The effect of PIKfyve and PI4KIIIβ inhibitors on platelet spreading

Švorinić, Korina

Master's thesis / Diplomski rad

2023

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Rijeka / Sveučilište u Rijeci**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:193:823389

UNIVERSITY OF RIJEKA

DEPARTMENT OF BIOTECHNOLOGY

Master's program

Biotechnology in medicine

Korina Švorinić

The effect of PIKfyve and PI4KIIIβ inhibitors on platelet spreading

Master's thesis

Rijeka, 2023.

UNIVERSITY OF RIJEKA
DEPARTMENT OF BIOTECHNOLOGY
Master's program
Biotechnology in medicine

Korina Švorinić

The effect of PIKfyve and PI4KIIIβ inhibitors on platelet spreading

Master's thesis

Rijeka, 2023.

Mentor: Assoc. prof. Antonija Jurak Begonja, PhD

SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Diplomski sveučilišni studij
Biotehnologija u medicini

Korina Švorinić
Učinak PIKfyve i PI4KIIIβ inhibitora na adheziju trombocita
Diplomski rad

Rijeka 2023.
Mentor: dr.sc. Antonija Jurak Begonja, izv.prof.

Acknowledgment

I would like to thank my mentor, associate professor Ph.D. Antonija Jurak Begonja for the support, for the given opportunity, and for the knowledge she provided.

I would also like to thank current and past members of the laboratory, Ana Bura, Ivana Bertović, Sara Čabrijan, Klaudia Liker, and Robert Kolman who each helped in their own way by donating samples and giving advice and support. Thank you for the wonderful work atmosphere.

I thank my parents for always believing in me, for their love and unconditional support, and my friends who were always there for me.

Master's thesis was defended on September 26th, 2023 in front of the committee:

1. Asst. prof. Nicholas Bradshaw, PhD
2. Asst. prof. Milan Mesić, PhD
3. Assoc. prof. Antonija Jurak Begonja, PhD, mentor

Thesis has 66 pages, 21 figures and 34 references.

Financial support

This work was supported by University of Rijeka support grant No. 18-188-1343 and American Society of Hematology (ASH) Global Research Award-2019.

Abstract

Platelets are small anucleated blood cells whose primary role is hemostasis. Lately, platelets have been associated with other functions like their involvement in innate immunity, regulation of tumor growth and viral infection, gaining increasing scientific interest. In addition, molecular mechanisms that lead to platelet activation and downstream changes are still not completely understood. After activation, platelets adhere to the damaged vessel wall or activable surface, undergo cytoskeletal reorganization, and degranulation, which leads to their complete spreading. These processes are mediated by diverse signaling molecules, among others, also with small lipids called phosphoinositides (PIs). Phosphatidylinositol 3, 5-bisphosphate [$PI(3,5)P_2$] produced by phosphatidylinositol-3-phosphate 5-kinase (PIKfyve) regulates membrane trafficking. Phosphatidylinositol 4-phosphate (PI4P) produced by phosphatidylinositol-4-kinase-III-beta (PI4KIIIβ) and other PI4Ks is a source of a key signaling molecule, $PI(4,5)P_2$, that also mediates actin cytoskeletal dynamics. Previous transcriptomic and proteomic data show the expression of PIKfyve and PI4KIIIβ in both mouse and human platelets. In this work, we aimed to decipher if pharmacological inhibition of PIKfyve (inhibitor YM201636) or PI4KIIIβ (IN10) prevents human platelet spreading. In addition, we analyzed downstream signaling pathways in the presence of the inhibitors after activating collagen receptor (GPVI), namely phosphorylation of Syk kinase and acetylation of tubulin. Also, we evaluated the localization of PIKfyve and PI4KIIβ (another PI4K) in human platelets in resting and activated conditions by immunostaining. We conclude that PI4KIIIβ is potentially involved in platelet spreading by modulating the cytoskeleton, however, additional studies are needed to confirm this observation.

Key words: platelets, PIKfyve, PI4Ks, PI4IIIβ, platelet spreading

SAŽETAK

Trombociti (krvne pločice) su male krvne stanice bez jezgre čija je primarna uloga u hemostazi. U posljednje vrijeme trombociti se povezuju s drugim funkcijama te njihovu uključenost u urođeni imunitet, regulaciju rasta tumora, razvoj virusnih infekcija, čime se povećava znanstveni interes za ove stanice. Osim toga, molekularni mehanizmi koji dovode do aktivacije trombocita i nizvodnih promjena još uvijek nisu u potpunosti razjašnjeni. Nakon aktivacije, trombociti prianjaju na oštećenu stijenku žile ili površinu koja ih može aktivirati, podliježu citoskeletnoj reorganizaciji i degranulaciji, što dovodi do njihovog potpunog širenja (eng. *spreding*). Ti procesi su posredovani različitim signalnim molekulama, između ostalih, i malim lipidima koji se nazivaju fosfoinozitidi (PI). Fosfatidilinozitol 3, 5-disfosfat [$PI(3,5)P_2$] koji proizvodi fosfatidilinozitol-3-fosfat 5-kinaza (PIKfyve) regulira promet membrana. Fosfatidilinozitol 4-fosfat (PI4P) koji proizvodi fosfatidilinozitol-4-kinaza-III-beta (PI4KIIIβ) i druge PI4- kinaze izvor je ključne signalne molekule, $PI(4,5)P_2$, koja također posreduje u dinamici aktinskog citoskeleta. Prethodni transkriptomski i proteomski podaci pokazuju ekspresiju PIKfyve i PI4KIIIβ u mišjim i ljudskim trombocitima. U ovom smo radu željeli utvrditi sprječava li farmakološka inhibicija PIKfyve (inhibitor YM201636) ili PI4KIIIβ (IN10) širenje ljudskih trombocita. Uz to, analizirali smo nizvodne signalne putove u prisutnosti inhibitora nakon aktivacije kolagenskog receptora (GPVI), fosforilaciju Syk kinaze i acetilaciju tubulina. Također, imunobojanjem smo analizirali lokalizaciju PIKfyve i PI4KIIβ (još jedna PI4- kinaza) u ljudskim trombocitima u uvijetima mirovanja i aktivacije. Zaključujemo da je PI4KIIIβ potencijalno

uključena u širenje trombocita modulacijom citoskeleta, no potrebna su dodatna istraživanja kako bi se potvrdilo ovo opažanje.

Ključne riječi: trombociti, PIKfyve, PI4Ks, PI4IIIβ, širenje trombocita

Table of contents

1. Introduction

1.1 Platelets

Platelets are, after erythrocytes, the second most abundant blood cell type [1]. They are anucleated small blood cells whose primary function is hemostasis, preventing blood loss during vessel damage [2]. Besides hemostasis, platelets play an important role in the development of many diseases. Their size averages from 2.0 to 5.0 μm in diameter and 0.5 μm in thickness which makes the smallest cells in circulating blood [3]. The platelet count in a healthy adult human is 150-400 $x10^9$/L while their lifespan in the blood is 8-10 days [2].

Circulating platelets arise from megakaryocytes (MK) in the bone marrow. MKs arise through the commitment of hematopoietic stem cells (HSC) to differentiate into MK lineage, proliferation of progenitors and lastly maturation of MKs (Figure 1.) [4]. PLT lifespan is controlled by apoptosis which triggers platelets cell death after 10 days if they do not activate and recruit forming a blood clot [4].

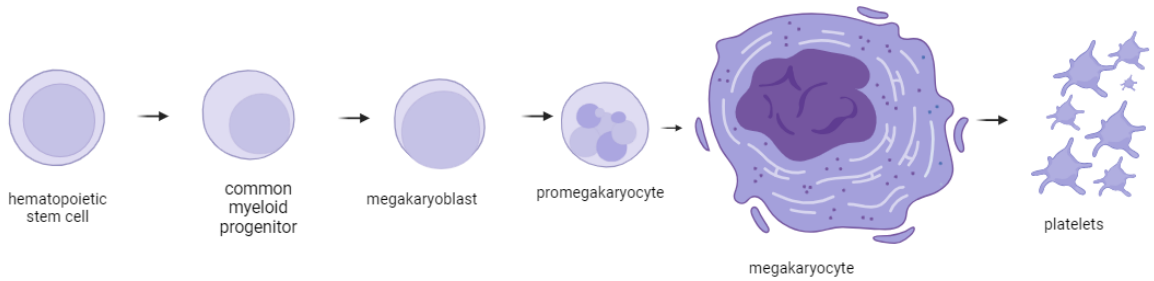

Figure 1. Production of platelets. Platelets arise from megakaryocytes. By commitment of the hematopoietic stem cell (HSC) to a common myeloid progenitor, megakaryocyte progenitors proliferate and differentiate into megakaryocytes that eventually give rise to circulating platelets. Image was made using Biorender.

1.2 Activation and platelet shape change

Platelets circulate in the blood in the resting state and upon vessel damage they activate by adhering to the site of injury and gradually they form a platelet aggregate [3]. By adhering to the damaged site, they activate different signaling pathways, reorganize the cytoskeleton, and release the content of their intracellular granules [3].

Resting platelets circulate in the blood in a discoid shape. Their shape and cell integrity are maintained by the cytoskeleton which consists of actin, tubulin and spectrin [2]. Cytoskeleton is a dynamic system that has the ability to remodel upon activation so platelets can change shape and aggregate during hemostasis [2].

Microtubules keep the discoid shape of platelets, they are organized in bundles of 3-24 microtubules forming microtubular coils, a recognizable component of resting platelets (Figure 2A-B). If the coil is disrupted, platelets lose their discoid shape and become spherical. Microtubule coils can re-polymerize so platelets can regain their discoid shape [2].

Actin microfilaments (F-actin) are thin, flexible fibers and polymers of actin monomer that have the ability to polymerize [2]. Their role is similar to microtubules, they provide structural support to resting platelets that are subjected to different forces in circulation [2]. Filaments are organized in meshwork that provide a matrix that attaches other structural components of platelets (Figure 2C-D) [2].

