diameter of a circle with the same area as that of the platelet) (Gaertner et al., 2017). On stiff substrates, $87.3 \pm 3.3\%$ (median $\pm$ MAD) of all platelets migrated, compared to $18.6 \pm 4.2\%$ on intermediate and 0% on soft PDMS substrates (ANOVA + post-hoc Tukey's test, stiff vs. intermediate $P = 1.14 \cdot 10^{-5}$, intermediate vs. soft $P = 0.022$, stiff vs. soft $P = 2.97 \cdot 10^{-6}$).

Next, we investigated the straightness of platelet migration. Platelets on stiff or intermediate PDMS tend to migrate in approximately the same direction as their initial direction of movement (similar to platelets migrating on glass (Seifert et al., 2021)), while platelets on soft PDMS showed only small, random movements in all directions, which is mostly due to measurement noise (Figure 4.6A). The straightness of migration (defined as the ratio of the start-to-end

distance to the accumulated distance, see Figure 4.6B) on stiff (0.72 ± 0.14) and intermediate (0.62 ± 0.14) PDMS was not significantly different ($P = 0.054$) (Figure 4.6C), indicating that the straightness of the migration is not influenced by the PDMS substrate stiffness.

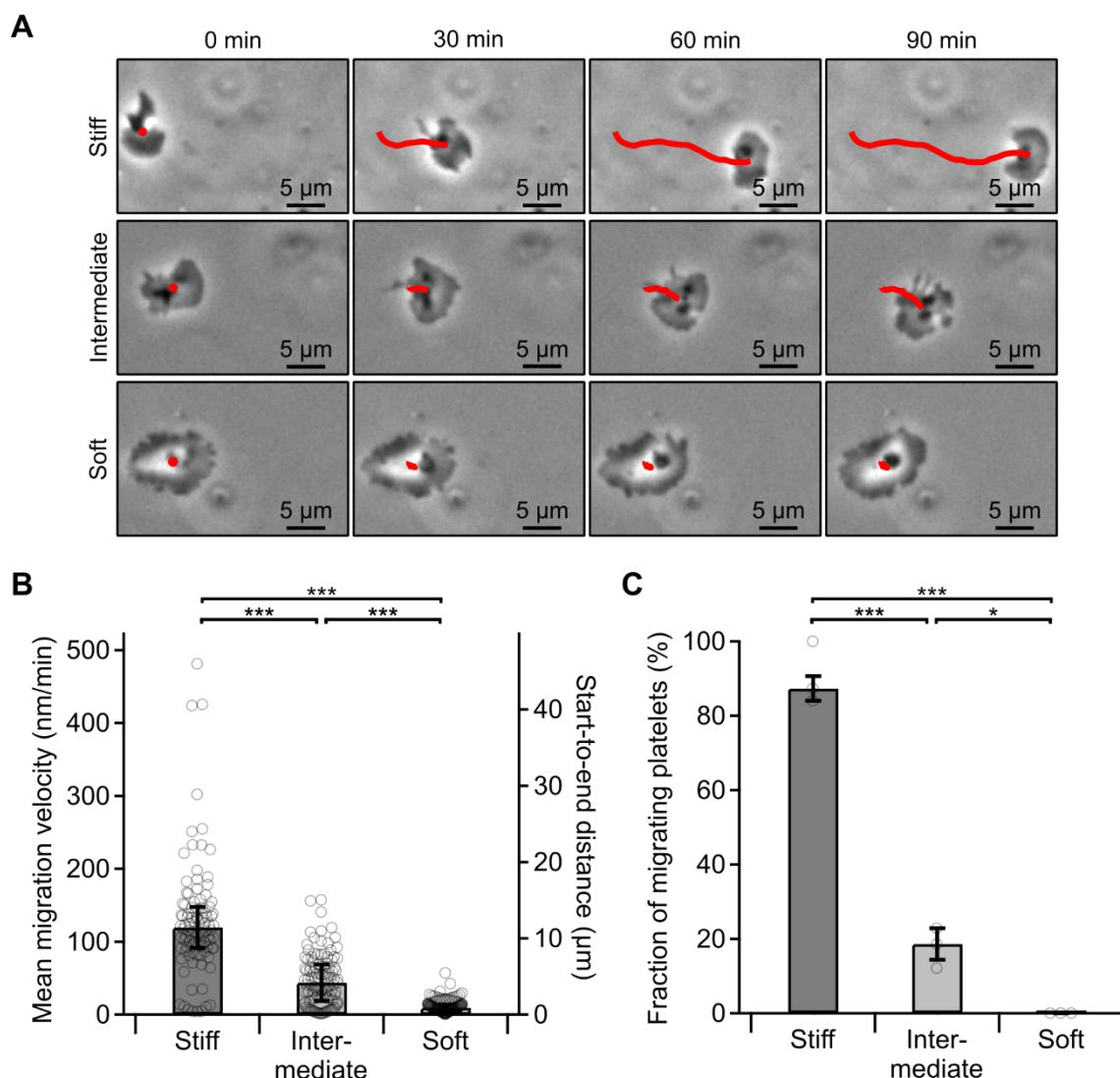


Figure 4.4: Platelet migration velocity and fraction of migrating platelets depend on substrate stiffness. (A) Time sequence of phase-contrast light microscopy images of platelets migrating on either stiff (Young's modulus approx. $E = 2$ MPa, top), intermediate (approx. 100 kPa, middle), or soft (approx. 5 kPa, bottom) PDMS substrates. The red trace indicates the movement of the platelet center over time. The full image sequence, which contains one image every 90 s, can be found in the Supplemental Information. (B) Mean migration velocity (left axis) and start-to-end distance (right axis) and (C) fraction of migrating platelets for the different PDMS substrate stiffnesses. Bar plots show median $\pm$ MAD, data points represent individual platelets (98, 111, and 126 for stiff, intermediate, and soft PDMS, respectively) from 3 independent experiments pooled together (B+E) or median values for each individual experiment (C). A donor-by-donor analysis for panel B is provided in Figure 4.5. Scale bar: 5 μ m. * for $P < 0.05$, *** for $P < 0.001$ for Dunn-Holland-Wolfe test (B) or Tukey test (C).

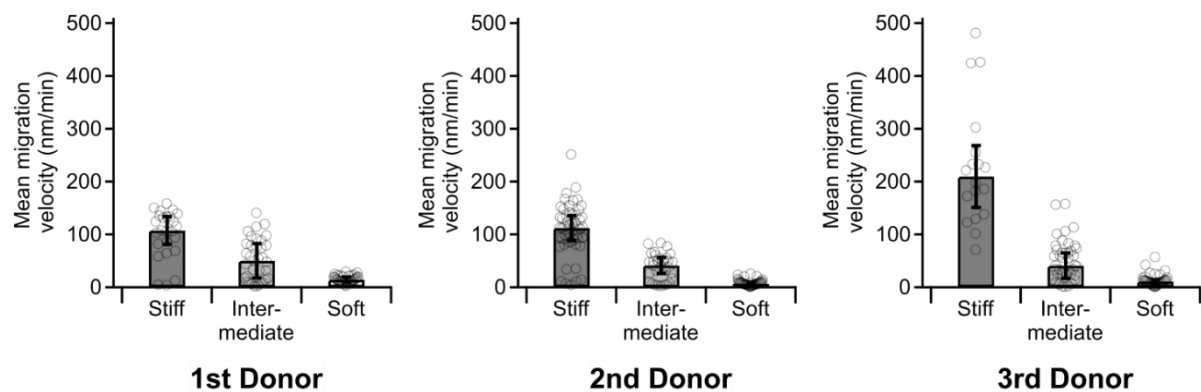


Figure 4.5: Analysis of individual donors for mean migration velocity (Figure 4.4). Bar plots show median $\pm$ MAD, data points show individual platelets from each donor (number of platelets from stiff to soft: 1st donor: 25, 35, 36; 2nd donor: 55, 33, 50; 3rd donor: 18, 43, 40).

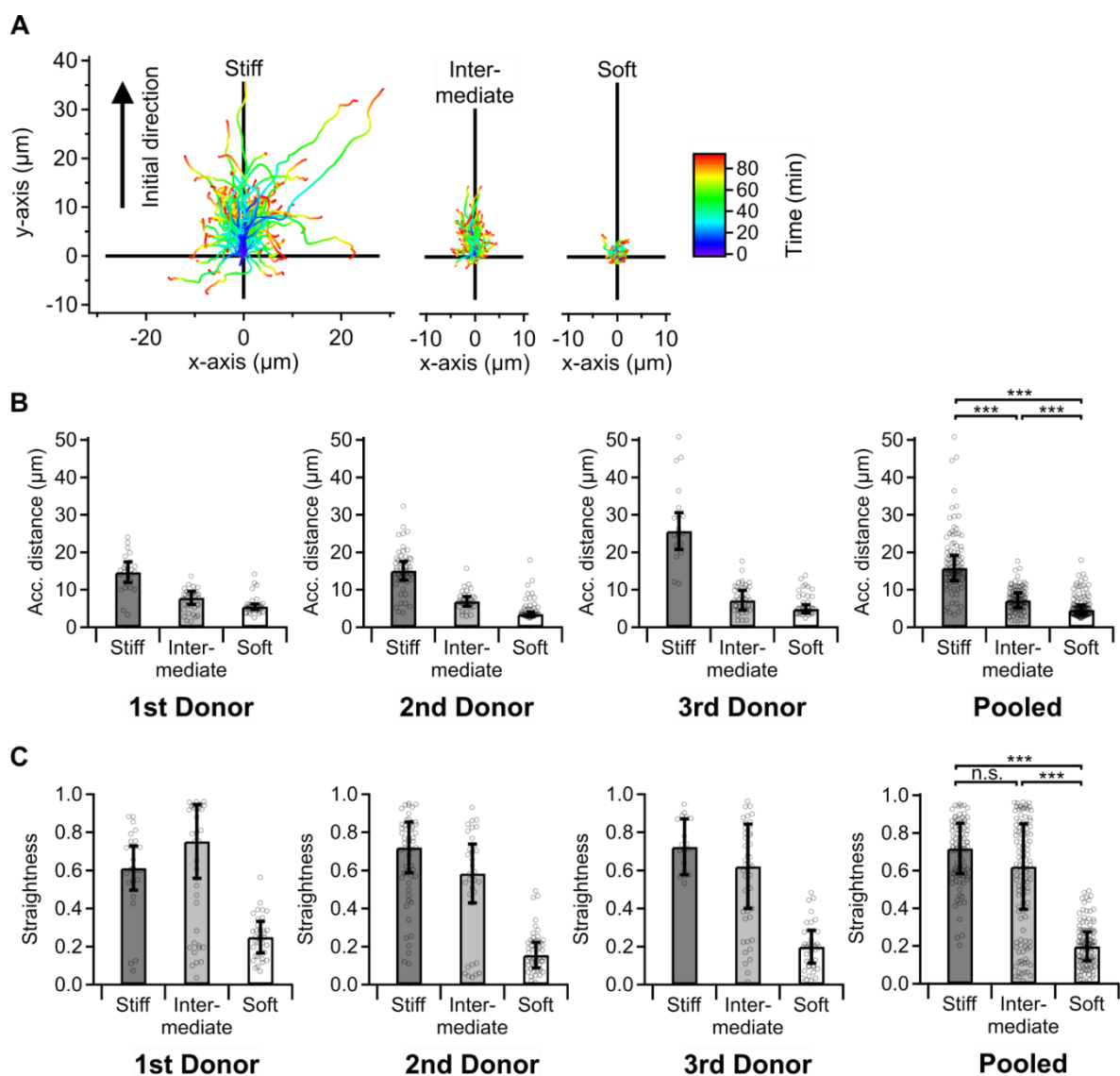


Figure 4.6: The straightness of platelet migration movement does not differ between stiff and intermediate PDMS. (A) Migration tracks of individual migrating platelets on stiff, intermediate, and soft PDMS. The initial direction of migration was aligned along the y-axis. (B) Accumulated distance and (C) straightness (ratio of the start- to end-point distance) for individual donors and pooled data.

to-end distance to the accumulated distance) of the migrated tracks (donor-by-donor and pooled). A straightness of 1 indicates a perfectly straight movement. Bar plots show median $\pm$ MAD, data points represent individual platelets (98, 111, and 126 individual platelets for stiff, intermediate, and soft PDMS, respectively). Scale bar: 5 μ m. * for $P < 0.05$, *** for $P < 0.001$ for Dunn-Holland-Wolfe test (B,C).

We also tested whether platelets preferentially move towards regions of higher or lower stiffness by preparing PDMS substrates with a sharp boundary between soft and stiff PDMS, thereby creating a high local stiffness gradient (see Materials and Methods and Figure 4.7A). We then imaged platelets adhering, spreading, and moving on the boundary between the stiff and the soft PDMS (Figure 4.7B) and found that these platelets moved towards the side with the soft PDMS (Figure 4.7C).

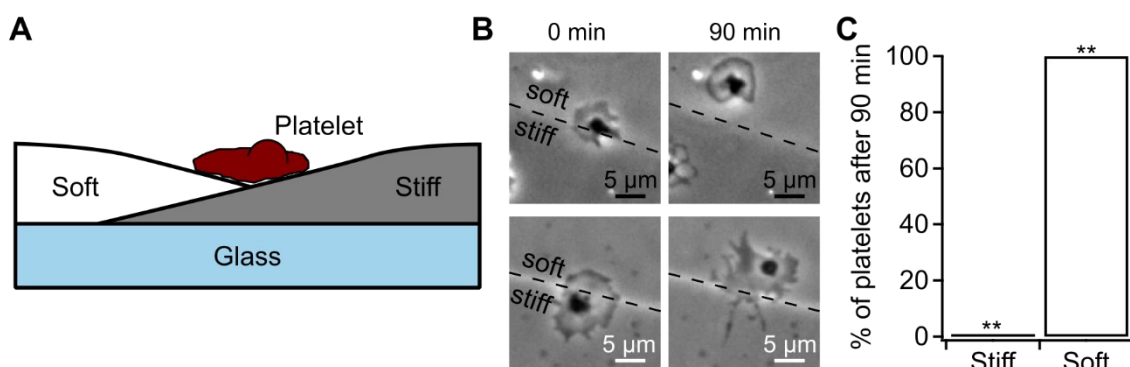


Figure 4.7: Platelets tend to move towards soft PDMS. (A) Platelets moved on PDMS with a sharp gradient in stiffness prepared by first polymerizing a wedge of stiff PDMS and then a wedge of soft PDMS on top. (B) Representative phase contrast images of platelets at the beginning of the measurement (0 min, left) and after 90 min (right). Initially, the platelets were located across the boundary between the stiff and soft PDMS (dashed lines). (C) Percentage of platelets that had moved towards the side with either stiff or soft PDMS at the end of the measurement, indicating that platelets preferably moved towards soft PDMS. A platelet was counted if it initially covered each side with at least 20% of its area and if the center of the area moved by at least 2 μ m perpendicularly to the boundary towards one side. Bar plots show 8 platelets from 4 independent experiments. ** for $P < 0.01$ for two-sided binomial test against probability 0.5 ($P = 0.0078$).

4.3.2 SICM reveals a difference in three-dimensional platelet shape

We previously showed using SICM that migrating platelets have a distinct three-dimensional, anisotropic shape; for example, high areas such as the pseudo-nucleus are shifted towards the trailing edge, while the shape of non-migrating platelets is isotropic (Seifert et al., 2022). To investigate whether the three-dimensional shape of migrating platelets is affected by substrate stiffness, platelets were fixed and imaged using SICM (Figure 4.8; a donor-wise breakdown is shown in Figure 4.9). Platelets migrating on stiff PDMS (Figure 4.8A, top row) showed the typical polarized, anisotropic (“half-moon”) shape of migrating platelets (Gaertner et al., 2017), while platelets on soft PDMS (Figure 4.8A, bottom row) were round (isotropic), like non-migrating platelets on stiff PDMS (Figure 4.2). To quantify the degree of three-dimensional polarization, we measured the relative shape anisotropy, defined as the distance from the center of volume to the center of area of the platelet divided by the effective radius of the platelet area (see Methods). The relative shape anisotropy was significantly larger for platelets migrating on stiff (Figure 4.8B, 0.096 ± 0.032) than for platelets on soft PDMS (0.050

± 0.017 , $P = 8.2 \cdot 10^{-5}$). Surprisingly, platelets migrating on stiff PDMS had a significantly larger volume (Figure 4.8C, 11.9 ± 2.8 fL) compared to those on soft PDMS (8.5 ± 1.7 fL, $P = 0.0034$).