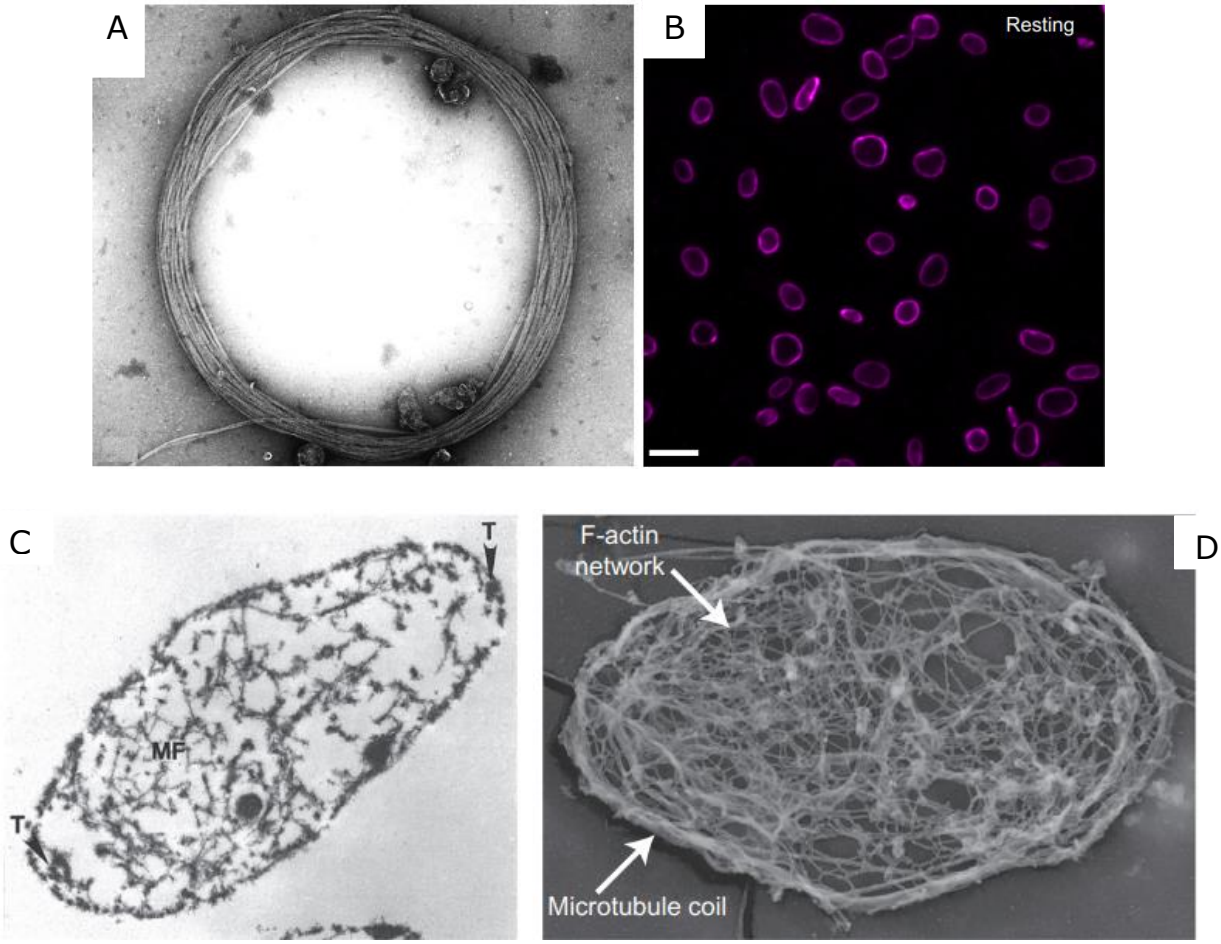

Figure 2. Cytoskeleton components of resting platelet. (A) Electron micrograph showing microtubule coil around platelet disc. (B) Resting platelets stained for α-tubulin in order to display microtubule coil. (C) The Meshwork of actin filaments (MF) and microtubule coil (T). (D) Cytoskeleton of resting platelet. Image taken from: Thomas SG. „The Structure of Resting and Activated Platelets" *Platelets* 2019 [2].

As mentioned above, there are many changes that occur following platelet activation. Platelets become activated *via* surface receptors that stimulate a number of complex and coordinated processes to clot them. Platelet responses include activation of different transduction pathways, secretion of granule contents, activation of surface glycoproteins, etc. [2]. The response that is the focus of this thesis is platelet shape change that is mediated by changes in the cytoskeleton. These changes are potentially mediated by lipids that will be described below, phosphoinositides.

During platelet activation, the main step in platelet shape change is the spreading of the platelet. Polymerization of actin filaments drives the spreading process while microtubular remodeling drives shape change from disc to sphere [2]. When platelets make contact with the surface, they change shape to a sphere from which filopodia is protruded [2]. Filopodia formation is driven by the elongation of F-actin bundles [2]. Filopodia and its finger-like projections interact with the extracellular matrix. After filopodia structure, platelets begin to flatten out and form lamellipodia. During lamellipodia formation, organelles and granules of platelets centralize and spread platelets to form a distinctive „fried egg" shape (Figure 3.) [2].

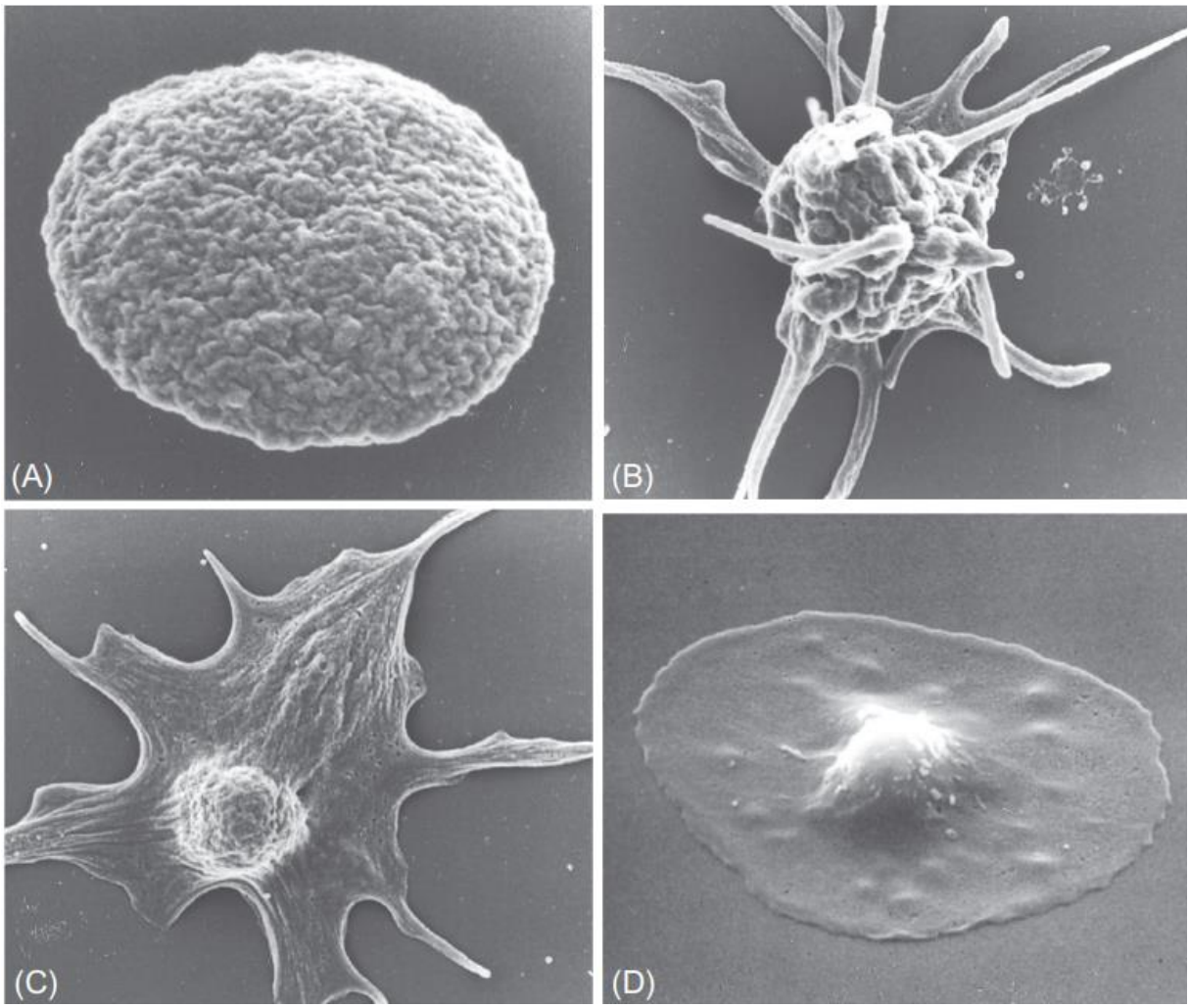

Figure 3. Activation of platelet and changes in shape that follow. (A) Resting platelet that circulates in blood has a discoid shape. (B) Early

spreading and activation of platelets with filopodia extensions. (C) Formation of lamellipodia during further activation and spreading. (D) Fully activated and spread platelet. Image taken from: Thomas SG. „The Structure of Resting and Activated Platelets" *Platelets* 2019 [2].

Fluorescence imaging allowed to follow reorganization of F-actin and microtubular coil during activation and spreading. In the initial adhesion state, PLTs form dynamic actin nodules while actin cytoskeleton reorganizes into dendritic arrays or bundles [2]. Later, during the fully spread shape, the formation of actin-myosin stress fibers is present to support the PLT shape (Figure 4A). The microtubular coil is in the center and forms a small coil, later it depolymerizes and forms microtubular arrays in the fully spread and activated state (Figure 4B).

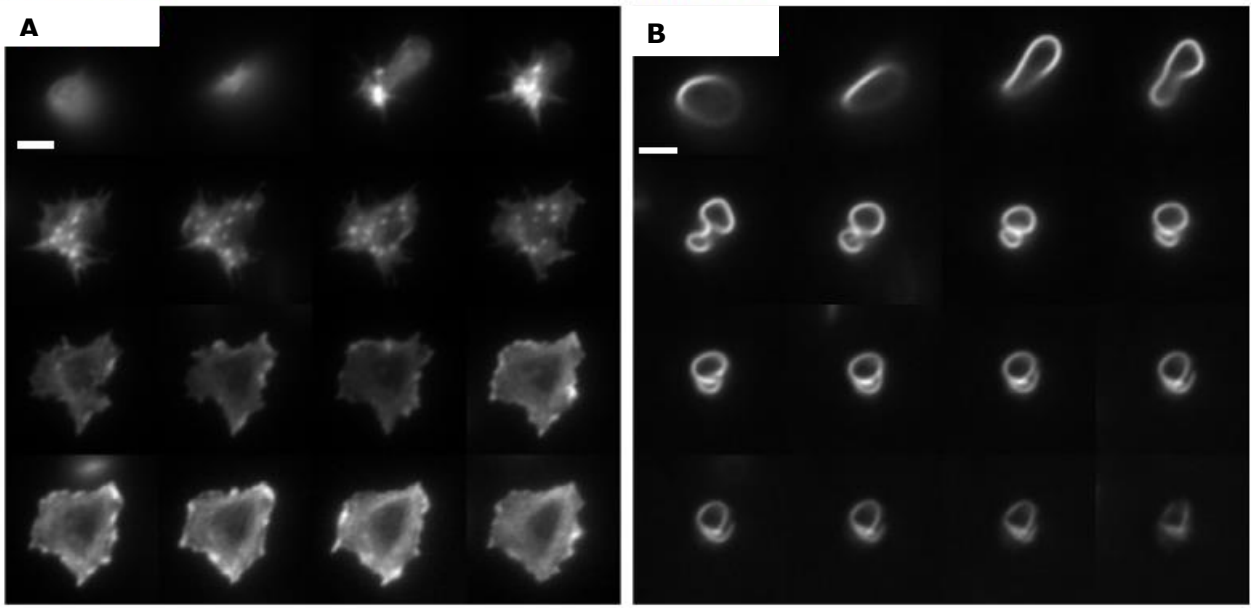

Figure 4. Morphological and cytoskeletal changes that occur during the spreading and activation of platelets. (A) Actin cytoskeleton changes in spreading platelets. (B) Microtubule coil changes in spreading platelets. Image taken from: Thomas SG. „The Structure of Resting and Activated Platelets" *Platelets* 2019[2].

1.3 Phosphoinositides

Activation of platelets influences various signal transduction pathways that modulate the cytoskeleton, one of which are phosphoinositides (PIs) [2]. Phosphoinositides are abundant and complex components involved in intracellular trafficking and signal transduction as well as other important functions like cytoskeletal reorganization, and cell mobility [5,6]. Metabolism of PIs is in control of many different kinases, phosphatases and phospholipases [5]. Keeping homeostasis in the metabolism of PI is important since its distortion is suggested to be involved in oncogenesis, infections, neurodegeneration, etc.