A varying degree of polarization of migrating platelets was also reflected in their two-dimensional shape: platelets migrating on stiff PDMS had a high aspect ratio (Figure 4.8D, 1.3 ± 0.14) and a low circularity (Figure 4.8E, 0.42 ± 0.15), consistent with previous observations of platelet migration on glass (Gaertner et al., 2017; Seifert et al., 2022). In contrast, platelets on soft PDMS had a significantly lower aspect ratio (Figure 4.8D, 1.1 ± 0.06) and higher circularity (Figure 4.8E, 0.65 ± 0.14 , $P = 4.9 \cdot 10^{-4}$ and $7.4 \cdot 10^{-4}$, respectively). Interestingly, the platelet area did not differ between PDMS substrates (Figure 4.10, $P = 0.45$).

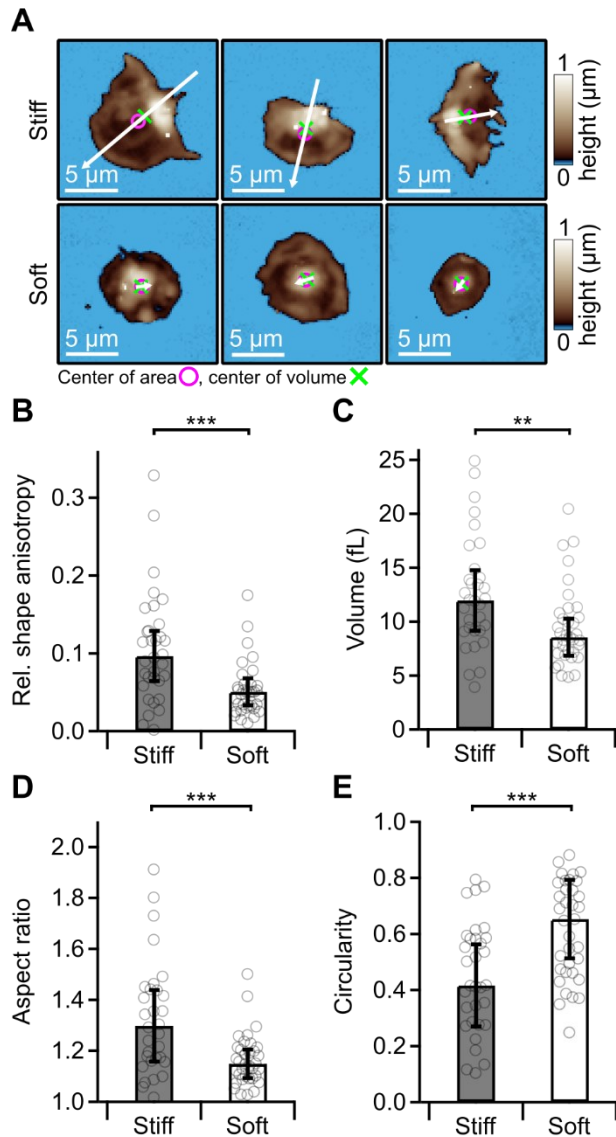


Figure 4.8: Platelets on soft PDMS substrates do not display the typical asymmetric migration shape. (A) SICM topography images showing representative platelets which were fixed during migration on either stiff (top) or on soft (bottom) PDMS substrates. The distance between the center of area (marked with a purple circle O) and the center of volume (marked with a green cross X) defines the platelet shape anisotropy, illustrated by white arrows (enlarged 10x for visibility). (B) Relative shape anisotropy, (C) volume, (D) aspect ratio, and (E) circularity of migrating platelets measured by SICM. Bar plots show median $\pm$ MAD, data points show individual platelets from 3 independent experiments pooled together (32 and 35 individual platelets for stiff and soft PDMS, respectively). A

donor-by-donor analysis for panels B-E is provided in Figure 4.9. n.s. for $P > 0.05$, *** for $P < 0.001$ for Mann-Whitney-U test.

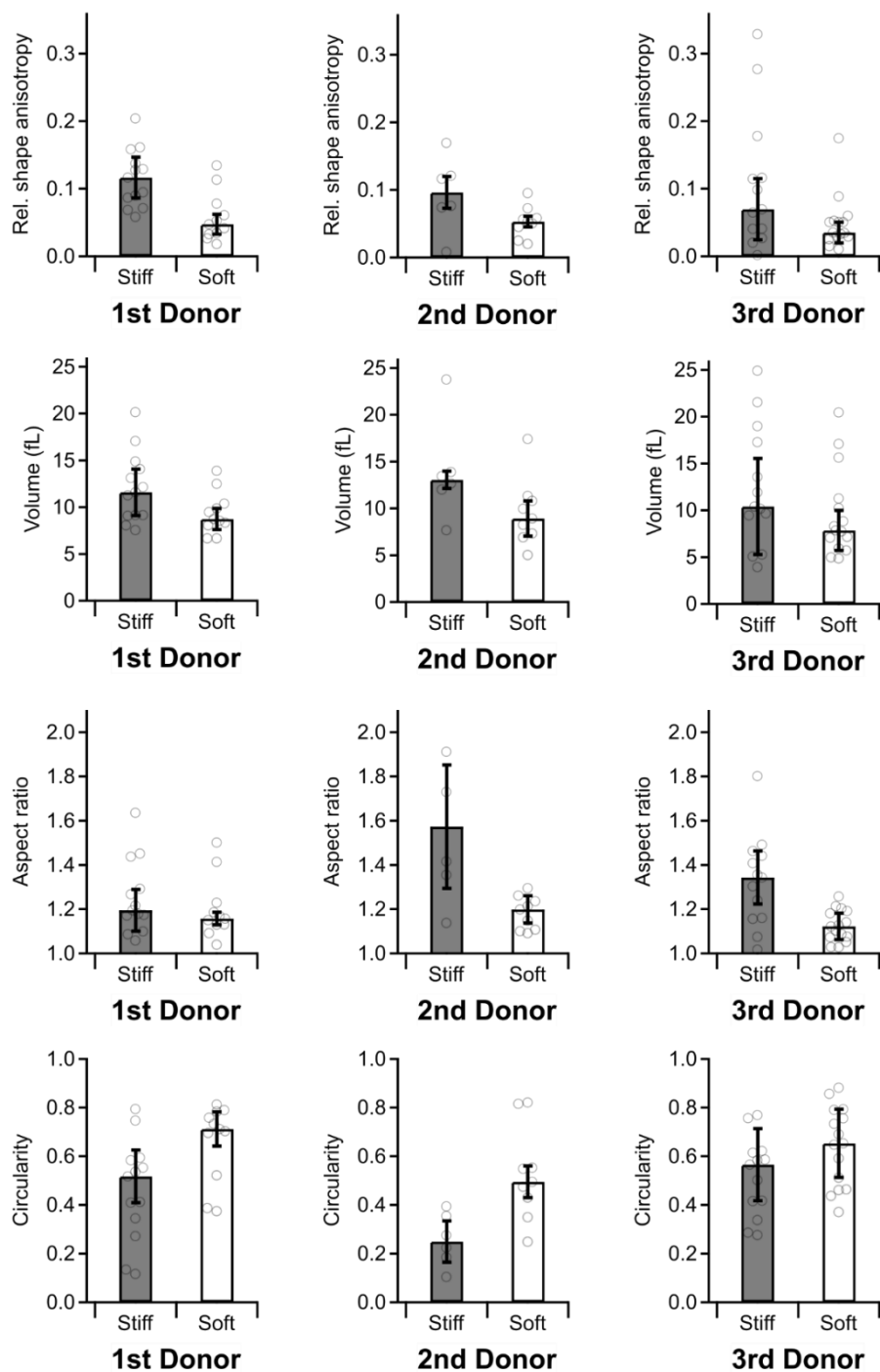


Figure 4.9: Analysis of individual donors for platelet shape (Figure 4.8B-E). Bar plots show median $\pm$ MAD, data points show individual platelets from each donor (number of platelets from stiff to soft: 1st donor: 13, 11; 2nd donor: 6, 9; 3rd donor: 13, 15).

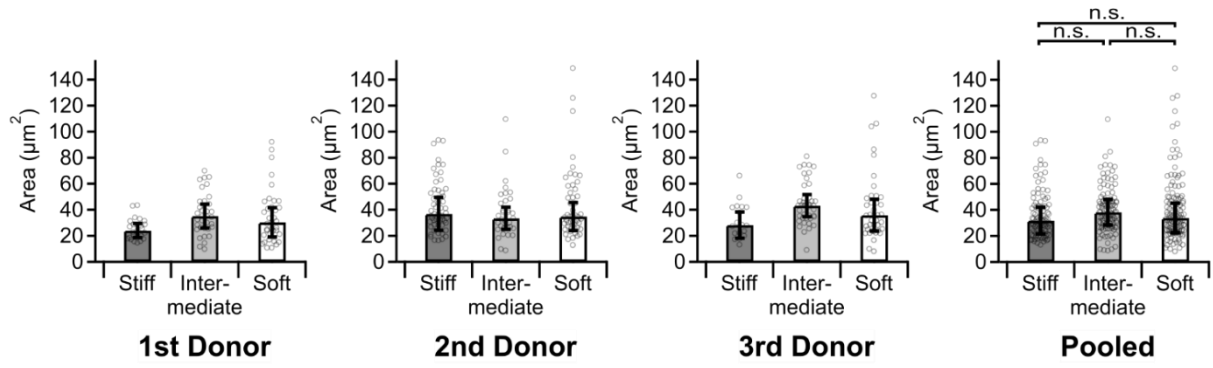


Figure 4.10: Platelet spreading area does not depend on PDMS stiffness. Bar plots show median $\pm$ MAD (donor-by-donor and pooled), data points represent individual platelets (98, 111, and 126 individual platelets for stiff, intermediate, and soft PDMS, respectively). n.s. for $P > 0.05$ for Mann-Whitney-U test.

4.3.3 Platelets migrating on soft PDMS substrates create a smaller fibrinogen density gradient

Removal of fibrinogen from the substrate is necessary for haptotactic platelet migration (Sarkar et al., 2020). We therefore used fluorophore-labeled fibrinogen to investigate the removal of fibrinogen by platelets on different PDMS substrates (Figure 4.11A). Platelets on stiff and intermediate PDMS showed fibrinogen removal (Figure 4.11A, left and center image, dark tracks), essentially “vacuuming” the fibrinogen coating as previously reported (Gaertner et al., 2017). On soft PDMS, platelets showed no tracks of removed fibrinogen, except for a small ring of slightly darker intensity at the platelet edges (Figure 4.11A, right image).

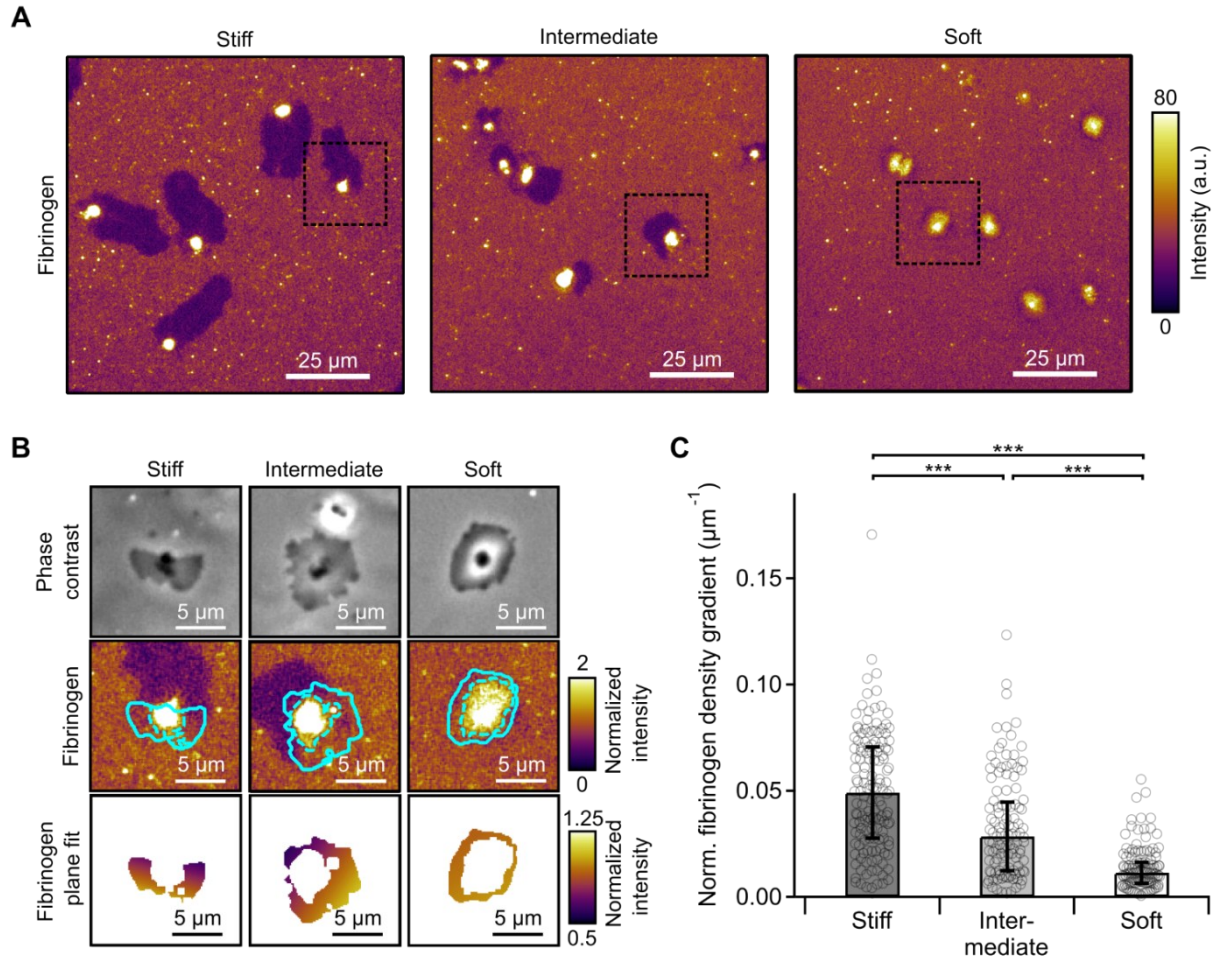


Figure 4.11: Platelets do not create a fibrinogen density gradient on soft PDMS substrates. (A) Fluorescence images of fibrinogen removal by platelets migrating on PDMS substrates with different stiffnesses. Dashed boxes indicate the platelets shown in (B). (B) Phase contrast (top row) and fibrinogen fluorescence (middle row) images of migrating platelets as well as the plane fit of the fibrinogen fluorescence under the platelets (bottom row). The solid cyan lines indicate the platelet contours, while the dashed cyan lines indicate areas excluded from the plane fit. (C) Normalized fibrinogen density gradient under platelets on stiff, intermediate, and soft PDMS substrates. Bar plots show median $\pm$ MAD, data points show individual platelets from 3 independent experiments pooled together (140, 114, and 119 individual platelets for stiff, intermediate, and soft PDMS, respectively). A donor-by-donor analysis for panel C is provided in Figure 4.12. n.s. for $P > 0.05$, *** for $P < 0.001$ for Dunn-Holland-Wolfe-Test.

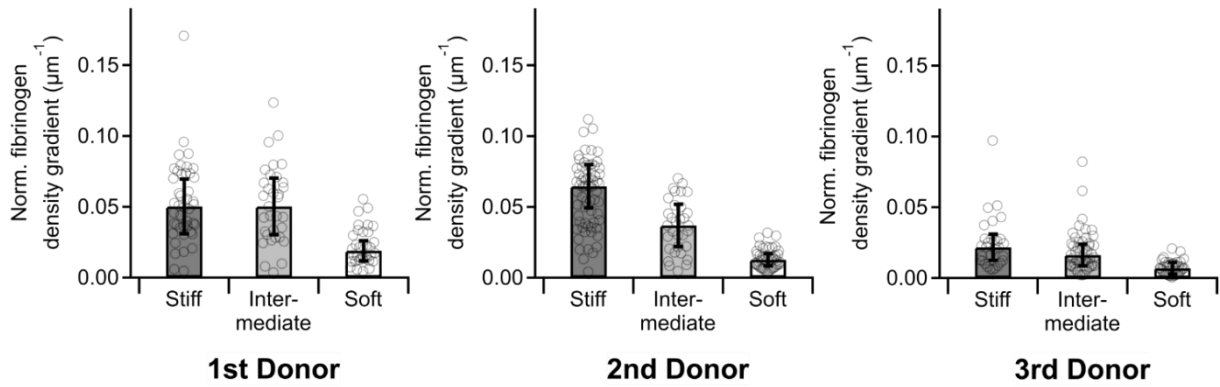


Figure 4.12: Analysis of individual donors for normalized fibrinogen density gradient (Figure 4.11C). Bar plots show median $\pm$ MAD, data points show individual platelets from each donor (number of platelets from stiff to soft: 1st donor: 44, 33, 34; 2nd donor: 66, 32, 48; 3rd donor: 30, 49, 37).

We quantified the platelet-generated fibrinogen density gradient under each single platelet (Figure 4.11B, top and center rows) using two-dimensional plane fitting (see Methods for details) (Figure 4.11B, bottom row). Platelets migrating on stiff and intermediate PDMS showed a higher normalized fibrinogen density gradient (Figure 4.11B, bottom row, left and center images) than platelets on soft PDMS (Figure 4.11B, bottom row, right image). On average, platelets on stiff and intermediate PDMS exhibited a significantly higher normalized fibrinogen density gradient (Figure 4.11C, 0.049 ± 0.021 and 0.028 ± 0.016 , respectively, $P = 3.16 \cdot 10^{-5}$) than those on soft PDMS (0.010 ± 0.0048 , stiff vs. soft $P = 3.26 \cdot 10^{-15}$ and intermediate vs. soft $P = 1.36 \cdot 10^{-10}$, respectively). A donor-wise breakdown is provided in Figure 4.12).

4.4 Discussion

Platelets and their biochemical interactions have been studied extensively (Valone et al., 1974; Lowenhaupt, 1978; Czapiga et al., 2005; Kraemer et al., 2010; Schmidt et al., 2011; Schmidt et al., 2012), but biomechanical interactions have only recently become an aspect of platelet research (Qiu et al., 2014; Kee et al., 2015; Hansen et al., 2018). To our knowledge, *in vitro* platelet migration experiments so far have been performed on glass or plastic substrates (Gaertner et al., 2017; Seifert et al., 2021; Seifert et al., 2022), which have a stiffness in the gigapascal range - much higher than that of most tissues in the body (Butcher et al., 2009). Hydrogel substrates with tunable stiffness are often used when studying the mechanobiology of cells (Tse & Engler, 2010). Hydrogel substrate fabrication and functionalization is technically demanding and heavily reliant on the user's expertise, leading to variations in stiffness both locally and across different batches (Protick et al., 2022). PDMS substrates are increasingly used as an alternative to hydrogel substrates (Daliri et al., 2021). We chose PDMS substrates because they are easy to reproduce, store, and use (Yoshie et al., 2018). The Young's modulus of PDMS is easily tunable (Palchesko et al., 2012; Rodriguez et al., 2019) and its ionic conductivity is much lower than that of hydrogels, which is beneficial for SICM

measurements. PDMS substrates with different stiffnesses have been used in mechanobiology to study, for example, matrix-rigidity-dependent cell polarization (Prager-Khoutorsky et al., 2011), neurite extension and muscle differentiation (Palchesko et al., 2012), cell adhesion and contraction (Yang et al., 2022), and cell traction forces (Beussman et al., 2023). Other studies have focused on the mechanosensing aspect of platelet adhesion, revealing that higher substrate stiffness leads to higher platelet activation, for example in terms of integrin $\alpha\text{IIb}\beta 3$ activation (Qiu et al., 2014). We therefore hypothesized that substrate stiffness also alters platelet migration. Our results demonstrate for the first time a link between platelet migration behavior and substrate stiffness.

Previous reports of platelets migrating on glass found average migration velocities of around 100 nm/min (Gaertner et al., 2017; Seifert et al., 2021; Seifert et al., 2022), which is similar to our result for platelets migrating on stiff PDMS (Figure 4.4B). It is therefore not likely that there is a stiffness “optimal” for maximum migration velocity above that of stiff PDMS. However, we cannot exclude that there might be such an optimal stiffness between the stiff and intermediate PDMS; further studies will be needed to fully investigate the interaction of platelets with the vast stiffness range of tissues found in the body (Guimarães et al., 2020).

Cell migration is also guided by the substrate stiffness - a process called durotaxis (Lo et al., 2000; Sunyer & Treppe, 2020). Most cell types exhibit positive durotaxis (migration towards stiffer substrates) while only a few cell types (e.g., U-251MG glioma cells (Isomursu et al., 2022; Ji & Huang, 2023), B16 melanoma cells (Huang et al., 2022), and neuronal axons (Flanagan et al., 2002; Moore & Sheetz, 2011)) exhibit negative durotaxis (migration towards softer substrates). However, it is not currently known whether platelet migration is also guided by durotaxis. Our result using substrates with a high local stiffness gradient that all platelets moved onto the side with the soft PDMS (Figure 4.7) may indicate that platelets undergo negative durotaxis. However, there are some limitations to this interpretation: First, since platelet migration is a haptotactic process, it is difficult to separate the effects of haptotaxis and durotaxis on platelet migration. Second, it is likely that the platelets in this experiment stopped moving on the soft PDMS at a certain distance from the boundary, because the effective stiffness of the substrate decreases with an increasing distance from the boundary (as the contribution of the underlying stiff PDMS layer to the effective stiffness decreases). A more comprehensive study is required to provide a clearer picture of the potential effects of durotaxis on platelet migration.

We showed that platelets migrating on stiff PDMS substrates have a larger average volume compared to (non-migrating) platelets on soft PDMS substrates (Figure 4.8). This larger volume might be due to the uptake of removed fibrinogen into the pseudo-nucleus region, increasing platelet height (Gaertner et al., 2017). A larger volume might facilitate platelet

migration, similar to a process found in migrating neutrophils (Nagy et al., 2024). It might be possible that the observed difference in volume is caused by platelet mechanosensing rather than by platelet migration - two effects we cannot separate with our study. However, as previous reports for other cell types showed a decrease in cell volume with increasing substrate stiffness (Guo et al., 2017; Perez Gonzalez et al., 2018; Xie et al., 2018; Janmey et al., 2019; Wang et al., 2020), it seems likely that the observed increase in platelet volume is caused by migration and not by mechanosensing. A future investigation of platelet volume by decoupling migration and mechanosensing would be valuable.

For a platelet to migrate, it must be able to produce high enough forces to reliably remove and uptake the substrate-bound fibrinogen from the substrate via actomyosin contraction (Gaertner et al., 2017; Sarkar et al., 2020). Based on these and our findings, we propose the following working model (Figure 4.13): If the substrate under the fibrinogen is stiff and thus non-compliant, a small pulling distance is sufficient to produce enough force to detach fibrinogen from the substrate, and thus the platelet can generate a fibrinogen density gradient and polarize (Figure 4.13A), both necessary conditions for migration (Lauffenburger & Horwitz, 1996; Gaertner et al., 2017; Sarkar et al., 2020). If, however, the substrate is soft and thus compliant, a larger pulling distance and thus mechanical work is needed to reach the force necessary to detach fibrinogen from the substrate. Therefore, the platelet cannot detach the fibrinogen from the substrate and consequently does not self-generate a fibrinogen density gradient, does not polarize, and does not migrate (Figure 4.13B). In accordance with this model, Kaiser *et al.* (Kaiser et al., 2023) noted that a platelet is immobilized if the force per fibrinogen molecule cannot overcome the threshold necessary to remove that molecule from the substrate. This process requires the substrate to be coated with fibrinogen rather than other procoagulant coatings such as collagen or von Willebrandt factor, because platelet migration is specifically dependent on fibrinogen and $\alpha\text{IIb}\beta\text{3}$ activation (Gaertner et al., 2017). Migrating platelets encountering collagen stop migrating, turn procoagulant, and start “ballooning” (Kaiser et al., 2023).

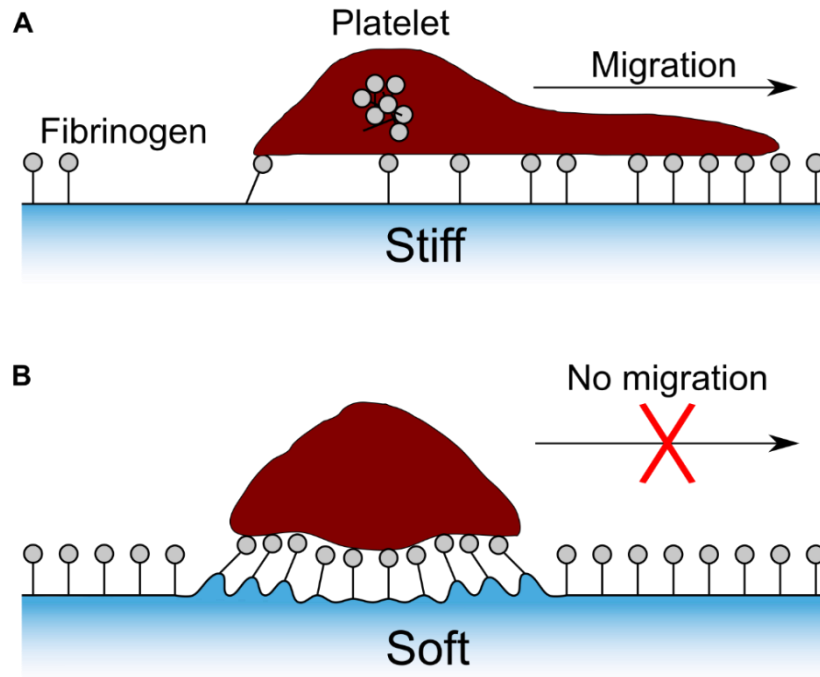


Figure 4.13: Working model explaining why platelets do not migrate on soft PDMS substrates. (A) Platelets seeded on stiff PDMS substrates self-generate a fibrinogen density gradient by removing fibrinogen from the substrate and undergo shape change, thereby enabling haptotactic migration. (B) Platelets seeded on soft PDMS substrates are not able to remove the fibrinogen from the PDMS due to the compliance of the soft PDMS, thus they are not creating the fibrinogen density gradient and the shape change necessary for haptotactic migration.

The stiffness of biological tissue varies greatly, ranging from a few pascals to many megapascals (Alonso & Goldmann, 2003; Kuznetsova et al., 2007; Levental et al., 2007; Arda et al., 2011; Garoffolo & Pesce, 2019; Guimarães et al., 2020). To hypothesize about the physiological function of the different migration behaviors, we used the “MechanoBase” database (Xiong et al., 2024) to determine typical stiffnesses that platelets might encounter *in vivo*. The inside of a blood vessel is layered by endothelial cells and tissue that show a rather low mean stiffness (Young’s modulus) (1-7 kPa) (Mathur et al., 2001; Lee, 2011; Guo et al., 2012), similar to our soft PDMS substrate. In contrast, other cell types and structures in the healthy arterial wall are much stiffer (10-40 kPa) (Asgari et al., 2022; Hooglugt et al., 2022; Petit et al., 2022). Atherosclerotic aortas even showed 16-60 kPa for the fibrous cap with 100 kPa or higher for the calcified zone of atherosclerotic plaques (Tracqui et al., 2011; Rezvani-Sharif et al., 2019).

In cases of acute and chronic inflammation or fibrosis, the stiffness of blood vessels and the surrounding tissue is increased (Mattace-Raso et al., 2004; Kusche-Vihrog et al., 2011; Park & Lakatta, 2012; Wells, 2013; Mozos et al., 2017; Yang & Plotnikov, 2021; Liu et al., 2022, 2024 #395). A stiffness increase is observed, for example, for fibrotic liver (healthy: 1 kPa, fibrotic: 10 kPa (Janmey et al., 2019) and lung (healthy: 2 kPa, fibrotic: 17 kPa (Booth et al.,

2012). Another example is the stiffening of cardiomyocytes caused by fibrosis after a myocardial infarct, where the stiffness of the myocardium increases from around 10 kPa (Engler et al., 2004) to between 35 and 70 kPa (Berry et al., 2006; Engler et al., 2008). Multiple studies suggest that such increases in stiffness facilitates immune cell activity via mechanosensing (Yang & Plotnikov, 2021; Du et al., 2023; Saha et al., 2023), for example, by increased macrophage phagocytosis (Patel et al., 2012; Sridharan et al., 2019), increased dendritic cell metabolism (Chakraborty et al., 2021), T-cell activation (Saitakis et al., 2017; Meng et al., 2020), or by activating immune cells by mechanical stimuli (Solis et al., 2019). Furthermore, it is known that platelet migration is involved in the early immune response. For example, migrating platelets collect bacteria (Gaertner et al., 2017) and prevent micro-bleedings from immune cell lesions in inflammation (Nicolai et al., 2020). Given these previous findings and our current results, we hypothesize that the increased stiffness of inflamed tissue facilitates haptotactic platelet migration, while soft, healthy tissue impedes haptotactic platelet migration. Platelet migration might therefore be a component of the immune response to changes in tissue stiffness, adding platelet migration to the field of “mechanoimmunology” (Du et al., 2023).

Our results have several biomedical implications. For instance, medical implants such as vascular heart stents need a high biocompatibility (Xiaohong, 2013). Much effort has been put into modifying the surface properties of stents, which are usually metal-based, to reduce inflammation, bacterial infection, thrombosis, and implant rejection (Jaffer et al., 2015; Small et al., 2018; Benčina et al., 2021). Modification of the implant surface with a soft coating that inhibits platelet migration may be disadvantageous, particularly with regard to bacterial infection, as migrating platelets collect bacteria from the surface during migration and a reduced platelet migration lowered the number of platelet-bundled bacteria and platelet-neutrophil interaction time in mice (Gaertner et al., 2017). On the other hand, migrating platelets can become procoagulant (Kaiser et al., 2023), which should be avoided to reduce the risk of thrombosis. In conclusion, the influence of substrate biomechanical properties on platelet mechanosensing and migration in biomedical devices is an important aspect to consider in the future.

5 Investigation of cGMP influence on thrombi using SICM

5.1 Introduction

Hemostasis, i.e. the platelet-facilitated formation of blood clots or thrombi at the site of injury, is necessary for proper wound healing. In certain adverse cardiovascular events, however, such as acute coronary syndrome or ischemic stroke following atherosclerosis, antiplatelet drugs such as aspirin or clopidogrel are administered to inhibit excessive thrombosis threatening to occlude blood vessels (Awtry & Loscalzo, 2000; Plosker & Lyseng-Williamson, 2007; Udel et al., 2015; Stanger et al., 2023). Current antiplatelet drugs inhibit thrombosis by severely disrupting platelet activity, potentially leading to internal bleeding with prolonged use (Godier et al., 2020; Swan et al., 2020; Geisler et al., 2022). However, as thrombus size is correlated with disease severity (Parr et al., 2011; Joundi & Menon, 2021), a reduction in thrombus size, while maintaining the native hemostasis function of platelets is greatly desired.