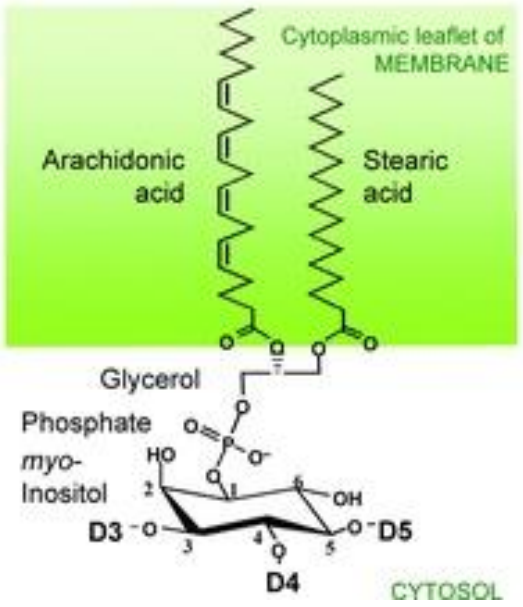

Figure 5. Structure of phosphoinositides. Positions D3, D4 and D5 can be phosphorylated. Glycerol backbone is esterified to two fatty acids and a phosphate. Image taken from: Li Y ping *et al.* „Research progress of phosphatidylinositol 4-kinase and its inhibitors in inflammatory diseases" *Eur J Pharmacol* 2021[7].

PIs structure consists of a glycerol backbone esterified with two fatty acid chains and a phosphate that is attached to a polar head which extends into the cytoplasm (Figure 5). [8]. Polar head group is the cyclic polyol *myo*-

inositol, $(CHOH)_6$ [8]. The inositol ring of PI contains five hydroxyl groups where positions D3, D4 and D5 can be phosphorylated by different lipid kinases [7]. By phosphorylating these positions by appropriate kinases, seven kinds of PIs can be produced (Figure 6).

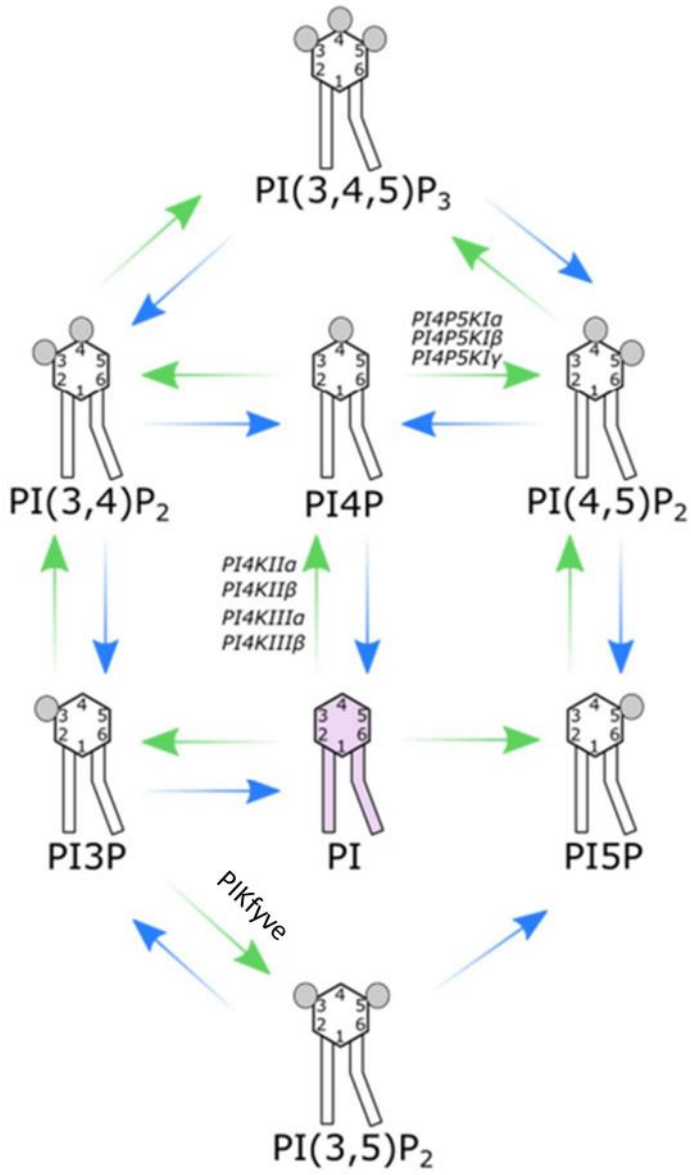

Figure 6. Seven different kinds of phosphoinositides are formed from the phosphorylation of hydroxyl groups on the head of PIs by different kinases. The source of PI5P is still not establish, PI4P is synthesized by four

different types of PI4Ks. Image was taken from: Pip TS, Bura A, Cabrijan S, Đuri I. „A Plethora of Functions Condensed into Tiny Phospholipids“ 2023 [5].

1.4 PI kinases (PIK)

Specific lipid kinases produce seven different phosphorylated phosphoinositides. Less abundant PIs are phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 5-phosphate (PI5P), phosphatidylinositol (3,4)-bisphosphate [$PI(3,4)P_2$] and phosphatidylinositol (3,5)-bisphosphate [$PI(3,5)P_2$].

A kinase that synthesizes $PI(3,5)P_2$ is 1-phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) [9]. It phosphorylates PI3P to produce $PI(3,5)P_2$ [9]. Unlike other PIs, $PI(3,5)P_2$ is low abundant so there are some limitations in understanding its function [10]. It plays a role in cellular homeostasis and in adaptation to stimuli where it acts as a signalling molecule [11].

One of the most important kinases, phosphatidylinositol 4-kinases (PI4K) catalyzes the production of phosphatidylinositol 4-phosphate (PI4P), and therefore the first step in the biosynthesis of four other (out of seven) PI lipids [12]. PI4P is an important regulator of membrane traffic [12] and also a precursor for other two signaling PIs, phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] and phosphatidylinositol 3,4,5- trisphosphate [$PI(3,4,5)P_3$] [12]. PI4P and $PI(4,5)P_2$ are the most abundant PIs in cells. There are four types of PI4K, type III and type II PI4Ks. Type III is the more studied type of PI4K and it produces PI4P at the Golgi and plasma membrane while type II was identified more recently as integral membrane proteins [12].

1.5 PIKfyve

Another important kinase is phosphatidylinositol-3-phosphate 5-kinase (PIKfyve) which is essential for the synthesis of phosphatidylinositol-3,5-bisphosphate [$PI(3,5)P_2$] via PI3P phosphorylation [9]. There are several

reports saying that PIKfyve catalyzes the synthesis of phosphatidylinositol 5-phosphate (PI5P) but the PI5P pools synthesized by PIKfyve are still poorly described [13]. PIKfyve and its phosphorylated product play an important role in membrane homeostasis as well as in vesicle trafficking [9]. It was shown that in mice, depletion of PIKfyve in platelets leads to arterial thrombosis and multiorgan defects that impair fertility and development [9]. This confirms that PIKfyve plays an important role in the biogenesis and function of platelet granules [9]. The most potent inhibitor for PIKfyve is YM201636.

1.6 PI4Ks

PI4K is divided into two classes, type II and III [14]. These two types have very different evolutionary histories, but both types have evolved to generate PI4P [14]. Type II kinases are composed of PI4KIIα and PII4KIIβ. This family regulates the growth of nerve terminals and plays a role in the recovery of synaptic vesicles [15]. Type III kinases are composed of PI4KIIIα and PI4KIIIβ, two isoforms important for Golgi compartment [15].

1.6.1 Type III PI4K

Type III PI4Ks play essential roles in many cellular processes, although it is still unknown how these enzymes are regulated[16]. These are large enzymes with several protein-binding partners [16]. PI4KIIIα is an enzyme that primarily localizes at the plasma membrane and is the main source of PI4P pools that later generate $PI(4,5)P_2$ and $PI(3,4,5)P_3$ [14]. Meanwhile, PI4KIIIβ is mainly localized at the Golgi and is part of the trans-Golgi network (TGN)[14]. It plays a role in lipid transport and membrane trafficking[14]. It generates PI4P sub-pools in the lysosomal and outer mitochondrial membrane and ER[5]. PI4KIIIβ and its involvement in viral infection, has raised interest in the development of inhibitors that could be used as anti-

viral therapeutics [14]. There are many potent inhibitors of PI4KIIIβ, one of which is IN-10.

1.6.2 Type II PI4K

The structures of the two isoforms of PI4KIIs are very similar. Both kinases have N-terminal and C-terminal regions separated by long non-conservative insert regions as well as the catalytic domain with a conservative palmitoylation motif [15]. The N-terminus of PII4KIIβ is acidic and interacts with adapter protein-1 while its C-terminal region can bind the molecular chaperon heat shock protein 90 (Hsp90) which is important for stabilizing PI4KIIβ in cytoplasm [15]. PIFKIIβ (55 kDa) is the less studied and less active of the two PI4Ks [5]. This kinase is, to a smaller extent, linked to the plasma membrane, endoplasmic reticulum (ER), trans-Golgi network (TGN) or clathrin-coated vesicles, but it is mostly cytosolic [5]. Although it is less studied than PI4KIIα, it was shown that PI4KIIβ synthesizes PI4P pools between the TGN and endosomes and that it is associated with intracellular signaling and phagocytosis[5]. Interestingly, it plays a role in cancerogenesis and inflammation where its downregulation promotes invadopodia structures and cancer metastasis [5]. Since there is evidence of the role of PI4KIIβ in different cellular processes and diseases, there are a number of new studies on its structure and attempts to design selective inhibitors.

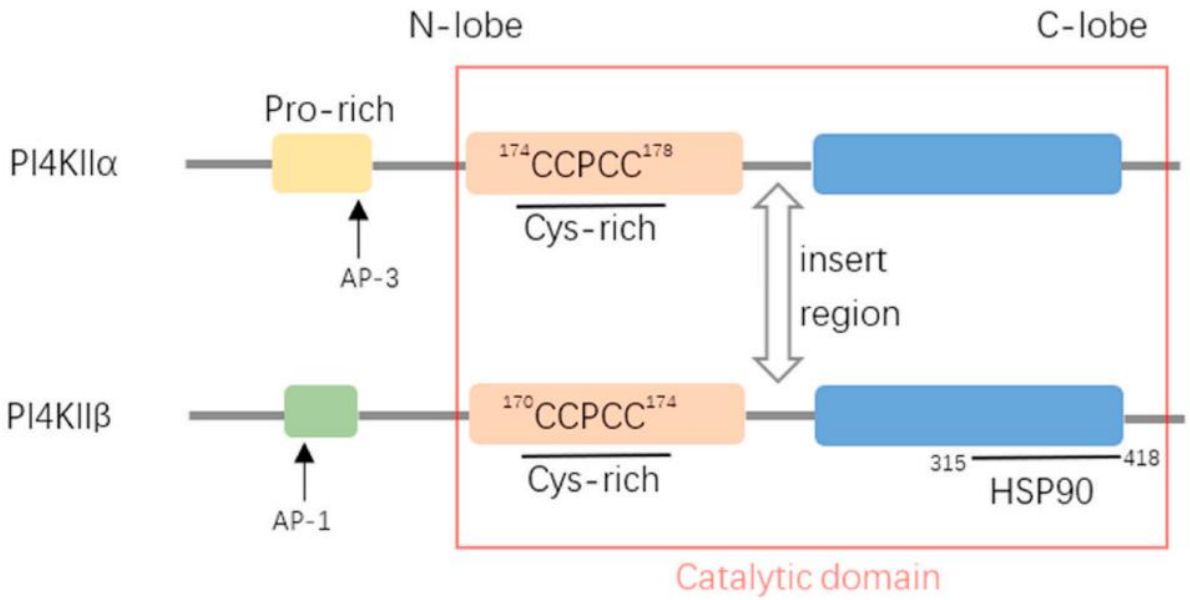

Figure 7. Structures of two isoforms of PI4IIKs. Both isoforms have an N-terminal and C-terminal separated by an insert region. HSP90 binds to PI4KIIβ in the region between residues 315–481 in the C-lobe. Image taken from: Waugh MG. „The Great Escape: How phosphatidylinositol 4-kinases and PI4P promote vesicle exit from the Golgi (and drive cancer)" *Biochem J* 2019 [15].