Therefore, endogenous mechanisms inhibiting thrombosis are investigated as a promising alternative. Even in a healthy individual, platelet activation and aggregation have to be tightly controlled to prevent the spontaneous formation of thrombi and vessel occlusion. One of the endogenous mechanisms involves nitric oxide (NO) (Makhoul et al., 2018; Gambaryan, 2022)(Figure 5.1). NO released by the endothelium activates the NO-sensitive soluble guanylyl cyclase (NO-GC) in platelets (Koesling et al., 1991; Garbers et al., 1994; Dangel et al., 2010; Koesling et al., 2016; Hofmann, 2020). This NO-GC activation increases the cyclic guanosine monophosphate (cGMP) levels in platelets and subsequently increases activity of the protein kinase G (PKG), ultimately resulting in a decreased thrombotic activity without fully disrupting thrombosis (Denninger & Marletta, 1999; Emerson et al., 1999; Smolenski, 2012). This NO-GC-cGMP-PKG cascade in platelets is shear-dependent, meaning platelets at thrombus surface are more likely to be inhibited, thus halting thrombus growth (Wen et al., 2017; Wen et al., 2018). While this effect of the NO-GC-cGMP-PKG cascade on the shape and biomechanics of individual platelets has been recently investigated (Balmes et al., 2024), the effect has not yet been investigated at the level of individual thrombi.

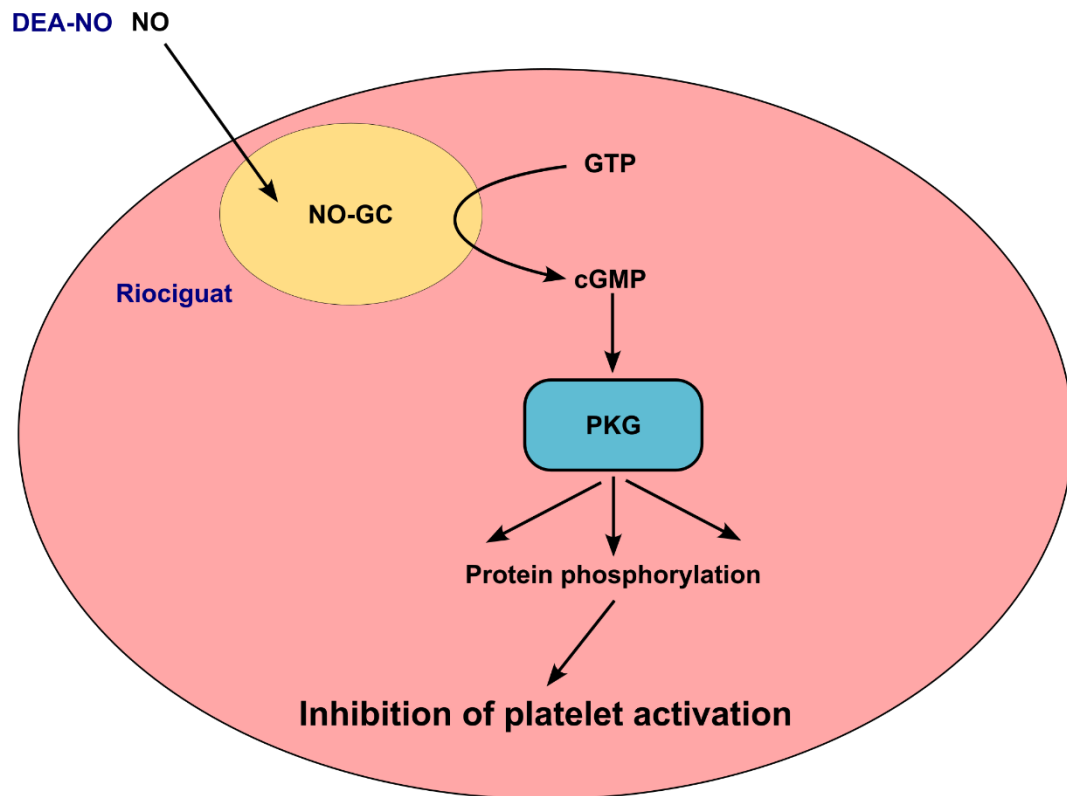


Figure 5.1: Inhibition of platelet activation by NO-GC-cGMP-PKG cascade. Nitric oxide (NO) released by the endothelium translocates into platelet cytosol, reacting with NO-sensitive soluble guanylyl cyclase (NO-GC). NO-GC in turn converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). Increased cGMP levels activate protein kinase G (PKG), phosphorylating a number of effector proteins, ultimately leading to inhibition of platelet activation. Riociguat is stimulating NO-GC, while diethylamine nonoate (DEA-NO) can be used as an alternative NO-donor.

Over the years, several drugs targeting this signaling cascade have been developed (Sandner et al., 2024). The drug riociguat (BAY 63-2521), an NO-GC stimulator, increases the sensitivity of NO-GC to NO and stimulates NO-GC at the same time, independently of NO (Benza et al., 2017; Klinger et al., 2021), thus increasing cGMP-levels and decreasing thrombotic activity. Diethylamine nonoate (DEA-NO) is a NO-donor (Keefer, 2003, 2011), that mimics the effect of NO released by the endothelium (Moncada & Higgs, 2006; Russo et al., 2023). Both NO-GC stimulant riociguat and NO-donor DEA-NO reduce platelet activity (Makhoul et al., 2018), but their influence on the three-dimensional thrombus shape is less clear. In the following chapter SICM was used to explore the effect of these drugs on thrombus shape parameters like area, volume, height, aspect ratio or surface roughness (shape parameters quantification explained in chapter 2.4).

5.2 Material & Methods

5.2.1 Preparation of thrombi samples made from human blood

Thrombi samples were procured by Michael Böttcher, Liubov Unger, and Daniel Pinto Quintero from the Interfaculty Institute of Biochemistry (IFIB) in Tübingen in the following manner:

Rectangular coverslips were coated with 100 μ l of 200 μ g/ml collagen type I and allowed to rest over night at 4°C. Human blood drawn from healthy volunteers was mixed with platelet Tyrode buffer (10 mM HEPES, 137 mM NaCl, 12 mM NaHCO₃, 2.7 mM KCl, 5.5 mM D-glucose, pH 7.4; supplemented with 0.1% bovine serum albumin; BSA) in a ratio of 2:1. Diluted blood was then preincubated with riociguat (200 μ M for chapter 3.3.1 or 200 nM for all the rest of chapter 3, respectively) and/or DEA-NO (10 μ M for all experiments) for 15 min before perfusion.

Thrombi were made by perfusing the diluted blood through a flow chamber (μ -slide I 0.2, Ibidi) covering a collagen-coated coverslip at room temperature at different shear rates, utilizing a syringe pump (B-Braun).

The thrombi were subsequently fixed using 4% paraformaldehyde for 10 min. Afterwards, coverslips with fixed thrombi were transferred into a PBS-filled petri dish and transported to the SICM set-up or stored for later experiments (storage at 4°C for 1 week max). Thrombi were then visualized using SICM.

Shear rate at the blood vessel wall ranges from 20-200 per second for veins, 300-800 per second for large arteries, 500-1600 per second for arterioles, and 800-10,000 per second for stenotic vessels (Kroll et al., 1996). Based on these values, a shear rate was used to simulate large arteries to generate thrombi in the flow chamber (500/s shear rate for chapter 5.3.1 or 1000/s shear rate for all other chapters).

5.2.2 Thrombi SICM Imaging

A custom-built SICM setup was used (described previously (Seifert et al., 2022)). Thrombus topography was recorded using SICM in hopping-mode with a pipette with an inner radius of $\approx$ 50 nm. Images were acquired with a vertical approach and retract speed of 200 μ m/s and different horizontal scan area sizes; usually, a pixel size of 200-250 x 200-250 nm² was used. First order plane flattening corrected sample tilt and offset. Only pixels exceeding a height value of at least 50 nm were counted as objects.

The area of spread platelets is usually between 20-40 μ m² (Sakurai et al., 2015; Seifert et al., 2017; Lickert et al., 2018; Paknikar et al., 2019; Seifert et al., 2021; Kempster et al., 2022). The height of a spread platelet is around 0.5-1 μ m (Rheinlaender et al., 2015; Seifert et al., 2017; Cebo et al., 2022; von Eysmond et al., 2025) and the volume of a spread platelet is around 10-12.5 fL (Krutzke et al., 2024). In order to include only thrombi and not single platelets in the analysis, only objects that exceeded all three of the following criteria were included in the analysis as thrombi:

1. An area of at least 40 μ m²,

2. a height of at least 1 μm
3. and a volume of at least 12.5 fL.

Quantification of two- and three-dimensional thrombi shape was done in IGOR Pro 9 (WaveMetrics, Lake Oswego, OR, USA), using the methods detailed in chapter 2.4.

5.2.3 Surface roughness calculation

To quantify a difference in thrombus surface roughness, the following method (shown similarly in (Simeonov, 2020)) was used (Figure 5.2):

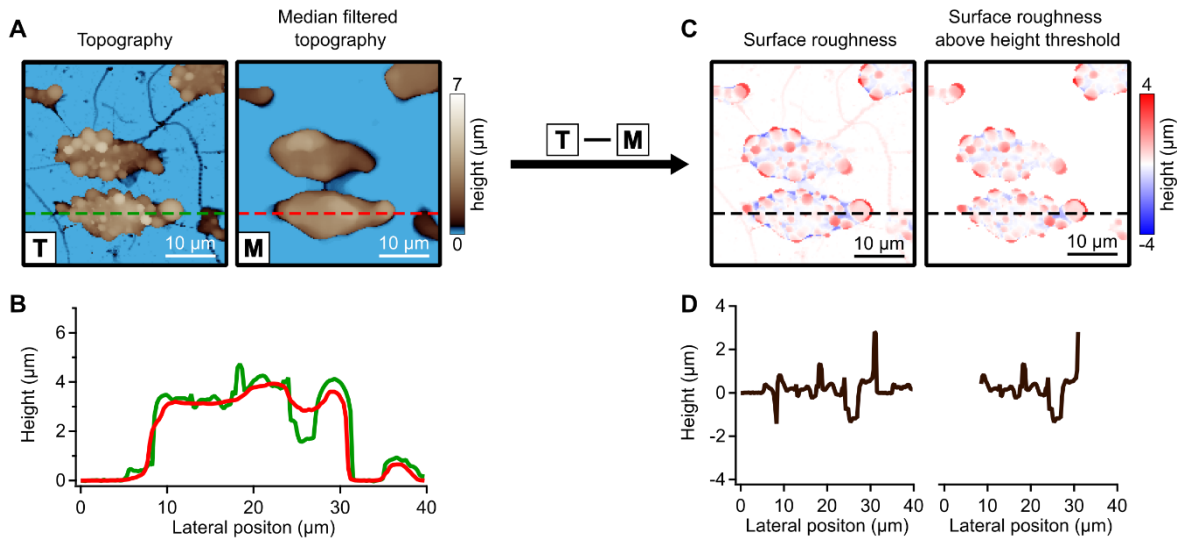


Figure 5.2: Schematic of surface roughness calculation of thrombi. (A) The thrombus topography (T) was processed using a median filter (here: $4 \times 4 \mu\text{m}^2$ or 15×15 pixels), approximating the size of surface features, to generate a smoothed version (M) representing the bulk thrombus shape. (B) Height profiles along the dashed lines in (A) showing the unfiltered topography (green) with large height variations, while the median-filtered image (red) appears smoother. (C) Subtracting the filtered image from the original yields a surface roughness image (left image), isolating local protrusions and grooves. For quantitative analysis, only features $\geq 1 \mu\text{m}$ in height were considered (right image). (D) Height profiles along the dashed black lines in (C) of surface roughness and analysis image, respectively, show height variations along the thrombus surface. Adapted with own data from (Simeonov, 2020).

First, the topography image (denoted as T in Figure 5.2A) was median filtered, i.e. a median value of all surrounding pixels within a chosen filter size ($4 \times 4 \mu\text{m}^2$ or 15×15 pixels for this image) was calculated for each of the pixels in the topography image. The filter size was chosen to be roughly the size of the surface “bubbles” (diameter of ca. $3\text{--}4 \mu\text{m}$). The median filtered topography (denoted as M in Figure 5.2A) shows the main body of the thrombus, while finer structures were removed. The unfiltered topography shows height profile with finer variations in height (green line in Figure 5.2A+B). In contrast, the median filtered topography is rather smooth (red line in Figure 5.2A+B). To obtain these height variations over the whole thrombus surface, the median-filtered topography was then subtracted from the original topography, resulting in the “surface roughness” image (Figure 5.2C, left) showing only small protrusions and grooves on the thrombus surface (Figure 5.2D, left graph). Similar as for the

other shape parameters, only surface features located at a height of 1 μm or higher in the original topography image were considered for analysis (Figure 5.2C, right).

The three-dimensional surface roughness S_a was then quantified as

$$S_a = \frac{1}{N} \sum_{i=1}^N |z_i - \bar{z}| \quad (12)$$

where N is the total number of pixels of the thrombus area and $\bar{z}$ is the arithmetic mean of the height variations. The relative surface roughness $S_{a,rel}$ was calculated by dividing S_a by the maximum height of the thrombus.

The schematic in Figure 5.2 shows only two single thrombi. To obtain a significant amount of data for thrombi surface roughness, it is beneficial to obtain as many thrombi in one SICM image as possible. However, since SICM acquires an image pixel by pixel, this comes with a tradeoff of either significantly increased time consumption or significantly lowered pixel resolution, which could cause problems with the filtering necessary to obtain a robust surface roughness. To check the robustness of the pixel resolution on thrombi surface roughness obtained from the images with a lot of thrombi (called overviews), the same thrombi were analyzed for surface roughness in the lower resolution overviews and the single higher-resolution thrombi images (called zoom-ins, where available, Figure 5.3A). Filter size was again chosen as $4 \times 4 \mu\text{m}^2$, corresponding to 15×15 pixels in the zoom-in and 3×3 pixels in the overview images.