1.7 PIK inhibitors

IN-10 or Compound 10 is currently the most potent inhibitor of PI4KIIIβ with IC_{50} ~ 3.6 nM [17]. It was shown that it has minor off-target inhibition of other kinases related to PI4KIIIβ [17].

YM201636, the inhibitor of PIKfyve, is the ATP-competitive compound with the IC_{50} 33 nM [18]. This compound inhibits PIKfyve catalyzed PI(3,5)P_2 synthesis so it is widely used to investigate the relevance of PIKfyve in different cellular processes [13,19]. Interestingly, it was shown that inhibition of PIKfyve with YM201636 has a positive effect in cancer treatment since it suppresses liver cancer growth inducing autophagy [18, 20].

1.8 Phosphoinositides in platelets

In platelets, PIs mediate membrane remodeling and vesicular trafficking that are needed for platelet activation [21].

Phosphatidylinositol 3-phosphate (PI3P) is produced by vacuolar protein sorting 34 (Vps34, PIP3, class III) and is a key component of vesicular trafficking [19]. It was discovered that by depletion of PI3P or specific pharmacological inhibition of Vsps34 decreased proplatelet formation [19]. PI3P is the substrate for PIKfyve that produces $PI(3,5)P_2$. Other studies confirmed how its inhibition reduces proplatelet formation *in vitro* which indicates that proplatelet production depends on both PI3P and $PI(3,5)P_2$[19].

A demarcation membrane system (DMS) is a membrane reservoir for the formation of proplatelets and platelets[19]. During MK enlargement DMS expanses and enriches with plasma membrane $PI(4,5)P_2$ which is considered a marker for membranes of MKs [19]. $PI(4,5)P_2$ can directly regulate a few cytoskeletal proteins through interaction with the pleckstrin homology domain (PH) and few other $PI(4,5)P_2$ binding motifs [22]. It was shown how it can control the structure and the extent of the actin cytoskeleton by regulating proteins that are in charge of binding or crosslinking actin filaments or sequestering actin monomers [22].

Artificially increasing levels of $PI(4,5)P_2$, by overexpression of the kinase that generates it, induces actin polymerization [23]. $PI(4,5)P_2$ is mostly present at the plasma membrane where it is very abundant (2–5mol% of total cellular PIs) and where it acts as a signaling molecule [21]. Another study showed that OCRL, 5-phosphatase, that dephosphorylates $PI(4,5)P_2$ into PI4P, recruits actin-binding proteins which nucleate actin polymerization leading to platelet shape change [24].

Interestingly, glycoprotein VI (GPVI) activates platelets *via* protein tyrosine kinase cascade and tyrosine kinase Syk [25]. This signaling pathway includes phosphorylation of the Fc receptor by Src-family kinases and a GPVI signalosome, in which Syk phosphorylates and activates phospholipase C

(PLC)-γ2, that hydrolyses $PI(4,5)P_2$ and gives rise central second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [25]. Human platelet spreading on fibrinogen is impaired in GPVI deficient patients [26]. Interestingly, the formation of lamellipodia in platelets is blocked by the inhibitors of Syk tyrosine kinase while adhesion of platelets to collagen and fibrinogen induces phosphorylation of Syk [26].

With all of the involvement of different PIs in platelet or megakaryocyte biology, we wanted to describe two less studied kinases. We wanted to see how pharmacological inhibition of PIKfyve and PI4KIIIβ impacts the spreading of platelets. Also, we wanted to analyze if inhibition of PIKfyve and PI4KIIIβ changes signaling (phosphorylation of Syk) and modification of tubulin.

2. Aims of the thesis

Platelets are important cells for human health, they play a major role in haemostasis and are involved in the pathophysiology of different diseases. Many signaling and regulatory components are needed for their function, such as PIs. The aim of this thesis is to investigate if PIKfyve and PI4KIIIβ contribute to the spreading of human platelets. Our hypothesis is that both PIKfyve and PI4KIIIβ have a regulatory role in human platelet activation.

The specific aims of this study are:

1. To optimize immunostaining and analyze the localization of PIKfyve in resting and activated human platelets of different donors by immunofluorescence
2. To determine the effect of pharmacological inhibition of PIKfyve and PI4KIIIβ on platelet spreading on collagen by specific inhibitors (YM201636 and IN-10, respectively)
3. To analyze if inhibition of PIKfyve and PI4KIIIβ changes signaling (phosphorylation of Syk) and modification of tubulin in platelets activated with collagen-related peptide
4. To optimize and analyze the localization of PI4KIIβ in resting and activated human platelets of different donors by immunofluorescence

3. Materials and methods

3.1. Human platelets isolation

This study was approved by the ethics committee of the Department of Biotechnology (#644-01/21-01/03-01). Every blood donor signed informed consent in accordance with the Declaration of Helsinki and ethics committee approval. Blood was isolated from healthy adult donors. Peripheral blood was drawn in a syringe containing Aster-Jandl anticoagulant (85 mM tri-sodium citrate dihydrate, 69 mM citric acid, 111 mM D-glucose; 1:10; AJ). Blood was transferred into tubes 10 mL each and 50 µL of 3 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 1:200, *Carl Roth*) was added. First centrifugation was performed at 280xg for 20 minutes. Platelet-rich plasma (PRP) was gently transferred without the buffy coat in separate tubes and diluted with CGS buffer (120 mM NaCl, 12.9 mM tri-sodium citrate dihydrate, 10 mM D-glucose; pH 6.5; 1:1). Second centrifugation was performed at 380xg for 10 min to pellet platelets. Supernatant was discarded while platelet pellets were resuspended in CGS buffer (1:1) and apyrase was added (0.2 U/mL; 1:1000, Sigma Aldrich) to prevent activation with ADP during centrifugation. Third centrifuge was performed at 400xg for 5 minutes. The platelet pellet was resuspended in HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM HEPES, 5 mM D-glucose; pH 7.4). Platelet concentration was measured with PocH-100i analyzer. Platelet count was adjusted to 3×10^8 /mL with HEPES buffer, and platelets were left to rest for 30 minutes in a bath at 37°C before starting the experiment (spreading and/or inhibitor treatment).

3.2. Immunofluorescence of platelets

3.2.1. Coverslips coating

For immunofluorescence of resting platelets, we used poly-L-Lysin (*Sigma;* PLL) coated glass coverslips (12 mm diameter). A final 0.1 mg/ml concentration of PLL solution was prepared by adding 100 µL aliquots of 1

mg/ml of stock solution was diluted with 900 µL of distilled water (dH_20) and vortexed. Coverslips were placed on a plate covered with parafilm and washed 3x with dH_20. Next, each coverslip was covered with 100 µL of PLL and left for 30 minutes. Coverslips were washed 3x with phosphate-buffered saline (PBS) and left to dry until the experiment.

For activated platelets, coverslips were coated with collagen (10 µg/mL, Takeda). Briefly, coverslips were put on a plate covered with parafilm and washed 3x with dH_20. Collagen solution was prepared from 10 µL of collagen 10 µg/mL stock solution diluted with 990 µL of physiological glucose solution (1:100, *Takeda*). On each coverslip, 100 µL of solution was added and left to coat overnight at +4ºC. Coverslips were washed 3x with PBS and covered with 100 µL of BSA (5mg/ml, *Capricorn Scientific*) in PBS for an hour to block collagen-unbound areas. Washed coverslips were left in 500 µL of PBS until the experiment.

3.2.2. Deposition of resting platelets

Isolated platelets were left to rest for 30 minutes in a 37ºC bath. Previously prepared PLL coated coverslips were placed in the wells of a 24-well plate and filled with 500 µL of 4% paraformaldehyde solution (PFA). To capture unactivated, resting platelets, 3 µL of platelets were added directly into 4% PFA solution on coverslips and left to be fixed for 10 minutes. Next, centrifugation of plates was performed at 400xg for 5 minutes and wells with coverslips were washed 3x with 500 µL of PBS. Platelets were permeabilized with 300 µL 0.1% Triton X-100 in PBS (10 minutes) or 300 µL 0.5% Tween 20 in PBS (20 minutes). Coverslips with platelets were washed with PBS 3x and blocked with 300 µL of 5% goat serum (GS, Sigma) for 45 minutes.

3.2.3. Platelet spreading

Collagen-coated coverslips were placed in wells filled with 500 µL of pre-warmed HEPES buffer. To activate platelets, 3 µL of platelets that were resting for 30 min were added directly into HEPES buffer covering collagen-coated coverslips. Platelets were left to be activated and spread for 45 minutes in an incubator at 37ºC. After spreading, the HEPES buffer was removed and platelets were fixed with 4% PFA (300 µL) for 15 minutes. Coverslips were washed 3x with 500 µL of PBS. Permeabilization of cells was done by 300 µL of 0.1% Triton X-100 in PBS (10 minutes) or 300 µL 0.5% Tween 20 in PBS (20 minutes). Cells were blocked with 300 µL of 5% GS for 45 minutes. After blocking, cells were washed 3x in PBS and left in PBS until immunostaining was performed.

3.3. Analysis of pharmacological inhibitors on platelet spreading

Previously isolated platelets were aliquoted based on the number of conditions in the experiment and left to rest for 30 minutes in a 37ºC bath. In the experiments, we used the inhibitor of PIKfyve YM201636, the inhibitor of PI4KIIIβ IN-10 and the inhibitor of PI4KIIIα GSK-A1. Stock solutions of the inhibitors were as follows: IN-10 (10 mM) and YM201636 (10 nM). Inhibitor dilutions were prepared from stock. Final concentrations that were used for platelet treatment for YM201636 inhibitor were: 500 nM, 1 µM, and 5 µM. Dilutions were prepared by adding 1µL of stock solution and 399 µL of HEPES buffer (500 nM, 1:400), 1 µL of stock solution and 199 µL of HEPES buffer (1 µM, 1:200) and 1 µL of stock solution and 39 µL of HEPES buffer (5 µM, 1:40). The final concentrations for IN-10 inhibitor were: 100 nM, 1 µM, 5 µM and 10 µM. Dilutions were prepared by making serial dilutions to avoid excessive buffer usage by adding 1µL of stock solution and 99 µL of HEPES buffer and 1 µL of this solution into 19 µL of HEPES buffer (100 nM, 1:100+1:20), 1 µL of stock solution and 199 µL of HEPES buffer (1 µM, 1:200), 1 µL of stock solution and 39 µL of HEPES buffer (5 µM, 1:40) and 1 µL of stock solution and 19 µL of HEPES buffer

(10 µM, 1:20). As a negative control (with known inhibitory effect), we used, GSK-A1 inhibitor at a final concentration of 500 nM that was prepared from working solution (5 mM, 1:10000). Since inhibitor stock solutions were prepared in DMSO, control (CTRL) samples were treated with same dilutions of DMSO as the highest concentration of inhibitor used. Finally, 1 µL of the premade inhibitor from the working solution was added to 50 µL of resting platelets, gently mixed and returned into bath for 15 minutes of preincubation. Previously prepared PLL or collagen-coated coverslips for resting and activated PLTs, respectively, were filled with 500 µL of pre-warmed HEPES buffer or room temperature 4% PFA. Into each well was added 5 µL of appropriate inhibitor for indicated final concentrations and 5 µL of appropriate platelet pre-incubation mixture. For resting conditions, platelets were fixed for 10 min in 4% PFA, and for activation, platelets were let to spread for 45 min in the incubator at 37ºC. Experiments were further conducted following the earlier described resting/activated platelet protocol.

3.4 Immunostaining and fluorescence microscopy

On a plate covered with parafilm was placed 30 µL of primary antibody mix. The primary antibody solution was prepared with 5% GS in PBS. Primary antibodies used were mouse anti-a-tubulin (#SC5286 Santa Cruz Tubulin; 1:100), rabbit anti-PIKfyve (#HPA0402604 Atlas Antibodies; 1:100), rabbit anti-PI4KIIB (#PA1-30306 Invitrogen; 1:100) and phalloidin conjugated with Alexa Fluor (AF)-488 (#A12379 Invitrogen; 1:100), phalloidin conjugated with iFluor-555 (#A12379 Abcam; 1:100). On a 30 µL mix of primary antibody, coverslips were placed with cells facing the drop of the solution. Primary antibodies were left to incubate in dark for an hour at room temperature. Following incubation with primary antibody, cells were washed three times with PBS. The solution mix for the secondary antibody was prepared and diluted in GS (1:5000). The secondary antibodies used were goat anti-rabbit IgG-Alexa Fluor 488 (#A11070 Life technologies,

1:500) and goat anti-mouse IgG-Alexa Fluor 555 (#A21424 Life technologies, 1:500). After 2 hours, cells were washed 3x in PBS, mounted with glue on microscopic glass and left to dry overnight in the dark.

Images were obtained using a *Zeiss Axio Observer Z1* microscope with *Zeiss Axiocam 506* mono microscopic camera*, Zeiss apochromat 60× Oil* objective and *Zeiss HXP 200 C* fluorescent illuminator. Cell surfaces were measured using Fiji ImageJ software. Images were taken under the conditions to obtain maximal quality (at different exposure times).

3.5 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot Analysis

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method for obtaining high-resolution separation of mixtures of proteins [27]. SDS-PAGE denatures proteins that will undergo electrophoresis [27]. For the analysis of signaling pathways in the absence or presence of different concentrations of inhibitors, platelets were activated with 1 µg/mL collagen-related peptide (CRP). These experiments were performed with platelets in solution ($3x10^8$/ml), in the volume of 50-100 µL. Activation was stopped after 2 minutes of CRP activation by lysing platelets with 3% SDS-STOP/β-mercaptoethanol (200 mM Tris, 6% SDS, 15% glycerol, 10 mg bromophenol blue, pH 6.7). Lysates were denatured for 5 minutes at +95 °C and frozen at -20 °C. Resolving gel was prepared first, by adding 1.5 M TRIS-HCl (pH 8.8), 10% SDS, 30% acrylamide, 10% APS and TEMED. APS is added last to polymerase the gel and 150 µL of isopropanol is added to poured gel so the gel is evenly distributed. While the resolving gel is polymerizing, stacking gel is prepared with the same reagents as resolving gel but the 1M TRIS-HCl is used instead of 1.5 M. Gel is poured and the comb is added immediately.

Following the preparation of resolving and stacking gel, samples were vortexed and kept on ice. As a marker for gel electrophoresis, we used 3 µL

of protein ladder (PageRuler™ Plus Prestained Protein Ladder *Thermo Scientific*) and then 25 µL of platelet sample. Electrophoresis was run first 15 minutes at 70-80 V and then at 123-130 V for an hour. Following electrophoresis, proteins were transferred onto the nitrocellulose membrane (*Santa Cruz Biotechnology*). The transfer casket was made of layers that included sponge, filter paper, gel, nitrocellulose membrane, and again filter paper and sponge. All components of the casket were immersed in transfer buffer (100 mL of 10x Transfer buffer, 700 mL of distilled water, 200 mL methanol). The transfer casket was placed into a transfer tank filled with transfer buffer for 90 minutes at 0.5-1 A. The membrane was stained first with Ponceau S to visualize the correct transfer of proteins. Blocking of nitrocellulose membrane was completed by incubating the membrane in 3% BSA in Tris-balanced solution with Tween 20 (TBS-T, 0.1 M Tris, 1.5 M NaCl, 0,1% Tween 20; blocking solution) for 45 minutes. First antibody was diluted in 5 mL blocking solution (1:1000) containing sodium azide (5%) and incubated overnight at 4ºC on a shaker. The primary antibodies used were rabbit anti-phospho-Syk (#2710S, Cell Signaling), mouse anti-acetylated tubulin (SC-23950, Santa Cruz) and mouse anti-GAPDH (#MAB374, Merck). After overnight incubation, the membrane was washed 3x for 10 minutes each time in TBS-T. Secondary antibodies were diluted in 15 mL of blocking solution (1:2000) and incubated for an hour at room temperature on shaker. Secondary antibodies were anti-mouse IgG antibodies (#7076S, Cell Signaling) and anti-rabbit IgG (#7074S, Cell Signaling) conjugated with horseradish peroxidase (HRP). Before detection, the membrane was washed with TBS-T 3x for 10 minutes each time. Proteins were visualized using 100 µl of LUMI reagent (1:1, #12015200001, Roche) and 100 µL of Super Signal (1:1, #34095, Thermo Scientific) on BioRad ChemiDoc™ MP visualizing system.

3.6 Data Analysis

Immunostaining experiments were performed 3 times with inhibitor YM201636 and 4 times with IN-10. Western blot experiments were performed 4 times with the blood of different donors. Data are present as a mean ± standard error of the mean (SEM). Data were analyzed by one-way ANOVA test using Prism software (GraphPad) following Bonferroni test. The level of statistical significance is $p<0.05$ (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

4. Results

4.1. Optimization of PIKfyve immunofluorescence staining in human platelets

Previously, expression of PIKfyve was observed in human platelets (PLTs) by Western blot (unpublished data, Laboratory of hematopoiesis) or in literature [28]. Our goal was to visualize PIKfyve in both resting and activated PLTs by immunofluorescence. As described in detail in Methods, both resting and activated PLTs were permeabilized with 0.1% Triton X-100 or with 0.5% Tween 20 in PBS.

In the resting PLTs, the discoid shape is maintained by the internal cytoskeleton that consists of actin and tubulin [3]. Figure 1. shows that the microtubule ring stained by α-tubulin was preserved indicating resting PLTs. Interestingly, staining of the microtubular ring was more successful in Triton X-100 permeabilized cells (Figure 8.) rather than in Tween 20 permeabilized cells (Figure 8.). F-actin filaments in resting PLTs appear as a sparse fibrous network [3]. Permeabilization with Triton X-100 showed to be more efficient as immunostaining of PIKfyve was stronger than in the case of Tween 20. In most cases PIKfyve stained uniformly within resting PLTs without forming any specific structures (Figure 8.).

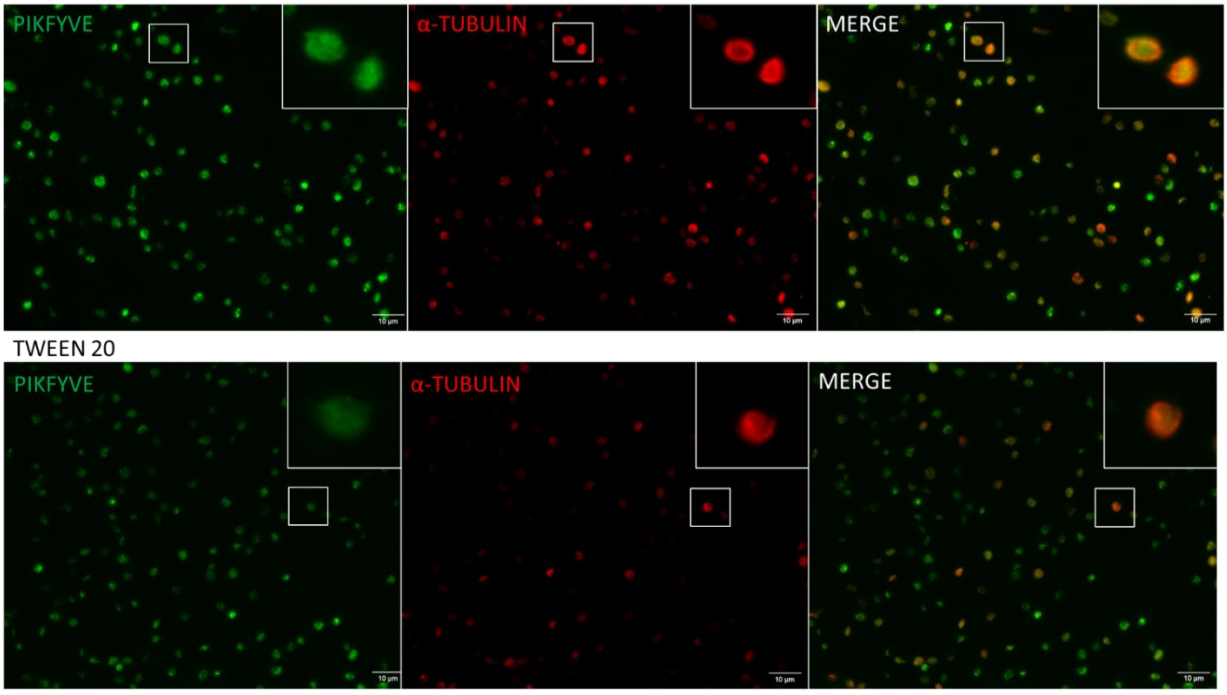

Figure 8. PIKfyve localization in resting human platelets. PLTs were isolated from human peripheral blood. PLTs were permeabilized with 0.1% Triton X-100 or 0.5% Tween 20 in PBS, and stained for PIKfyve (green) and α-tubulin (red). The scale bar for images is 10 μm.

Two major collagen receptors on PLTs, glycoprotein VI (GPVI) and integrin α2β1 (also known as GPIa-IIa), play significant hemostatic roles [3,29]. GPVI binds the collagen exposed to the extracellular matrix (ECM) after vessel injury. The affinity of integrin α2β1 for collagen rises as the platelet undergoes the process of activation. Together, GPVI and integrin α2β1, cause the platelet to tightly bind to collagen [29].

During PLT activation on collagen, PLTs reorganize its cytoskeleton which results in the microtubules depolymerization and re-polymerization which leads to PLT shape change [24]. In activated PLTs, α-tubulin is organized like a network (Figure 9.). In both, Triton X-100 and Tween 20 permeabilized cells, α-tubulin is visible and well conserved. Again, the signal for PIKfyve staining is better when PLTs were permeabilized with Tween 20. In fully spread PLTs PIKfyve is somewhat enriched at the edges at lamellipodia,

while in less spread PLTs (earlier stages), PIKfyve colocalizes with α-tubulin (observed in both Triton X-100 and Tween 20 treated PLTs) (Figure 9.).