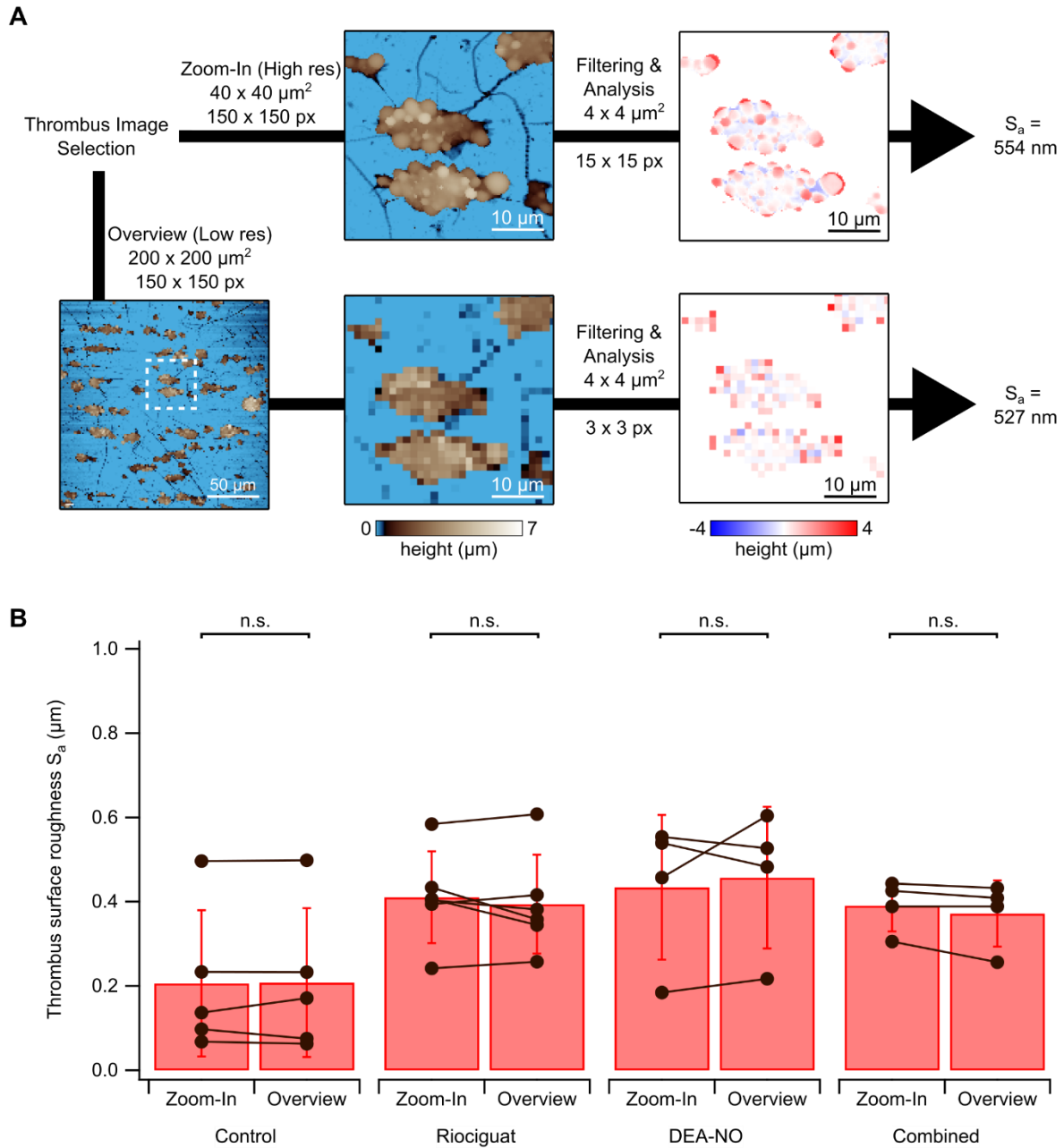


Figure 5.3: Comparison of robustness of surface roughness analysis between small and large pixel size SICM images. (A) Explanation of the procedure: To assess surface roughness analysis robustness for large overview SICM images, the same thrombi were analyzed, one in a high resolution image (Zoom-In, top path, 40 x 40 μm^2 , 150 x 150 pixels, pixel size 0.266 x 0.266 μm^2) and one in a lower resolution image (Overview, bottom path, 200 x 200 μm^2 , 150 x 150 pixels, pixel size: 1.333 x 1.333 μm^2). Both were filtered with a 4 x 4 μm^2 median filter, with corresponding filter size in pixels (15 and 3, respectively). The calculated surface roughness S_a for both is similar. (B) Comparing surface roughness values of all thrombi available in both zoom-in and overview images reveals no significant difference, regardless of treatment. Barplots show mean, error bars show standard deviation, connected data points are individual thrombi with both a high and low resolution image from 3 different experiments pooled together (n=5 for control, n=6 for riociguat, n=4 for DEA-NO and n=4 for the combined treatment). n.s. $P \geq 0.05$, Paired t-test.

The results show that there is no significant difference in calculated surface roughness S_a between the same thrombi, regardless of the pixel resolution of the image (Figure 5.3B). This is true for the control (Control Zoom-In: 206 $\pm$ 173 nm, mean $\pm$ standard deviation, Control Overview: 208 $\pm$ 176 nm, $P = 0.85$, paired t-test), riociguat (411 $\pm$ 109 nm and 394 $\pm$ 117 nm, respectively; $P = 0.40$), DEA-NO (433 $\pm$ 171 nm and 457 $\pm$ 168 nm, respectively; $P = 0.64$),

and the combined treatment (391 ± 51 nm and 371 ± 79 nm, respectively; $P = 0.17$). This means that a surface roughness analysis of thrombi using the lower-resolution overview images is robust enough; a higher-resolution zoom-in on the thrombi is not necessary.

5.2.4 Statistics

Statistical evaluation was done in Igor Pro 9 (WaveMetrics, Lake Oswego, OR, USA) using built-in functions. All data are tested using either the two-tailed Mann-Whitney-U test for two samples or the Dunn-Holland-Wolfe test for non-parametric multiple comparisons for three or more samples, with P -values below 0.05 considered significantly different (unless noted otherwise).

5.3 Results

5.3.1 The effect of riociguat on three-dimensional thrombus shape

Since NO-GC stimulant riociguat reduces platelet activity (Makhoul et al., 2018), it is likely that thrombus shape is affected, as well. Thrombi made from human platelets pre-incubated with 200 μ M riociguat or Tyrode's buffer (control) were imaged with SICM and their shape parameters quantified.

The results indicate a reduction of thrombus size by riociguat: Thrombi were seen in both control samples and samples treated with 200 μ M riociguat (Figure 5.4A). However, untreated thrombi were slightly taller than those treated with riociguat (5.00 ± 1.13 μ m vs. 4.14 ± 0.87 μ m, median $\pm$ median absolute deviation, MAD, $P = 0.033$, Wilcoxon rank test). Untreated thrombi were also larger in area (104.7 ± 42.1 μ m² vs. 57.3 ± 28.4 μ m², $P = 1.19 \cdot 10^{-5}$) and volume (0.24 ± 0.12 pL vs. 0.12 ± 0.07 pL, $P = 2.63 \cdot 10^{-4}$). No significant difference was found in the circularity of thrombi (0.16 ± 0.04 pL vs. 0.18 ± 0.06 , $P = 0.11$).

5.3.2 Influence of riociguat and DEA-NO on thrombus shape

The influence of riociguat on thrombus formation in the arterioles was investigated. Shear rate was increased to 1000/s to simulate arterioles and the riociguat concentration was adjusted to a typical blood concentration of a patient medicated with riociguat (200 nM). In addition, the influence of DEA-NO on thrombus formation alone or in combination with riociguat was studied.

Our results indicate only a small effect of therapeutic riociguat and DEA-NO on the three-dimensional shape of thrombi at a shear rate of 1000/s (Figure 5.5). There was no significant difference in the number of thrombi (Figure 5.5B) or the area covered by these thrombi (Figure 5.5C) between treatments, except for a small significant increase for number of particles with the 10 μ M DEA-NO treatment ($P = 0.0164$). Of these particles, all particles exceeding a height of 1 μ m and an area of 40 μ m² were considered for further thrombus shape analysis.

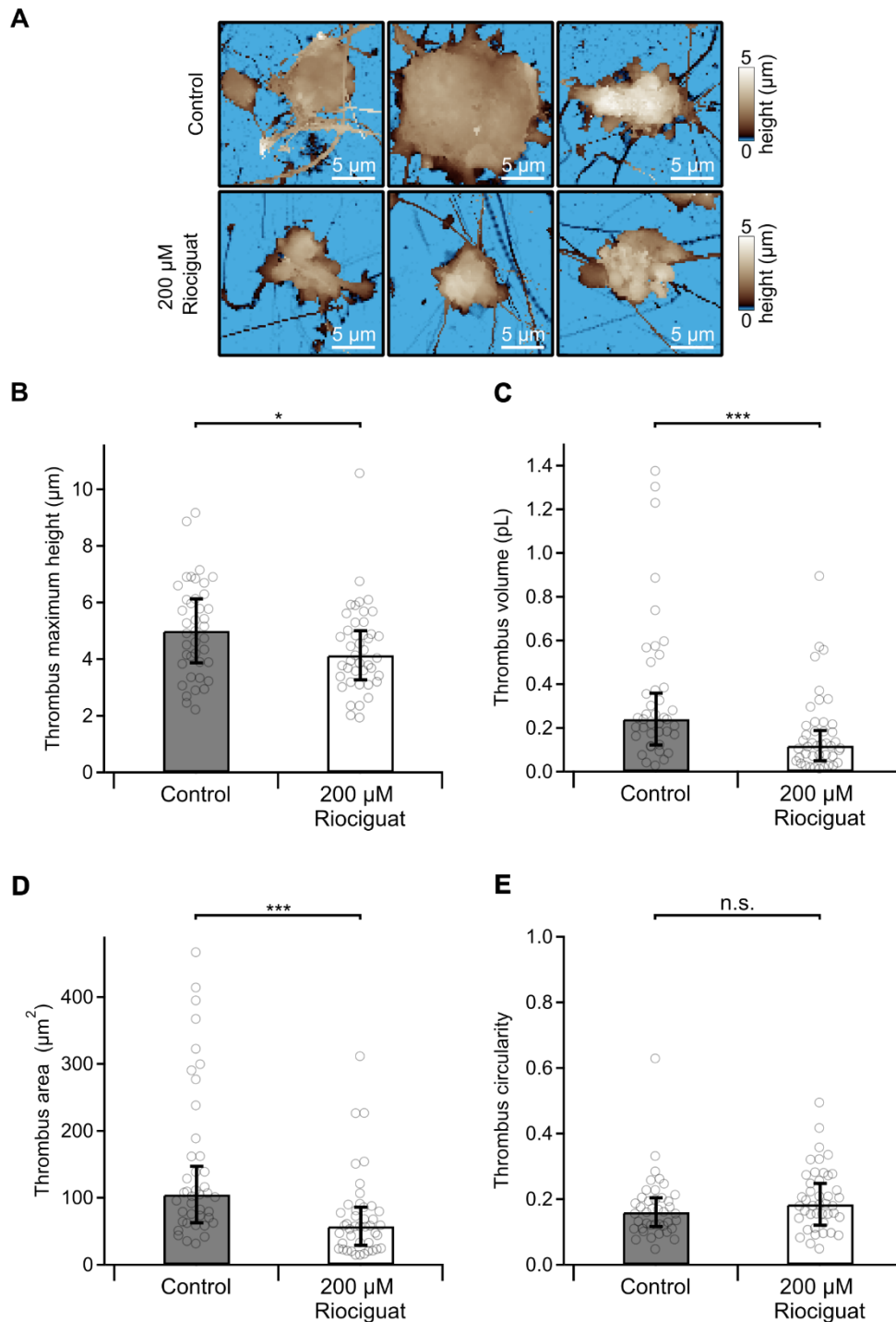


Figure 5.4. Riociguat decreases three-dimensional thrombus size. (A) Representative images of thrombi either untreated (Control) or treated with 200 μ M riociguat (15 min preincubation). (B) Maximum height, (C) volume, (D) area and (E) circularity of untreated and treated thrombi. Bar plots show median $\pm$ MAD, data points represent individual thrombi (41 and 43 for Control and Riociguat, respectively) from 3 independent experiments pooled together. Scale bar: 5 μ m. * for $P < 0.05$, *** for $P < 0.001$ for Wilcoxon rank test. Thrombi samples were procured by Michael Böttcher.

In contrast to our previous results with 200 μ M riociguat, 200 nM riociguat treatment did not significantly change thrombus area (Figure 5.5D, $108.4 \pm 49.7 \mu\text{m}^2$, median $\pm$ MAD, $P = 0.676$) or volume (Figure 5.5E, $0.24 \pm 0.14 \text{ pL}$, $P = 0.999$), while treatment with 10 μ M DEA-NO

lowered thrombus area and volume significantly ($80.0 \pm 32.0 \mu\text{m}^2$, $P = 1.99 \cdot 10^{-6}$ and $0.16 \pm 0.07 \text{ pL}$, $P = 0.000496$, respectively), compared to control ($127.1 \pm 70.2 \mu\text{m}^2$ and $0.27 \pm 0.20 \text{ pL}$, respectively). Treatment with both riociguat and DEA-NO is different again, resulting in smaller reduction in thrombus area ($91.7 \pm 39.9 \mu\text{m}^2$, $P = 0.018$) and no significant difference in thrombus volume vs. control ($0.17 \pm 0.09 \text{ pL}$, $P = 0.0885$). Surprisingly, thrombus maximum height (Control: $3.18 \mu\text{m} \pm 1.27$, Figure 5.5F) was significantly enhanced with riociguat treatment ($4.17 \pm 1.67 \mu\text{m}$; $P = 3.79 \cdot 10^{-5}$ vs. Control), while there was no significant difference in thrombus height with the other treatments ($3.38 \pm 0.70 \mu\text{m}$ and $3.26 \pm 1.1 \mu\text{m}$, for DEA-NO and combination, respectively, $P = 0.99$ vs. Control for both). Treated thrombi were more elongated than control thrombi, quantified by their significantly higher aspect ratio (Figure 5.5G, riociguat: 1.72 ± 0.40 , $P = 0.00829$, DEA-NO: 1.81 ± 0.43 , $P = 5.95 \cdot 10^{-6}$ and Combination: 2.00 ± 0.54 , $P = 4.42 \cdot 10^{-9}$, respectively), compared to untreated thrombi (1.48 ± 0.31).

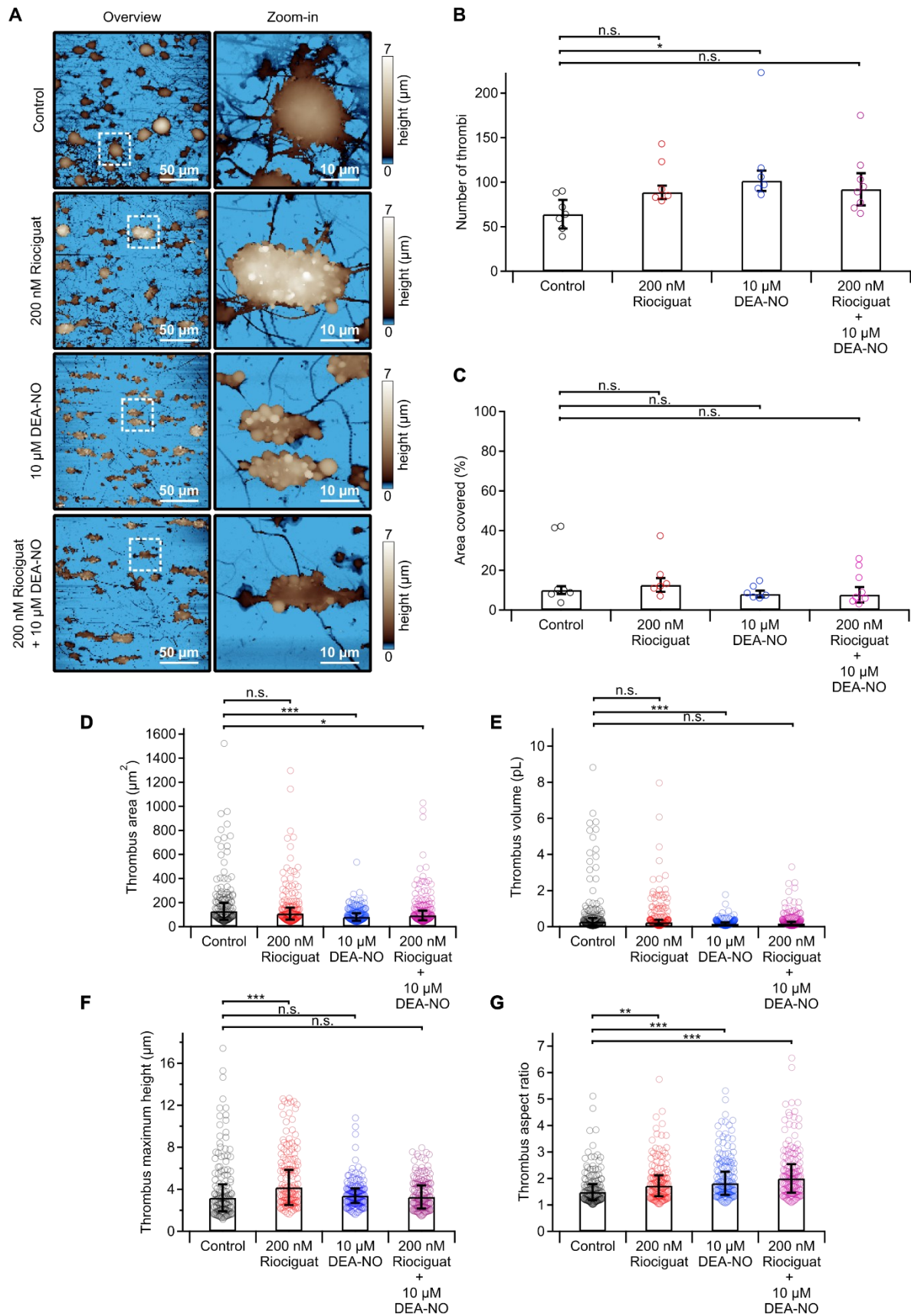


Figure 5.5: Riociguat and DEA-NO elongate three-dimensional thrombus morphology. (A) Representative topography images captured with SICM of untreated (top row), 200 nM riociguat-treated (second row), 10 μ M DEA-NO-treated (third row) and both riociguat and DEA-NO-treated (bottom row) thrombi samples. All treatments were done using 15 min of preincubation. Left column shows overview images at $200 \times 200 \mu\text{m}^2$ image size (150×150 pixels; pixel size $1.33 \times 1.33 \mu\text{m}^2$), right column shows zoom-in of a single thrombus at $40 \times 40 \mu\text{m}^2$ image size

(150 × 150 pixels; pixel size 266.67 × 266.67 nm²), indicated by the white-dashed square in the left column. (B) Number of thrombi and (C) area covered by the thrombi. Shape parameters (D) thrombus area, (E) thrombus volume, (F) thrombus mean height and (G) thrombus aspect ratio of untreated (black), 200 nM riociguat-treated (red), 10 μM DEA-NO-treated (blue) and both riociguat and DEA-NO-treated (purple) thrombi samples. Barplots show median ± MAD, data points represent either individual overview images (B+C, 7, 6, 6 and 8 images, for control, riociguat, DEA-NO and combination, respectively) or individual thrombi (D-G, 128, 141, 146 and 152 for control, riociguat, DEA-NO and combination, respectively) from 3 independent experiments pooled together. Scale bar left column: 50 μm, scale bar right column: 10 μm. * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ for Dunn-Holland-Wolfe test. Thrombi samples were procured by Frank Regler and Liubov Unger.