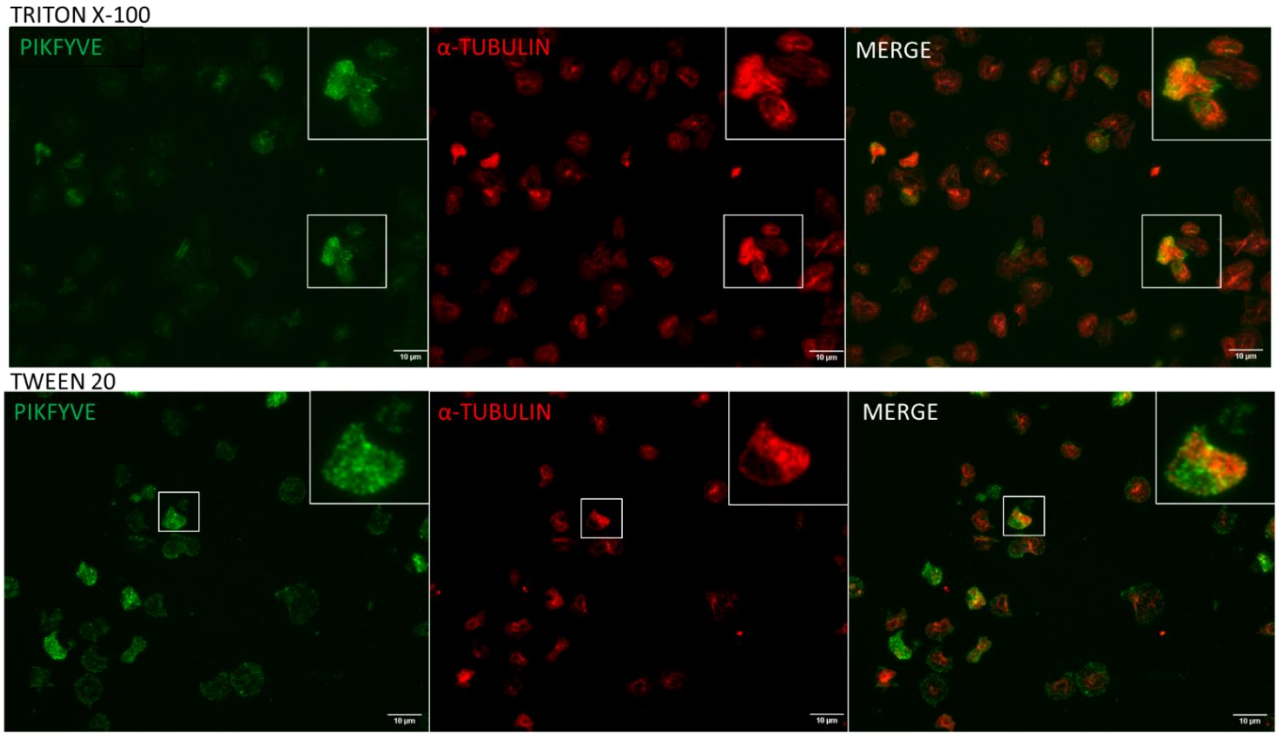

Figure 9. PIKfyve localization in human platelets activated on collagen. PLTs were permeabilized with 0.1% Triton X-100 or 0.5% Tween 20 in PBS and stained for PIKfyve (green) and α-tubulin (red), colocalization is evident in merged pictures (yellow). The scale bar for images is 10 μm.

4.2. Pharmacological inhibition of PIKfyve has no effect on resting or activated human platelets

As staining of PIKfyve and its colocalization with α-tubulin was visible in resting and activated PLTs, we wanted to examine if inhibition of PIKfyve would affect platelet function. With a pharmacological approach, using different concentrations of PIKfyve specific inhibitor YM201636, we analyzed its effect on resting PLTs and PLTs activated and spread on collagen.

Firstly, we wanted to be sure that the inhibitor by itself has no effect on PLTs, e.g. that it does not activate PLT or in any other way changes morphology of the PLT cytoskeleton. As a control (CTRL), we used untreated resting PLTs that showed microtubular ring structure on the edges and F-actin staining in the center of the cell (Figure 10a). Throughout experiments, inhibitor GSK-A1 was used as a negative control (Figure 10b) since it was shown as very potent inhibitor (IC50~3.1) targeting PI4KIIIα [5] (inhibits production of PI4P), and strongly inhibits PLT activation (unpublished data, Laboratory of hematopoiesis, Sara Čabrijan).

Resting PLTs in suspension were preincubated 15 minutes with 500 nM, 1 μM or 5 μM inhibitor YM201636 or with 500 nM inhibitor GSK-A1. PLTs were then collected on coverslips, fixed and stained for α-tubulin and F-actin. As compared with control (CTRL) untreated PLTs, inhibitor-treated PLTs still had preserved microtubular rings and preserved F-actin network (Figure 10). There was no significant inhibition in either 500 nM or 5 μM concentrations of inhibitor (Figure 10) indicating that the YM201636 inhibitor itself does not affect PLT cytoskeleton. The same was observed for GSK-A1. Occasionally, some microtubular ring disruption and more spherical shape of PLTs could be seen on PLTs exposed to 1 μM concentration of inhibitor (Figure 10).

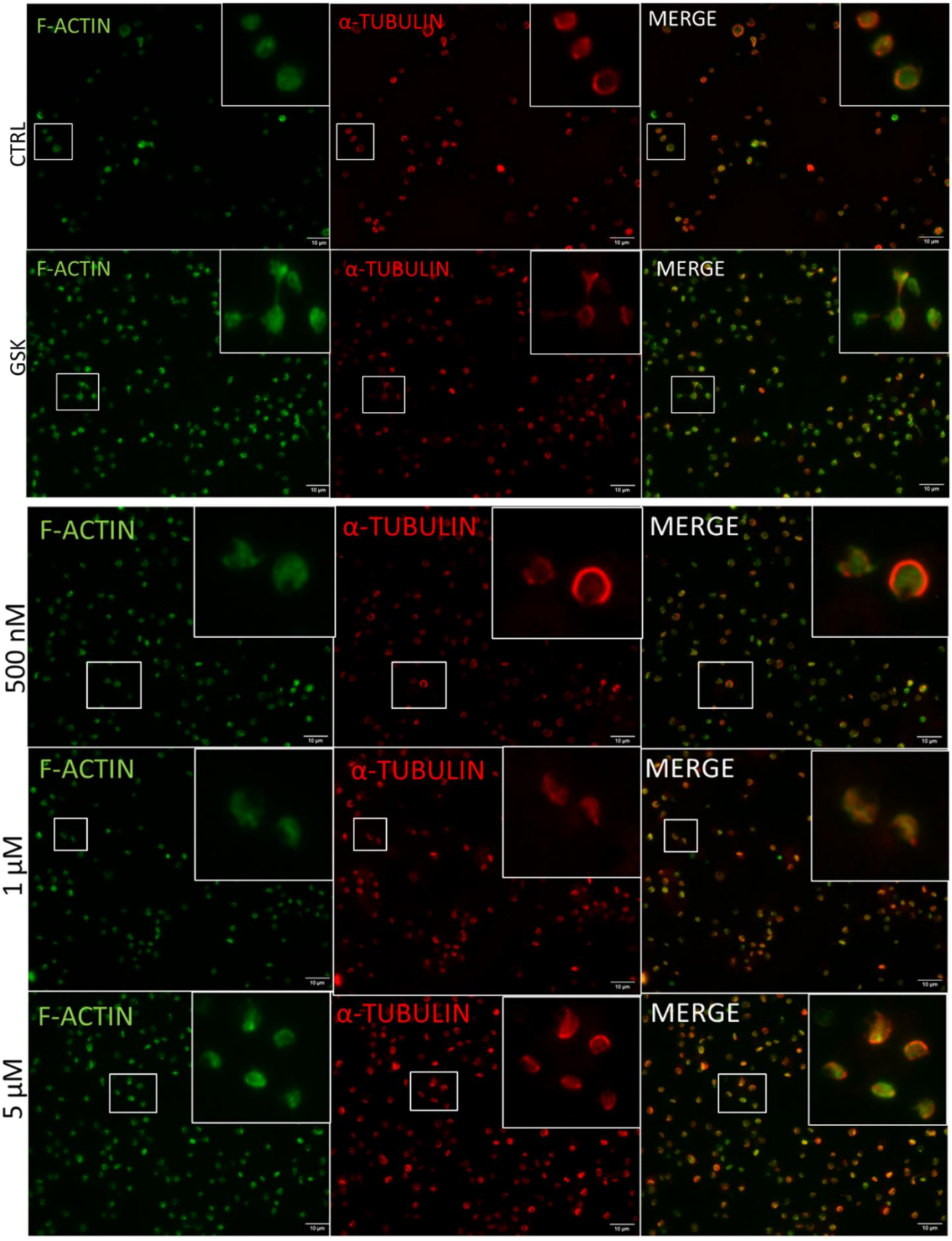

Figure 10. Positive and negative control in resting PLTs and PIKfyve inhibition with YM201636 inhibitor in resting platelets. PLTs were isolated from human peripheral blood and stained for α-tubulin and F-actin. PLTs were

untreated (CTRL), or inhibited with 500 nm GSK-A1 (negative control), or inhibited with 500 nM, 1 μM or 5 μM of PIKfyve inhibitor YM201636 for 15 minutes in suspension. Then PLTs were collected into 4% PFA and spun down on coverslides and stained for α-tubulin (red) and F-actin (green). The scale bar of the images is 10μm.

For activation, PLTs were spread on collagen. In activated condition (CTRL), PLTs are adhered and fully spread so as a result they form lamellipodia (Figure 11). Same as in resting PLTs, GSK-A1 was used at a concentration of 500 nM, and YM201636 inhibitor at 500 nM, 1 μM or 5 μM PLTs were first inhibited in suspension for 15 min, and then allowed to spread on collagen for 45 min in presence of inhibitors.

GSK-A1-inhibited PLTs form filopodia, finger-like projections as extensions from cell periphery (Figure 11). PLTs exposed to GSK-A1 inhibition do not spread properly. F-actin is elongated and forms dendritic-like arrays.

The lowest concentration of YM201636 inhibitor, 500 nM, does not affect PLTs spreading and their structure is mostly lamellipodia and a recognizable „fried egg"shape. Two higher concentrations, 1 μm and 5 μm have extremely modest influence on platelet structure. Lamellipodia structure prevails predominantly with few filopodia included. All three concentrations show a similar structure as in CTRL structures.