5.3.3 Influence of riociguat and DEA-NO on thrombus surface roughness

Following this, the surface roughness for each thrombus was quantified (Figure 5.6A). The results support the first qualitative analysis of the thrombi: While untreated thrombi have a low surface roughness of 118 ± 55 nm, the surface roughness of treated thrombi was significantly higher, with 350 ± 104 nm for 200 nM riociguat (Median ± MAD, $P = 6.4 \cdot 10^{-15}$, Dunn Holland Wolfe test), 356 ± 124 nm for 10 μM DEA-NO ($P = 6.4 \cdot 10^{-15}$) and 336 ± 86 nm for the combined treatment ($P = 1.0 \cdot 10^{-11}$), respectively (Figure 5.6B). The effect even persists when normalizing the surface roughness by dividing it by the maximum height of the respective thrombus to account for different thrombus heights (Figure 5.6C). Control thrombi showed a normalized roughness of 0.064 ± 0.023 , while treated thrombi displayed a normalized roughness of 0.138 ± 0.034 for 200 nM riociguat ($P = 6.4 \cdot 10^{-15}$), 0.186 ± 0.050 for 10 μM DEA-NO ($P = 6.4 \cdot 10^{-15}$) and 0.164 ± 0.029 for the combined ($P = 6.4 \cdot 10^{-15}$) treatment. Interestingly, the combined treatment did not have a synergistic effect, rather, the sole DEA-NO treatment gave the highest normalized surface roughness. Taken together, these results demonstrate that the treatment of thrombi with a NO-GC stimulator and activator seemingly increases the thrombi surface roughness.

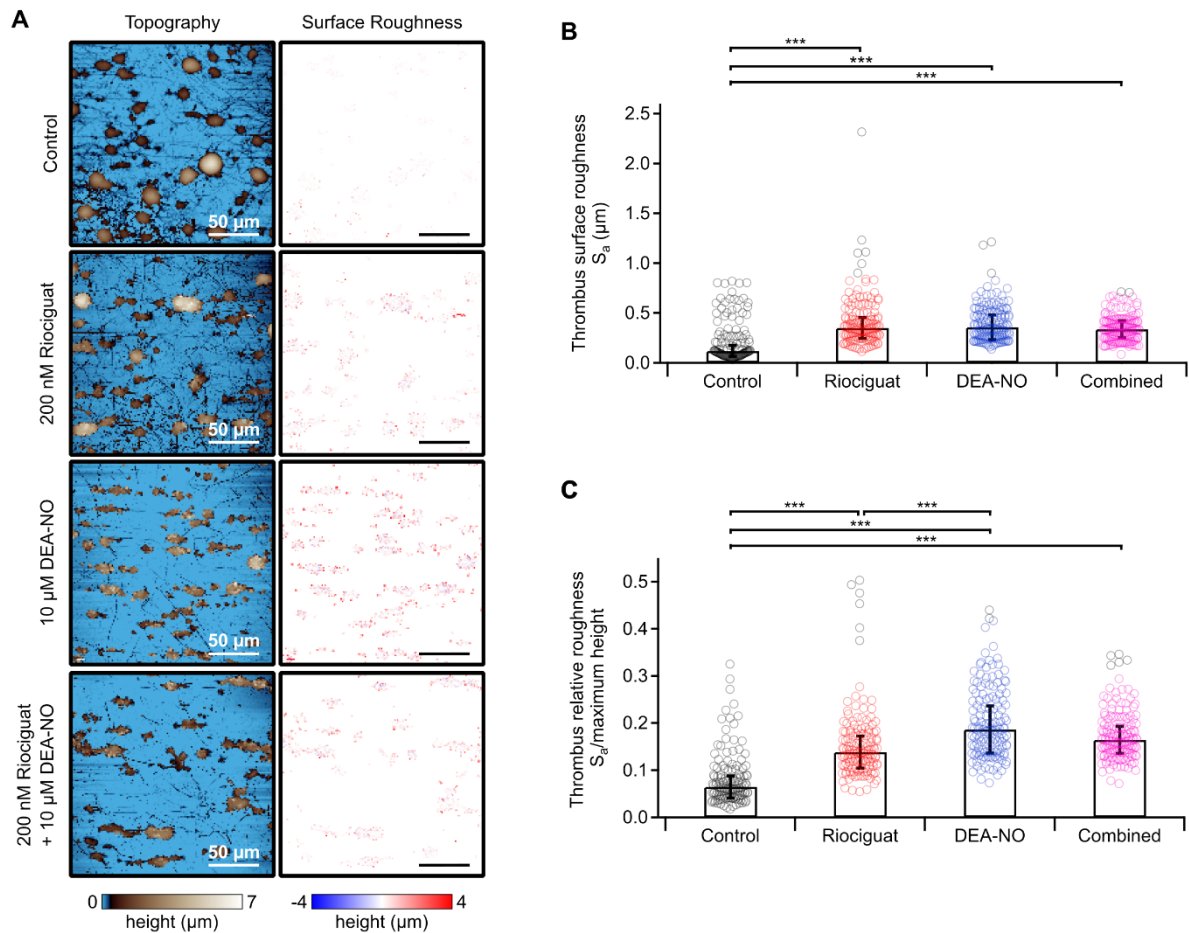


Figure 5.6: Riociguat and DEA-NO increase thrombi surface roughness. (A) Representative raw topography and processed surface roughness images of thrombi with either no treatment (top row) or treatment with 200 nM riociguat (second row), with 10 µM DEA-NO (third row) or a combined treatment of both (bottom row). 200 × 200 µm² images, pixel resolution 150 × 150 pixels. Quantification of (B) thrombi surface roughness and (C) surface roughness normalized by maximum height depending on the treatment (untreated (black), 200 nM riociguat-treated (red), 10 µM DEA-NO-treated (blue), and both riociguat and DEA-NO-treated (purple)). All treatments were done using 15 min of preincubation. Barplots show median ± MAD, data points represent individual thrombi (128, 141, 146 and 152 for control, riociguat, DEA-NO and combined treatment, respectively) from 3 independent experiments pooled together. Scale * for P < 0.05, ** for P < 0.01, *** for P < 0.001 for Dunn-Holland-Wolfe test. Thrombi samples were procured by Frank Regler and Liubov Unger.

5.4 Discussion

To ensure the efficient closure of wounds without causing severe thrombosis and blood vessel occlusion, platelet activation and thrombus growth must be tightly controlled. NO acts a natural shear-dependent brake to thrombosis using the NO-GC-cGMP-PKG cascade (Wen et al., 2018), playing a central role in this control process. The shape, composition and size of thrombi are of particularly high importance in this context, as these characteristics are directly linked with severity of cardiovascular diseases (Parr et al., 2011; Undas & Ariëns, 2011; Joundi & Menon, 2021). Although the size of thrombi causing acute cardiovascular events *in vivo* are often at the scale of mm³ and cm³ (Parr et al., 2011; Riedel et al., 2011; Yoo et al., 2018; van Voorst et al., 2023), the *in vitro* µm³-scale preformed thrombi generated in this study provide insight into early thrombogenic processes in the presence of NO. Previous studies show that

thrombus size is negatively correlated with NO concentration (Atkinson et al., 2018; Wen et al., 2018; Li et al., 2025). However, the precise three-dimensional thrombus structure in the context with NO is still poorly understood. There is previous research investigating platelet and thrombus structure with high resolution electron microscopy (Tomaiuolo et al., 2020), but it is lacking in the area of the influence of NO on thrombus size and structure (only one study was mentioned in a review (Groves et al., 1993)). To fill this gap, SICM was used in this study to quantify the influence of NO on thrombi on the nanometer scale.

This present study shows that treatment of thrombi with 200 μ M riociguat leads to a reduction in thrombus height, area, and volume, while thrombus circularity was not affected, showing that stimulation of the NO-GC with riociguat inhibits thrombus growth. However, this was only seen for 200 μ M riociguat. At 200 nM riociguat (around 84 μ g/L) a typical blood concentration of administered riociguat (Grimminger et al., 2009; Michaličková et al., 2020), there was no difference in thrombus area or volume compared to control when no NO was additionally administered. The significant changes in thrombi shape by treatment with 200 μ M riociguat compared to the minor changes at only 200 nM riociguat show that a critical concentration of riociguat might indicate that riociguat is capable of thrombus disruption on its own. There is precedence to this, as one study found that riociguat only inhibited platelet activation in whole blood at concentrations of above 50 μ M, compared to only 100 nM in washed platelets (Reiss et al., 2015). Riociguat raised basal cGMP from $\sim$ 1.1 to $\sim$ 6.0 pmol per 10^9 platelets at 1 μ M. For comparison, the NO donor sodium nitroprusside at the same 1 μ M concentration raised cGMP to $\sim$ 8.4 pmol per 10^9 platelets, indicating that riociguat alone produces a substantial but somewhat smaller increase than a direct NO donor (Reiss et al., 2015). In the same study, platelets in whole blood flowing over collagen-coated channels, similar to the generated thrombi here, were only inhibited in their activation at $\geq$ 100 μ M of riociguat. As the plasma concentrations of riociguat is usually between 100-500 nM in patients (Frey et al., 2008; Frey et al., 2016), the effect of riociguat on thrombus size at therapeutic concentrations was expected to be negligible, which is supported by the results shown here. Previous work showed that erythrocytes in the blood could function as a buffer, reducing NO-inhibition of platelet activation with increasing number (Michelson et al., 1996), which might explain this difference in (diluted) whole blood vs. washed platelets. This circumstance hints that riociguat does not significantly inhibit normal physiological thrombus formation at prescribed concentration (similar to platelet aggregation as previously reported (Frey et al., 2016)). At higher concentrations, as shown in Figure 5.4, there is a significant decrease in thrombus area and volume, which might result in a heightened risk of bleeding. However, it is difficult to draw final conclusions without validation of riociguat induced cGMP-increases, for example with imaging of a cGMP-sensor or quantification via enzyme-linked immunosorbent assay (Wen et

al., 2018). Additional biochemical investigations are required to correctly interpret the results found here.

For prevention of vessel occlusion, the thrombus volume is the most important factor (Riedel et al., 2011). The results given here show that even on a small timescale, there is a significant reduction in thrombus volume with DEA-NO treatment. While 200 nM riociguat treatment showed no change in thrombus area and volume compared to control, these shape parameters significantly decreased with DEA-NO treatment, meaning there is an effect on thrombus size that is triggered by NO, likely the NO-cGMP signaling pathway. In contrast to riociguat, 10 μ M DEA-NO might be enough to diminish thrombosis. Spread platelets treated with 10 μ M DEA-NO are also markedly reduced in their spreading area (Atkinson et al., 2018), supporting the finding reported in this thesis. There is also the issue of NO desensitization. At 500 nM DEA-NO or above the platelets become desensitized to DEA-NO itself (Mullershausen et al., 2001; Wen et al., 2018), meaning the high DEA-NO used in this study may change the effect on platelet aggregation and thrombus formation. Interestingly, there is no synergistic effect when both riociguat and DEA-NO are used. This is contrary to the previously reported high rate of synergy between riociguat and DEA-NO (Schermy et al., 2008). In fact, riociguat increases NO-GC activity by stabilizing the bond between NO and NO-GC (Sandner, 2018). Again, the reason for this lack of synergy might be the usage of diluted whole blood rather than washed platelets (Reiss et al., 2015), reducing effective riociguat due to protein and red blood cell binding. In a conference abstract presenting a flow-cytometry studying this effect, 90 % inhibition of platelet activation by 1 μ M riociguat and 31 μ M DEA-NO, was reduced to 10 % inhibition by adding blood plasma (Y. Wu et al., 2018). This could mean a NO-donor is less readily available in whole blood, resulting in lower platelet activity. Indeed, previous studies confirmed platelet-derived NO is less available in whole blood, compared to washed platelets (Malinski et al., 1993). Further studies are required to investigate this buffering effect of whole blood.

A previous study looked at the shape of single platelets, when treated with riociguat, the NO-GC activator cinaciguat and 8-bromo cGMP, a cGMP analogue (Balmes et al., 2024). In contrast to the results with thrombi given in the present thesis, all three treatments increased the circularity of the single platelets compared to control, meaning that the aspect ratio decreased. In addition, platelet Young's modulus was halved when they were treated with riociguat. This could indicate that platelet biomechanics have an influence on thrombus structure. However, the concentration of riociguat used in the experiments was 10 μ M (Balmes et al., 2024) and the experiments were done with washed platelets instead of (diluted) whole blood (Balmes et al., 2024), making a direct comparison to the results presented here difficult.

Future research focusing on Young's modulus mapping of (non-fixed) thrombi using SICM could potentially reveal biomechanical aspects of thrombus structure.

Thrombus aspect ratio increased significantly with treatment. This could be explained by the thrombi being not as stable as control thrombi, meaning that platelets at the outer shell of the thrombi get ripped off it by the shear forces of flowing blood and then gather behind the thrombus, thus making the thrombus more elliptical than circular. One explanation could be different subpopulations of platelets. A study found that roughly 20 % of platelets possess a downregulated NO-GC-cGMP-PKG pathway (Radziwon-Balicka et al., 2017). These NO-GC-negative platelets are the primary platelets binding directly to collagen, while the NO-GC-positive platelets made up the bulk of the thrombus. In the case of additional NO-inhibition, the NO-GC-negative platelets might not be as affected as their NO-GC-PKG pathway is downregulated and still form basic thrombi. The NO-GC-positive platelets meanwhile might be inhibited in their activation and platelet-platelet adhesion via fibrinogen, meaning shear stress can more easily remove an attached platelet from a growing thrombus, carrying it further downstream and thus elongating the thrombus. Indeed, the activity of fibrinogen receptor $\alpha\text{IIb}\beta\text{3}$ upon stimulation by collagen was significantly reduced in NO-GC-positive platelets (Radziwon-Balicka et al., 2017). This reduction in $\alpha\text{IIb}\beta\text{3}$ activity might result in a less secure thrombus architecture.

Another explanation could be thrombus composition. Thrombi are mainly composed of platelets as well as red and white blood cells, tightly wrapped by a fibrin network (Alkarithi et al., 2021). The exact composition is different depending on the location of the thrombus in the body, with thrombi causing acute ischemic stroke in the brain characterized by a rich red blood cell and platelet region within a fibrin network, while venous thrombosis thrombi are composed of mainly red blood cells and fibrin with only some platelets and white blood cells (Alkarithi et al., 2021). The inside of the thrombus ("core") is usually composed of densely packed red blood cells, while the outer edges ("shell") of the thrombus is covered in a layer of fibrin-crosslinked platelets (Cines et al., 2014; Risman et al., 2022). The core region of the thrombus is thought to be enriched by thrombin, while NO can permeate into the shell region of the thrombus (Stalker et al., 2014). The shell region of the thrombus is also more exposed to the shear forces of the surrounding blood flow, enabling the endogenous shear-dependent NO-cGMP signaling cascade to further inhibit thrombus growth (Wen et al., 2018). Curiously, this hypothesis should result in a reduction in thrombus height and area as shown before (Atkinson et al., 2018; Wen et al., 2018), but that is not the case in the data shown here. Rather, the thrombus maximum height of thrombi seems to even increase with the treatment of 200 nM riociguat compared to control. There was also no significant difference in thrombus maximum height for the other two treatments. This in stark contrast to previous reported results (Atkinson

et al., 2018), where NO significantly decreased the height of preformed thrombi. However, the studies are slightly different. In one study (Wen et al., 2018), thrombi were formed from human blood in the presence of 10 μ M DEA-NO at a shear rate of 500/s rather than the shear rate of 1000/s used here. In another study (Atkinson et al., 2018), a different NO-donor (S-Nitrosoglutathione, 1 μ M) was added after allowing platelet to form thrombi at 1000/s shear rate. Here, the surface coverage did not change significantly, but the thrombus height lowered. Further studies using fluorescence and SICM will be necessary to fully elucidate the full impact of NO on a thrombus.

The surface roughness of thrombi was also quantified. The results indicate that untreated thrombi are smoother, with a low surface roughness. In contrast, thrombi created from blood treated with a NO-GC-stimulator/NO-donor often feature bubble-like protrusion, resulting in a higher surface roughness. A possible explanation for the increased surface roughness might be a reduced clot contraction (Lam et al., 2011): Platelets treated with NO-GC stimulators/activators are reduced in their activity (Denninger & Marletta, 1999; Emerson et al., 1999; Smolenski, 2012; Benza et al., 2017; Klinger et al., 2021), possibly leading to reduced contraction forces, as inhibited platelets show diminished contractile forces (Ting et al., 2019). Red blood cells are usually closely packed in contracted thrombi (Cines et al., 2014), acting as a counterforce to the contraction (Tutwiler et al., 2016). In addition, NO directly lowers fibrin network density (Helms et al., 2017). A reduction in platelet contraction forces might result in a decrease in red blood cell compression and an increased clot permeability, resulting in some red blood cells “poking out” of a more porous fibrin network and thus creating a higher thrombus surface roughness. Fluorescent labeling of distinct blood cell types within the thrombus may offer valuable insights into its composition and help clarify this aspect.

As a last note, the thrombi investigated here were fixed using 4% paraformaldehyde prior to SICM imaging and morphology analysis. Formaldehyde-based fixation is known to cause morphological changes in both single cells and whole tissues (Rautiainen et al., 2010; Schnell et al., 2012; Chatterjee, 2014; Idziak et al., 2023), such as platelets swelling (Silver & Allen Gardner, 1970) or bacteria shrinking (Zhu et al., 2021) as well as an increase in cell Young’s modulus (Kim et al., 2017; Iwashita et al., 2020). These artifacts might influence the interpretation of the data presented here, for example the surface roughness data. However, as all samples were fixed using the same procedure, any potential fixation-induced morphological artifacts can be assumed to have affected all samples in a comparable manner and therefore should not have influenced the comparison between the four treatment groups. Nevertheless, SICM imaging of live thrombi, including the assessment of their Young’s modulus, represents a potential avenue for future research. Alternatively, a different fixation

protocol that better preserves the native sample morphology, such as glyoxal fixation, could be employed (Richter et al., 2018).

In summary, the results of this section demonstrate that NO donors and NO-GC modulators like DEA-NO and riociguat exert significant effects on thrombus morphology and stability. While therapeutic concentrations of riociguat did not significantly alter thrombus formation, higher doses and NO donors such as DEA-NO reduce thrombus volume and increase surface roughness, suggesting cGMP signaling impaired thrombus compaction and increased vulnerability to shear forces. These effects are possibly mediated by platelet subpopulations with differential NO sensitivity, as well as changes in clot contraction and fibrin structure.

Future work should focus on dissecting the roles of blood cell types in thrombus stability under NO and NO-GC modulation. Furthermore, integrating fluorescent cell tracking and live imaging under flow could provide deeper insight into thrombus composition and dynamics in response to NO signaling. Finally, SICM Young's modulus mapping of live thrombi could be used to study thrombus biomechanics.

6 Outlook

Platelets have been revealed as a critical component in cardiovascular inflammation in recent years (Boulaftali et al., 2025). Their nonthrombotic functions, such as platelet migration (Gaertner et al., 2017; Nicolai et al., 2020; Kaiser et al., 2023; von Eysmond et al., 2025) and immune cell function (Nicolai et al., 2019; Nicolai et al., 2024), are still not fully understood and part of active research. In addition, platelets are the primary target of therapies for preventing intravascular thrombosis and resulting critical health conditions such as myocardial infarction and stroke in CVD. These platelet-focused anti-aggregation therapies have significant advantages: Platelets lack a nucleus and are only available in the vasculature, preventing systemic or transcriptional/translational side-effects (Boulaftali et al., 2025). However, the available techniques also have several disadvantages. There is an inherent increased risk of bleeding (Sørensen et al., 2009) and no broad clinical application. For example, integrin receptor $\alpha\text{IIb}\beta 3$ blockage leads to efficient thrombosis prevention, but also results in severe bleeding and can thus only be used in the short term (Byrne et al., 2023). The influence of these therapies on the nonthrombotic functions of platelets are still quite poorly understood, e.g. on the biomechanical aspects of platelet behavior.

So, there is a need for effective treatment of platelet hyperreactivity and increased risk of thrombosis. This thesis demonstrates two potential candidates for a point of attack: The cGMP-cascade and the receptor ACKR3. In addition, this thesis demonstrates the significant influence of biochemical and biomechanical cues on thrombus formation, platelet activation and spreading and even nonthrombotic functions such as platelet migration.

ACKR3 stimulation, as demonstrated in chapter 3, reduced platelet Young's modulus and changed actin cytoskeleton architecture, pointing towards turning platelets toward a more anti-thrombotic phenotype. In addition to reducing platelet hyperreactivity, ACKR3 also changes the platelet lipidome itself to reduce hyperlipidemia (Cebo et al., 2022). This means ACKR3 signaling is a possible link between platelet biomechanics and lipidome. Importantly, the inability of ACKR3 to fully reverse the effects of OxLDL activation shows that there might be a difference between the lipid-driven and receptor-driven signaling pathways. Together, these findings show that ACKR3 is an attractive therapeutic target, as it simultaneously attenuates platelet activation and influences lipid-driven platelet hyperreactivity, thereby addressing two key drivers of CVD.

Secondly, platelet migration remains incompletely understood. As outlined in chapter 4, platelet migration is heavily influenced by biomechanical factors, however there are still significant questions, which remain unresolved. While significant effort has been made to link the molecular pathways of both platelet haptotactic migration and platelet mechanosensing in recent years (Schoen et al., 2024), the physiological relevance and therapeutic potential is still

not fully explored. As the Young's modulus of tissue vastly changes during inflammation (Engler et al., 2004; Janmey & Miller, 2011; Booth et al., 2012), platelet migration is potentially a context-dependent immune function, based on the status of inflammation of the surrounding tissue.

Lastly, as shown in chapter 5, NO-donor DEA-NO and NO-GC stimulator riociguat demonstrably change the platelet behavior not only on the single platelet level (Balmes et al., 2024), but also on the thrombus level, by lowering thrombus volume and increasing thrombus surface roughness, indicating a loss of stability in thrombi. This might render thrombi more susceptible to shear forces while preserving baseline hemostasis. This circumstance makes NO-based signaling a highly valuable target for minimizing pathological thrombosis and bleedings risk at the same time.

6.1 Future directions to study platelet behavior in inflammation

The inflamed blood vessel is a complex tissue and still not fully understood. Various aspects can be considered, as the diverse topics in this thesis can attest. Biological life is complex and thus the results presented in this thesis are somewhat limited: First, they are all *in vitro* studies and, except for the platelet migration experiments in chapter 4, only a static record of a highly dynamic environment. Future directions could thus focus on combinations of methodical approaches presented in this thesis and *in vivo* and dynamic studies.

6.1.1 Combination of methodical approaches

The experimental aspects of this thesis are highly varied (see chapter 3.2, 4.2 and 5.2), ranging from the influence of cGMP on thrombi, the regulation of lipid-loaded platelets by ACKR3 and the influence of substrate Young's modulus on platelet migration. As inflammation is fairly complex, one might speculate the overlap of these distinct topics. For example, what is the influence of cGMP or ACKR3 stimulation on platelet migration? NO/cGMP can regulate the migration behavior of many different cell types, such as neurons (Mandal et al., 2013; Knipp et al., 2025), breast cancer cells (Schwappacher et al., 2013), vascular smooth muscle cells (Sarkar et al., 1996; Holt et al., 2016) and dendritic cells (Tang et al., 2025). It is of note that an increase of intracellular cGMP levels increased dendritic cell motility (Tang et al., 2025) and breast cancer cell invasion (Schwappacher et al., 2013). Therefore, it is easy to speculate that an increase of cGMP might increase platelet migration velocity. Platelet haptotactic migration requires an interplay between Rac1-activated lamellipodia formation by Arp2/3 at the platelet front and RhoA and myosin activation for retraction at the platelet rear (Schoen et al., 2024). In addition, the tyrosine kinase c-Src is required to maintain platelet polarization (Schoen et al., 2024). In a model with the amoeba *Dictyostelium discoideum*, increasing intracellular cGMP levels elevated myosin and actin activity (Pfannes et al., 2013). Stimulation of HEK293 cells with either NO or 8-bromide-cGMP resulted in Rac1 activation (Hou et al., 2004).

Riociguat increased the activity of vasodilator-stimulated phosphoprotein (VASP), an enhancer of the Arp2/3 complex (Reiss et al., 2015). On the other hand, NO decreased RhoA activation in platelets (Aburima et al., 2017). On the ACKR3 and hyperlipidemia side, platelet chemotaxis is induced by CXCL14 via the receptor CXCR4, another chemokine receptor (Witte et al., 2020). Interestingly, OxLDL inhibits the cGMP-cascade in platelets (Magwenzi et al., 2015), potentially resulting in larger thrombi. Additionally, OxLDL activates RhoA (Wraith et al., 2013), meaning it could influence platelet migration as well. Indeed, previous research has shown that lipid-loaded platelets are inhibited in their migration (Seifert et al., 2021). ACKR3 stimulation could therefore have an influence on platelet haptotactic migration. Future biochemical experiments might reveal more interactions of both the NO-cGMP signaling cascade and OxLDL for platelet migration.

One interesting aspect of platelets in inflammation is the ongoing research regarding subpopulations of platelets, namely old and young (also called reticulated) platelets. Young/reticulated platelets are more reactive than old ones (Armstrong et al., 2017; Bongiovanni et al., 2022). Old platelets have shown more inflammatory functions, such as leucocyte and neutrophil interactions as well as bactericidal function, compared to young platelets (Anjum et al., 2025). As platelet migration is heavily involved in inflammation (Nicolai et al., 2020), a comparison of platelet migration or mechanosensing aspects of old and young platelets might be of interest.

The role of biophysical properties in biological systems is also still part of ongoing research. For example, a stiff substrate seemingly enhances breast cancer cell migration via CXCR4 (Ho et al., 2025). The adhesion of platelets is enhanced on stiff substrates (Qiu et al., 2014; Kee et al., 2015), potentially leading to changed thrombus size, morphology, and built-up. Knowledge about thrombus mechanical properties is also still limited (Domingues et al., 2022). On the macroscale, it is known that fibrin/platelet content increases thrombus Young's modulus (Boodt et al., 2021). A recent review agreed on the notion that thrombus structure and composition is a major determinant for thrombosis severity and outcome (Weisel & Litvinov, 2025). Therefore, biomechanical investigations of live thrombi on the micro- and nanometer-scale using SICM might be an interesting avenue for future biomechanical research.

6.1.2 *In vivo* and dynamic studies

A reductive *in vitro* environment in a petri dish can never fully replicate the conditions of live cells in their usual *in vivo* environment. For example, platelets are often confined between tissues and do not have the space available as in an *in vitro* assay. Capillaries are roughly 8 μm in diameter, confining the available space for platelet spreading significantly (Tu & Chao, 2018). Indeed, a spatial confinement spreading assay showed altered platelet adhesion, spreading and mechanics compared to control (Rodríguez et al., 2025). Therefore, future ex

vivo and *in vivo* assays need to be done to fully grasp the influence of the aspects presented here.

For platelet migration, multiple *in vivo* mouse model experiments have already been done (Gaertner et al., 2017; Nicolai et al., 2020; Kaiser et al., 2023). Similar investigations can be done to investigate the influence of inflamed tissue on platelet migration.

6.1.3 Conclusion

This thesis demonstrates that platelet behavior in inflammation is influenced by both biochemical and biomechanical regulation. High-resolution biophysical methods reveal the modulation of the NO-cGMP pathway alters thrombus architecture, while platelet Young's modulus and cytoskeletal organization are changed by oxidized lipids and ACKR3 activation. Platelet migration is gated by substrate Young's modulus, with effective platelet haptotactic migration only occurring on sufficiently stiff substrates. These results illustrate that platelet biomechanics are a critical part of platelet function and are thus a necessary consideration for future antithrombotic and anti-inflammatory therapies.

7 List of abbreviations

ACD – acid-citrate-dextrose

ACKR3 – atypical chemokine receptor 3

ADP – adenosine diphosphate

AFM – atomic force microscopy

AR – aspect ratio

Arp2/3 – actin related protein 2/3

BSA – bovine serum albumin

cGMP – cyclic guanosine monophosphate

CNN – convolutional neural network

CTCF – corrected total cell fluorescence

CXCR7 – C-X-C chemokine receptor type 7

DEA-NO – diethylamine nonoate

DMSO – dimethyl sulfoxide

F-actin – filamentous actin

HSA – human serum albumin

IFs – intermediate filaments

LDL – low-density lipoprotein

MAD – median absolute deviation

MFI – mean fluorescence intensity

MIF – macrophage migration inhibitor factor

MLC2 – myosin light chain 2

MLCP – myosin light-chain phosphatase

MTs – microtubules

NO – nitric oxide

NO-GC – NO-sensitive soluble guanylyl cyclase

NS8100 – Nusil 8100

OxLDL – oxidized low-density lipoprotein

PBS – phosphate buffered saline

PDMS – polydimethylsiloxane

PKG – protein kinase G

PRP – platelet-rich plasma

PS – phosphatidylserine

ROCK – Rho-associated kinase

ROS – reactive oxygen species

RT – room temperature

S184 – Sylgard™ 184

S527 – Sylgard™ 527

SDF-1 – stromal-derived factor 1

SICM – scanning ion conductance microscopy

SPM – Scanning probe microscopy

TxA₂ – thromboxane A₂

VASP – vasodilator-stimulated phosphoprotein

VSMCs – vascular smooth muscles cells

vWF – von Willebrand factor

WASp – Wiskott Aldrich syndrome protein

WAVE – WASp-family verprolin-homologous protein

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9 Publications, manuscripts and conference contributions

9.1 Publications

- **von Eysmond, H.**, Seifert, J., Rheinlaender, J., & Schäffer, T. E. (2025). Mechanosensing alters platelet migration. *Acta Biomaterialia*, 196, 213–221.
- **von Eysmond, H.**, & Schäffer, T. E. (2022). Scanning Ion Conductance Microscopy and Atomic Force Microscopy: A Comparison of Strengths and Limitations for Biological Investigations. In T. E. Schäffer (Ed.), *Scanning Ion Conductance Microscopy* (pp. 23–71). Springer International Publishing.
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- Seifert, J., **von Eysmond, H.**, Chatterjee, M., Gawaz, M., & Schäffer, T. E. (2021). Effect of Oxidized LDL on Platelet Shape, Spreading, and Migration Investigated with Deep Learning Platelet Morphometry. *Cells*, 10(11), 2932.