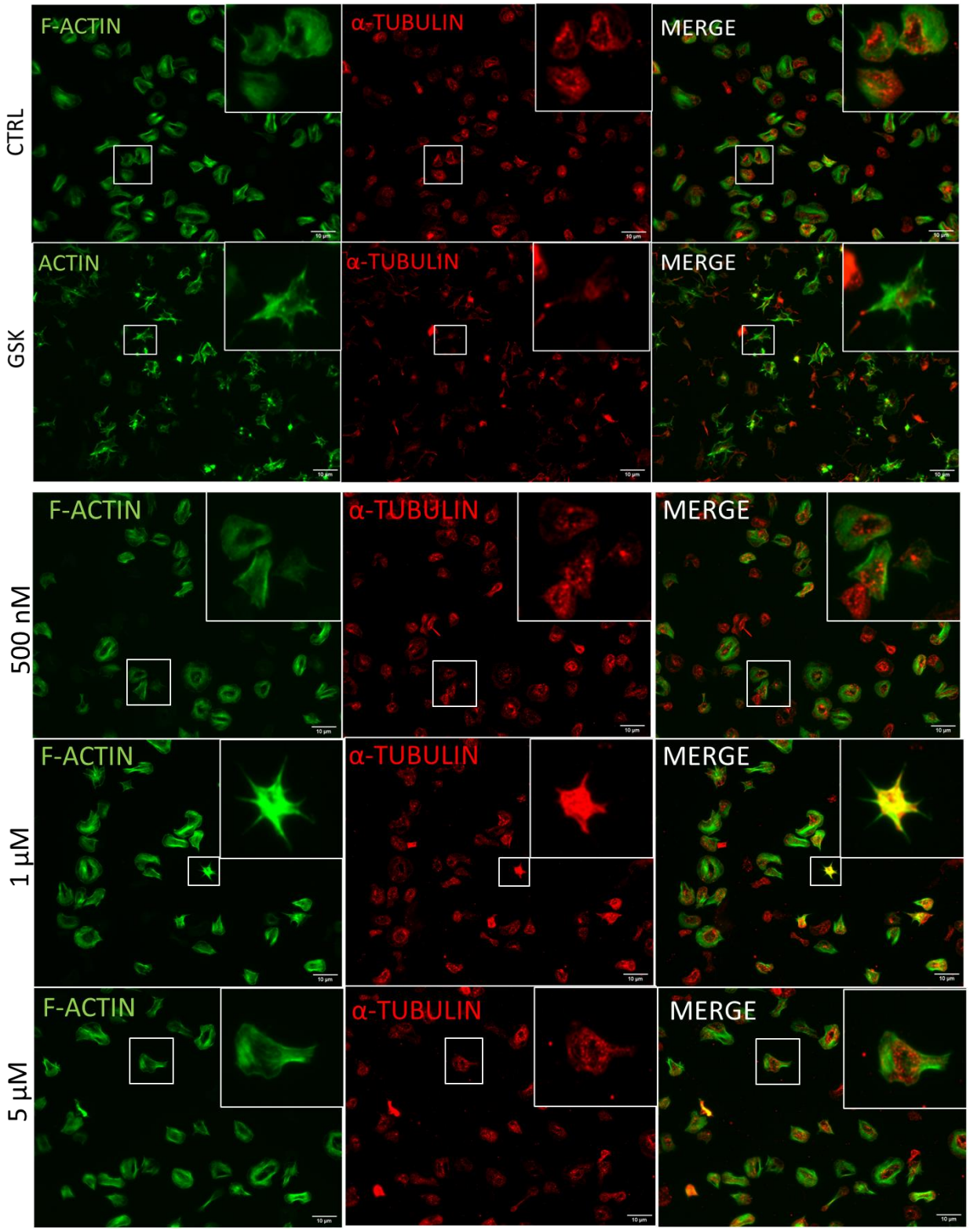

Figure 11. Positive and negative control in activated PLTs and PIKfyve inhibition with YM201636 inhibitor in activated platelets. PLTs were isolated from human peripheral blood and stained for α-tubulin and F-actin. The scale bar of the images is 10µm. (a) PLTs were inhibited with 500 nM PIKfyve inhibitor

YM201636. (b) PLTs were inhibited with 1 μM PIKfyve inhibitor YM201636. (c) PLTs were inhibited with 5 μM PIKfyve inhibitor YM201636.

4.2.1 Quantification of platelet spreading area and percentage of filopodia and lamellipodia after PIKfyve inhibition

Visual examination of immunofluorescently stained PLTs treated with PIKfyve inhibitor indicated no major changes. However, to objectively evaluate different visual fields of the slides and independent experiments, we measured the spreading area of PLTs by ImageJ program and presented quantified data as graphs. By comparing the spreading area of untreated fully activated and spread PLTs with PIKfyve inhibited PLTs, we confirmed that there was no significant difference between those. The only significant difference is the one between CTRL PLTs and PLTs inhibited with GSK-A1, as it was expected ****P<0.0001, ANOVA, GraphPadPrism).

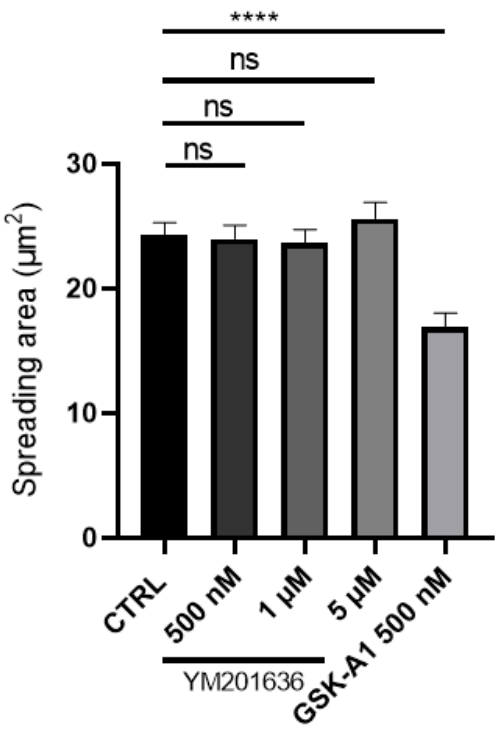

Figure 12. PIKfyve inhibition does not affect the platelet spreading area. Spreading area (μm2) was measured of untreated (CTRL), GSK-A1 (500 nM) or

YM201636 (500 nM, 1 µM, 5 µM) inhibited PLTs. Results are presented as a column graph of 3 independent experiments. The statistical analysis was performed using the Ordinary One-way ANOVA test. Data are presented as mean ± standard error of the mean (SEM). ns, non-significant; ****P<0.0001.

Previously, with immunofluorescence, we observed that exposing PLTs to three different concentrations of PIKfyve YM201636 inhibitor has little to no effect on the morphology of PLTs. Next, we wanted to objectively evaluate and quantify the percentage of PLTs that are fully spread and show lamellipodia state as well as incompletely spread PLTs holding filopodia, in order to see if there is any subtle difference in treated PLTs. As expected, there was a significant difference in number of lamellipodia and filopodia between CTRL and GSK-A1 PLTs (***P<0.001, ANOVA). In CTRL conditions, PLTs are fully activated and spread so there is an increased number of lamellipodia (80%). GSK-A1 inhibited PLTs form an increased number of filopodia rather than lamellipodia. As a consequence, it would be expected that YM201636 inhibited PLTs show similar characteristics as GSK-A1 inhibited PLTs. Surprisingly, PLTs treated with 500 nM YM201636 present more lamellipodia structures (74%) than filopodia (26%). Similarly, PLTs treated with 1 µM and 5 µM YM201636 inhibitor show increased number of lamellipodia (70% and 62%) than filopodia (30% and 38%). A slight decline in percentage of PLTs presenting lamellipodia as the concentration of inhibitor increased, however, this was not statistically significant. Accordingly, there was a modest increase in a number of filopodia as the concentration of inhibitor increased, however statistically insignificant. Altogether, there is no significant difference in number of lamellipodia between CTRL and three groups of PLTs exposed to YM201606 inhibitor. This correlates with the immunofluorescence images above (Figure 13) where most PLTs show lamellipodia in untreated CTRL as well as inhibitor exposed PLTs.

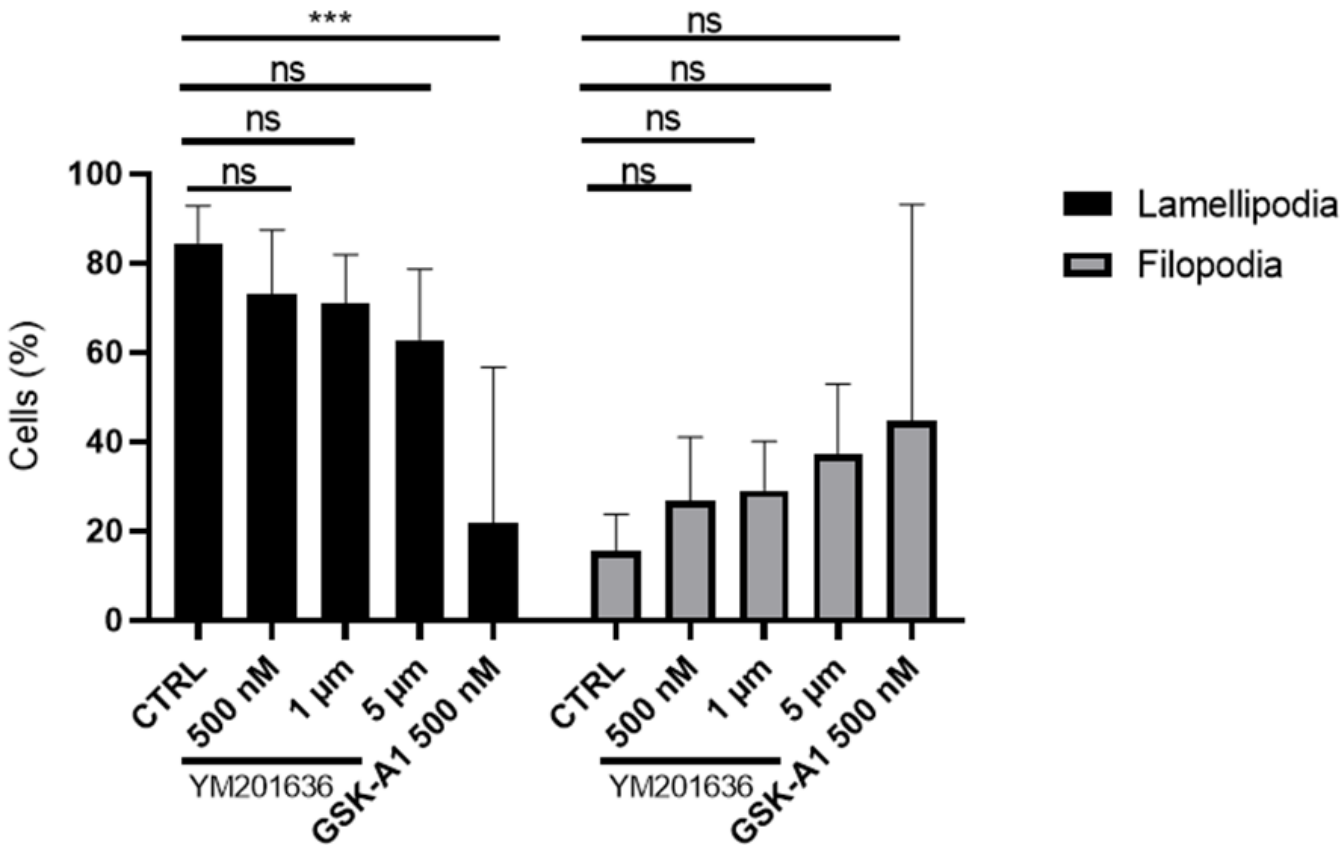

Figure 13. PIKfyve inhibition does not affect lamellipodia formation. Graph shows a comparison of percentage (%) of PLTs with lamellipodia or filipodia in untreated (CTRL), GSK-A1 (500 nM) or YM201636 (500 nM, 1 µM, 5 µM) inhibited PLTs. Results are presented as a column graph of 3 independent experiments. Data are presented as mean ± standard error of the mean (SEM). ns, non-significant; ***P<0.001. The statistical analysis was performed using the Two-way ANOVA test.

4.3. PI4KIIIβ inhibition partially impairs the spreading of activated human platelets

After analyzing the localization of PI4KIIβ, we wanted to see if inhibition of PI4KIIIβ influences the spreading of PLTs. For this purpose, we used PI4KIIIβ specific inhibitor, IN10.