9.2 Manuscripts

- Fu, X., Brun, A., Dittrich, K., Cebo, M., Lackner, M., Menig-Benzig, L.S., Bouzabia, B., Rheinlaender, J., Banks, H., **von Eysmond, H.**, Dötsch, T., Schwegmann, S., Bucio-Garcia, J.A., Nürnberg, B., Beer-Hammer, S., Schwab, M., Schaeffeler, E., Hofmann, U., Haag, M., Rath, D., Geisler, T., Gawaz, M., Bakchoul, T., Schäffer, T. E., Lämmerhofer, M., Chatterjee, M. Targeting ACKR3/CXCR7 Enhances Platelet Anticoagulant Acylcarnitines and Modulates Procoagulant Function (*accepted*)

9.3 Conference contributions

- **von Eysmond, H.**, Seifert, J., Rheinlaender, J., Schäffer, T. E. (2024, June 25th) Platelet migration behavior is dependent on substrate stiffness. [Conference Presentation] IUPAB 2024, Kyoto, Japan. (*Received the BPS-IUPAB Student Award*)

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11 Appendix

11.1 Custom code for shape analysis of lipid-loaded platelets

This custom code “20250603_Static_spread_single_analysis” was used in chapters 3.2.4 and 3.3.4 to perform shape analysis of platelets using ImageJ/Fiji.

20250603_Static_spread_single_analysis

```
//20230905 HVE Finished initial version of code
//This code was made to get scan all actin images in a folder and then do
both shape parameter analysis and fluorescence intensity analysis
//Holding ESC stops the running of the code!

/*
 * Macro template to process multiple images in a folder
 */
#@ File (label = "Input directory", style = "directory") input
#@ File (label = "Output directory", style = "directory") output
#@ String (label = "File suffix", value = ".nd2") suffix
processFolder(input); //processing the folder
var low_thresh=0; //define some global variables we want to extract (lower
threshold to 0)
//var particles=0
//var mean_bkg=0;
// function to scan folders/subfolders/files to find files in folder with
correct suffix, .nd2 files
function processFolder(input) {
    list = getFileList(input); //get list of all files in folder
    list = Array.sort(list);

    //Array.show(list); //activate this line to verify the list
    nd2files = newArray(0) //define a new array where the .nd2 files will
be loaded in
    //Array.show(nd2files) //activate this line to verify the list

    for (i=0; i<list.length; i=i+1) ///curate the list of all files to only
process .nd2 files
    {
        // count the number of .nd2 files
        if (endsWith(list[i], ".nd2")) {

            nd2files=Array.concat(nd2files,list[i]);
            //Array.show(nd2files); //activate this line to verify the list
        }

        else
            //Array.show(nd2files); //activate this line to verify the list
        }
    }

    img_number=0
    non_img_number=0
    total_number=nd2files.length;

    thresh = newArray(0);
    //Array.show(thresh); //only enable to control what the thresholds are
    //Array.show(tresh);

    //part_num=newArray(0); //only enable to control what the number of
particles are
```

```

//Array.show(part_num);

//bkg_array=newArray(0);
//Array.show(bkg_array);

for (i=0; i<nd2files.length; i=i+1){ //process all .nd2 files in the
folder
    //conditions for for-loop
    //name = nd2files[i]+"_Fluoresc_res";
    name1 = nd2files[i]+"_Results";
    name2 = nd2files[i]+"_ROIs";
    name3 = nd2files[i]+"_Summary";

    // count the number of .nd2 files
    img_number=img_number+1;

//print(img_number); /decommentize to control the image number and the right
suffix
    //print(suffix);

    print("Processing file "+img_number+" out of "+total_number+"
.nd2 files");
    processFile(input,output, nd2files[i]);

    //bkg_array[i]=mean_bkg;
    //Array.show(bkg_array);

    //part_num[i]=particles; //only enable to control what the number
of particles are
    //Array.show(part_num);

    // Array.show(thresh); //only enable to control what the
thresholds are
}

print("Processed "+img_number+" .nd2 files out of "+total_number+"
total files")

path = input;
foldername = File.getName(path);
print("Folder path: "+path);
print("Folder name: "+foldername);
exp_date = substring(foldername, 0, 8);
print("Date of Experiment: "+exp_date);
getDateAndTime(year, month, week, day, hour, min, sec, msec);
print("Date:"+day+"/"+month+"/"+year+" Time: " +hour+": "+min+": "+sec);
selectWindow("Log");
getInfo("Log");
save(output + "/" + "Analysis_log.txt");
close("Log");
}

function processFile(input, output, file){ //main process function

    //set batchmode
    setBatchMode("hide"); //batchmode on, so that images are processed in
the background
    //setBatchMode("false"); //batchmode off, if you want to check on images, use
waitForUser down below as well

```

```

//open file
open(input + File.separator + file); //open the file
print(file); //print into log
run("Next Slice [>]"); //go to the FITC channel of the file

//Start analysis
//reset image threshold
resetThreshold();

//shape thresholding
setAutoThreshold("Li dark");
call("ij.plugin.frame.ThresholdAdjuster.setMode", "Over/Under");

//store used threshold
getThreshold(lower, upper); //get the lower threshold
low_thresh=lower;
print("Lower threshold: "+lower);

//set shape measurements
run("Set Measurements...", "area mean integrated shape display
redirect=None decimal=2");

//shape particle analysis
run("Analyze Particles...", "size=5-Infinity display exclude clear
include summarize overlay");

//number of analyzed particles
print("Number of analyzed particles: "+nResults);
print(" "); //for printing an empty line in log

//Overlay.setStrokeColor("red"); //changes overlay color

//save results
selectWindow("Results");
saveAs("text", output + "/" + name1);
run("Close"); //closing results
saveAs("png", output + "/" + name2); //save png copy of image
close();
selectWindow("Summary of " + file);
saveAs("text", output + "/" + name3);
run("Close");

//waitForUser;
//mean_bkg= ;
}

```

11.2 Code for calculating a fluorescence gradient

The following code “20240516 Fluo_gradient Igor Procedure” was created to obtain the fluorescence gradient of migrating platelets in chapters 4.2.8 and 4.3.3. It was done in Igor Pro 9 (WaveMetrics, Lake Oswego, OR, USA).

20240516 Fluo_gradient Igor Procedure

```

#pragma TextEncoding = "UTF-8"
#pragma rtGlobals=3// Use modern global access method and strict wave
access
#pragma DefaultTab=3,20,4// Set default tab width in Igor Pro 9 and later

```

```
//function to extract a fluo gradient within a specified cell by masking
using CNN maskimage and flattening the image using JR iterative flattening
```

```
function fluo_cell_gradient(wave fluoimage, wave cnnimage, variable
flatten_threshold, variable cutout_width)
    //fluoimage: original fluorescent image to be analyzed; cnnimage:
maskimage made from cnn; flatten_threshold: fluorescence threshold (in a.u.)
used for iterative flattening
    //cutout_width: width in m of the cutout from the original picture,
centralized around cursor
    //needs JRiterativeflattening function to work

    //extract cursor positions from fluoimage
    variable x_center=xcsr(A)
    variable y_center=vcsr(A)
    variable cutout_half=cutout_width/2
    variable bottom= y_center-cutout_half; variable
top=y_center+cutout_half
    Variable left=x_center-cutout_half; variable
right=x_center+cutout_half

    //prepare the binary maskimage of the particle of interest and fill
any holes using analyze particles function
    duplicate /R=(left,right) (bottom,top) /FREE /O
cnnimage,particle_image
    duplicate /R=(left,right) (bottom,top) /FREE /O
particle_image,maskimage
    redimension /B /U maskimage
    maskimage=particle_image>32000 ? 0 : 255
    imageanalyzeParticles /NSW /Q /FILL /M=3 /A=(150*3) stats maskimage
    Wave M_particle
    //newimage /F M_particle

    //cut out the cells in the fluoimage using the mask
    duplicate /R=(left,right) (bottom,top) /O fluoimage, fluo_maskimage
    fluo_maskimage=M_particle<64 ? fluo_maskimage : nan
    Dowindow /K fluomaskimage; newimage /K=1 /N=fluomaskimage /F
fluo_maskimage

    //crop cell of interest out of rest of the image
    fluo_maskimage=((abs(x-x_center)<cutout_half) && (abs(y-
y_center)<cutout_half)) ? fluo_maskimage : nan
    SetAxis left bottom,top
    SetAxis bottom left,right

    //do the flattening with specified flattening image (while only
copying the relevant cutout and open the flattened image)
    duplicate /R=(left,right) (bottom,top) /FREE /O
fluo_maskimage, flat_image
    JRiterativeFlattening(flat_image, "plane", 1, flatten_threshold, 0, 0)
    wave JRtempimage

    Dowindow /K JR_flatimage; newimage /K=1 /N=JR_flatimage /F
JRtempimage
    SetAxis left bottom,top
    SetAxis bottom left,right

    //subtract the flattened image from the original fluoimage to
obtain gradient image
```

```

duplicate /O fluo_maskimage,gradient_image
gradient_image-=JRtempimage

Dowindow /K GradientImage; newimage /K=1 /N=GradientImage /F
gradient_image
SetAxis left bottom,top
SetAxis bottom left,right

//do a poly2d fit to obtain slope of gradient, and add absolute
values of both vector slopes to obtain gradient in intensity/m increase.
CurveFit/Q/M=2/W=0 poly2D 1, gradient_image/D
wave W_coef

variable incr=sqrt((W_coef[1]^2)+(W_coef[2]^2))
string incr_print=num2str(incr)

print "Gradient increase is "+incr_print+" per m"

end

function fluo_cell_gradient_loop(wave fluoimage, wave cnnimage,variable
flatten_threshold, variable cutout_width)
//This function extracts the local fluorescence gradient from each
cell in the fluoimage aand combines them into a wave, instead of just a
single cell as in fluo_cell_gradient
//fluoimage: original fluorescent image to be analyzed; cnnimage:
maskimage made from cnn;flatten_threshold:fluorescence threshold (in a.u.)
used for iterative flattening
//cutout_width: width in m of the cutout from the original picture,
centralized around cursor
//needs JRiterativeflattening function to work

//get x- and y-centers of particles from cnnimage
duplicate /FREE /O cnnimage,particle_image
duplicate /FREE /O particle_image,maskimage
redimension /B /U maskimage
maskimage=particle_image>32000 ? 0 : 255
imageanalyzeParticles /Q /E /EBPC /FILL /M=3 /A=(150*3) stats
maskimage
wave M_moments; variable num_particles; variable particle;// set
debugger stop here and stop function after executing for full functionality
of Hve_Display_Particle_Positions
num_particles=dimsz(M_moments,0)
Make /O /N=(num_particles) gradients;gradients=0
//return 0
for(particle=0;particle<num_particles;particle++)

//extract cursor positions from fluoimage
variable x_center_index=round(M_moments[particle][0])
variable y_center_index=round(M_moments[particle][1])
variable x_center=indextoscale(fluoimage,x_center_index,1)
variable y_center=indextoscale(fluoimage,y_center_index,0)
variable cutout_half=cutout_width/2
variable bottom= y_center-cutout_half;variable
top=y_center+cutout_half
Variable left=x_center-cutout_half;variable
right=x_center+cutout_half

```

```

        //prepare the binary maskimage of the particle of interest
and fill any holes using analyze particles function
        duplicate /R=(left,right) (bottom,top) /FREE /O
cnnimage,particle_image
        duplicate /R=(left,right) (bottom,top) /FREE /O
particle_image,maskimage
        redimension /B /U maskimage
        maskimage=particle_image>32000 ? 0 : 255
        imageanalyzeParticles /NSW /EBPC /Q /FILL /M=3 /A=(150*3)
stats maskimage
        Wave M_particle

        //Dowindow /K Particle_Image; newimage /N=Particle_Image /F
M_particle

        //cut out the cells in the fluoimage using the mask
        duplicate /R=(left,right) (bottom,top) /O
fluoimage,fluo_maskimage
        fluo_maskimage=M_particle<64 ? fluo_maskimage : nan

        //Dowindow /K fluomaskimage;newimage /K=1 /N=fluomaskimage
/F fluo_maskimage

        //crop cell of interest out of rest of the image
        fluo_maskimage=((abs(x-x_center)<cutout_half) && (abs(y-
y_center)<cutout_half)) ? fluo_maskimage : nan

        //SetAxis left bottom,top
        //SetAxis bottom left,right


        //do the flattening with specified flattening image (while
only copying the relevant cutout and open the flattened image
        duplicate /R=(left,right) (bottom,top) /FREE /O
fluo_maskimage,flat_image

        JRiterativeFlattening(flat_image,"plane",1,flatten_threshold,0,0)
        wave JRtempimage

        //Dowindow /K JR_flatimage; newimage /K=1 /N=JR_flatimage /F
JRtempimage

        //SetAxis left bottom,top
        //SetAxis bottom left,right


        //subtract the flattened image from the original fluoimage
to obtain gradient image
        duplicate /O fluo_maskimage,gradient_image
        gradient_image-=JRtempimage

        Dowindow /K GradientImage; newimage /K=1 /N=GradientImage /F
gradient_image

        //SetAxis left bottom,top
        //SetAxis bottom left,right


        //do a poly2d fit to obtain slope of gradient, and add
absolute values of both vector slopes to obtain gradient in intensity/m
increase.

```

```

CurveFit/Q/M=2/W=0 poly2D 1, gradient_image/D
wave W_coef

variable incr;incr=sqrt((W_coef[1]^2)+(W_coef[2]^2))

gradients[particle]=incr

//string incr_print=num2str(incr)
//print "Gradient increase is "+incr_print+" per m"

//if(particle==53)
//break
//endif
endfor
Dowindow /K Gradient_Table;edit /K=1 /N=Gradient_Table gradients
end

function Hve_Display_Particle_Positions(wave image[,variable
t_start,variable t_end])

t_start = ParamIsDefault(t_start) ? 0 : t_start
t_end = ParamIsDefault(t_end) ? inf : t_end
wave w_xcoords=W_spotx
wave w_ycoords=W_spotY
w_xcoords=(w_xcoords*dimdelta(image,0))+dimoffset(image,0)
w_ycoords=(w_ycoords*dimdelta(image,1))+dimoffset(image,1)
If(!WaveExists(w_xcoords))
    print "Run Fluo Gradient Procedure first."
    return 0
EndIf

variable index,ds_p=dimsize(w_xcoords,0),p_start,p_end
DoWindow/F BrowseImageGraph
RemoveFromGraph/ALL
SetDrawLayer/K UserFront

For(index=0;index<ds_p;index++)
    appendtograph w_ycoords[index][t_start,t_end] vs
w_xcoords[index][t_start,t_end]
    duplicate/O/RMD=[index][] w_ycoords temp
    [p_start,p_end]=GetDataLimitsDim1(temp)
    SetDrawEnv xcoord= bottom,ycoord= left,fsize= 8, textrgb=
(20000,40000,65535)
    DrawText
w_xcoords[index][p_start],w_ycoords[index][p_start],num2str(index)
EndFor
end

Static Function [variable p_min,variable p_max] GetDataLimitsDim1(wave
data) //copied from JS_Report_DisplayParticlePositions
variable ds=dimsize(data,1)
p_min=-1
Do
    p_min+=1
While(numtype(data[0][p_min])==2 && p_min < ds)
p_max=ds
Do

```

```

        p_max-=1
    While(numtype(data[0][p_max])==2 && p_max > 0)
        return [p_min,p_max]
End
//old stuff

//variable bottom_index=scaletoindex(fluo_maskimage,bottom,0)
//    variable top_index=scaletoindex(fluo_maskimage,top,0)
//    variable left_index=scaletoindex(fluo_maskimage,left,1)
//    variable right_index=scaletoindex(fluo_maskimage,right,1)
//    print top_index,bottom_index,left_index,right_index
//    print top,bottom,left,right

```