Isolated human PLTs were treated with inhibitor IN-10 in four different concentrations (100 nM, 1 μM, 5 μM and 10 μM) for 15 min in suspension. PLTs treated with 100 nM IN-10 inhibitor were not shown. The results did not show any significance since there was not any inhibition and PLTs showed characteristics of CTRL conditions. After that PLTs were fixed, spun onto coverslips and stained for F-actin and α-tubulin. PLTs that were exposed to 1 μM and 5 μM inhibitor IN-10 preserved α-tubulin coil and no change in F-actin staining indicating that inhibitor by itself has no effect on PLTs (Figure 14). However, treatment with the highest concentration 10 μM reduced the size and changed the shape of PLTs. Although in some PLTs treated with 10 μM of inhibitor, a preserved microtubular coil could be observed, in other PLTs it seem to be disturbed or lost. Since this was only observed in PLTs treated with 10 μM of inhibitor, to confirm results, the experiment needs to be repeated. This indicates that IN-10 in high concentration potentially could have a toxic effect and additional studies should be performed.

c

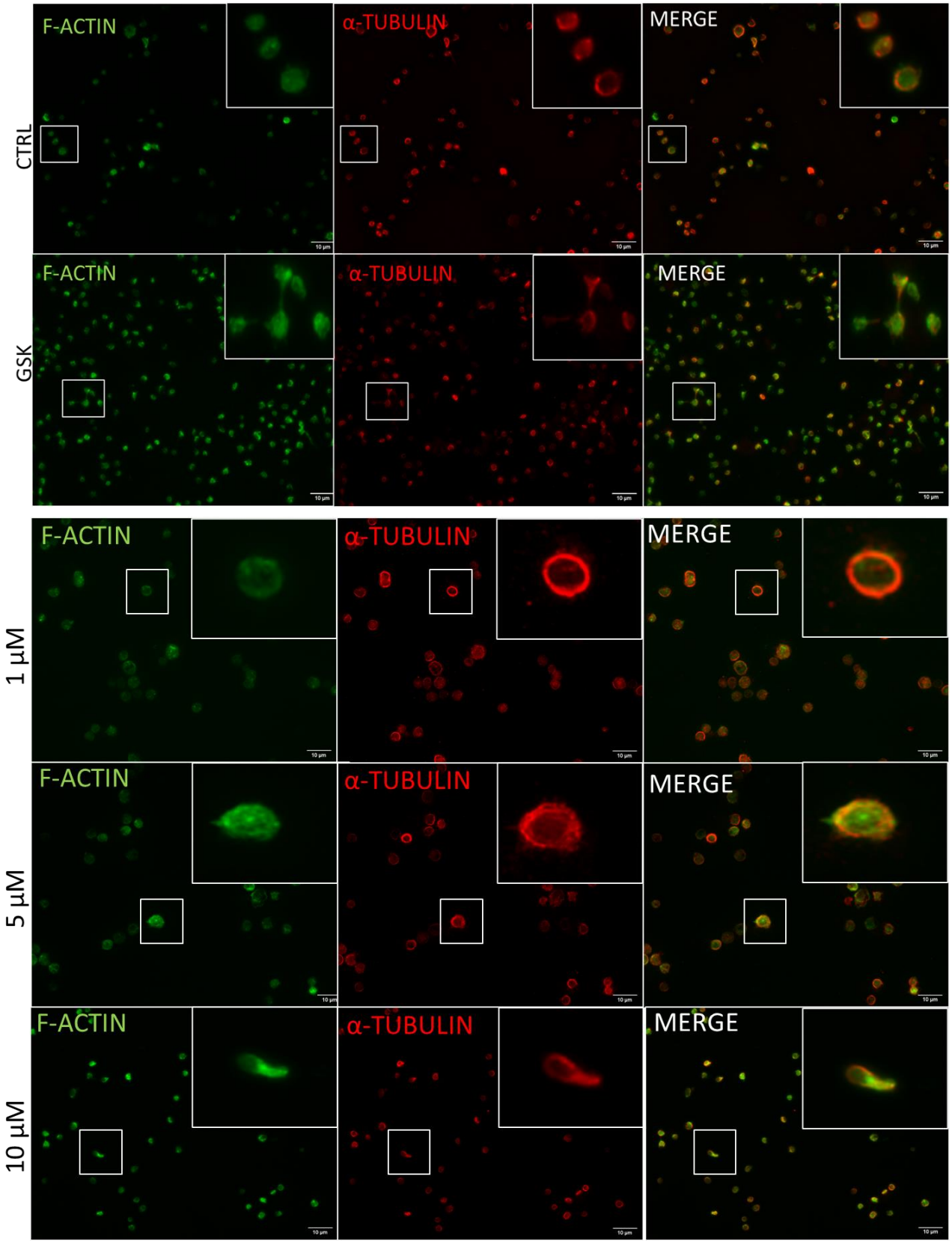

Figure 14. PI4KIIIβ inhibition with IN-10 inhibitor in resting platelets. PLTs were inhibited with PI4KIIIβ inhibitor IN-10 in concentrations 1 µM, 5 µM,

and 10 μM for 15 min in suspension. PLTs were stained for F-actin (green) and α-tubulin (red). The scale bar of the images is 10μm.

Activated PLTs were spread on collagen. Untreated PLTs that were spread on collagen were used as a positive control (CTRL). As in previous experiments for activation, fully spread PLTs adhere to the surface and form lamellipodia (Figure 15). Very potent inhibitor of PI4Ks, GSK-A1, was used as a negative control in concentration 500 nM for inhibition of spreading of activated PLTs. Again, a strong inhibition of PLT spreading was observed after inhibition of GSK-A1 (Figure 15).

PLTs were exposed to four different concentrations of IN-10 inhibitor, from the lowest 100 nm, 1 μM, 5 μM, and the highest 10 μM for 15 min in suspension and then let to be activated on the collagen coated surface in the presence of inhibitor. PLTs exposed to the lowest concentration of 100 nM show similar features as untreated sample. Lamellipodia mostly prevails and the PLTs are completely spread, and adhered. Modest changes could be observed in PLTs that were exposed to 1 μM and 5 μM IN-10 inhibitors (Figure 15). They are in the form of lamellipodia although their spreading area is somewhat smaller. At the highest concentration, 10 μM, PLTs display noticeable change in shape. Interestingly, spreading seems to be reduced than in the two lower concentrations and as well as lower number of PLTs was adhered. Lamellipodia structures are in the minority while filopodia prevail (Figure 15).

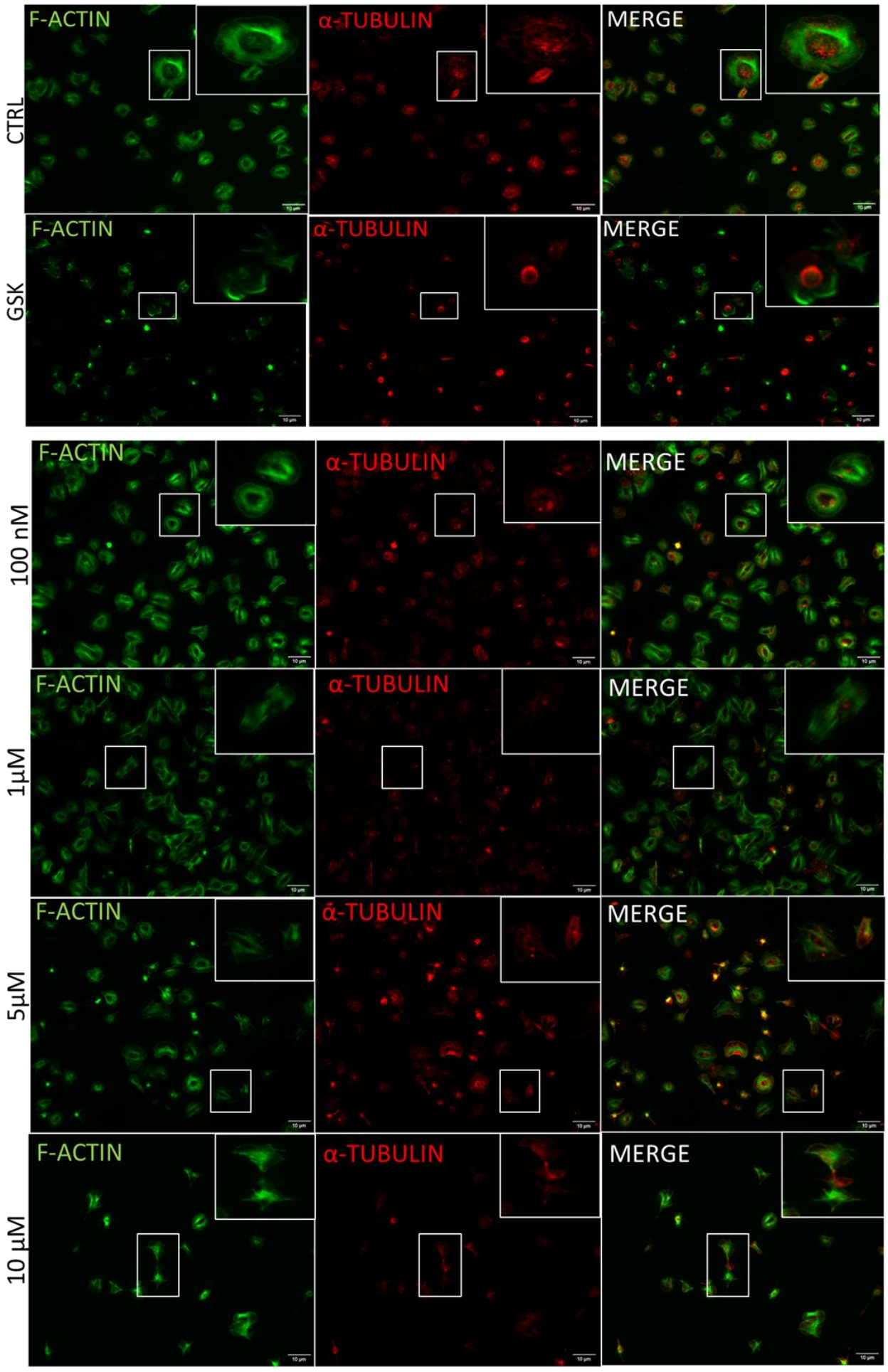
CTRL
F-ACTIN
α-TUBULIN
MERGE
GSK
F-ACTIN
α-TUBULIN
MERGE
100 nM
F-ACTIN
α-TUBULIN
MERGE
1µM
F-ACTIN
α-TUBULIN
MERGE
5µM
F-ACTIN
α-TUBULIN
MERGE
10 µM
F-ACTIN
α-TUBULIN
MERGE

Figure 15. Positive and negative control in activated PLTs and PI4KIIIβ inhibition with IN-10 inhibitor in activated platelets. PLTs were isolated from human peripheral blood and stained for α-tubulin and F-actin. The scale bar of the images is 10μm. PLTs were inhibited with 100 nM, 1 μM, 5 μM and 10 μM PI4KIIIβ inhibitor IN-10.

The experiment of PI4KIIIβ inhibition with IN-10 inhibitor in activated PLTs was performed three times with the blood of different donors. We did a fourth experiment, with the same protocol and different donors and gained different results than with the previous three experiments. In the previous three experiments, it was shown that inhibition with 100 nM of IN-10 inhibitor showed no results. In the last, fourth experiment, there is a slight inhibition where PLTs are not completely activated. Modest changes were observed in previous experiments where PLTs were exposed to 1 μM and 5 μM IN-10 inhibitors. Here, PLTs exposed to 1 μM and 5 μM IN-10 inhibitor form mostly filopodia. It could be observed how their activation is disturbed, spreading area smaller with a lot of protrusion of F-actin. In previous experiments, at the highest concentration, 10 μM, PLTs display noticeable changes in shape and adherence. In the last experiment, PLTs showed characteristics of PLTs that were treated with GSK-A1. Their spreading was completely disturbed, the spreading area shrank and their adhesion was almost non-existent. From the presented results, it can be observed how inhibition of PLTs in subject number four has stronger effect. It can be concluded how there could be individual differences in sensitivity to inhibitor between different donors. It is interesting how some PLTs do not polymerize actin and do not reorganize microtubules during activation at the concentration of 1 μM and completely retain the microtubular coil as in resting PLTs while some PLTs do. Further studies need to be done with expression of proteins to confirm these assumptions.

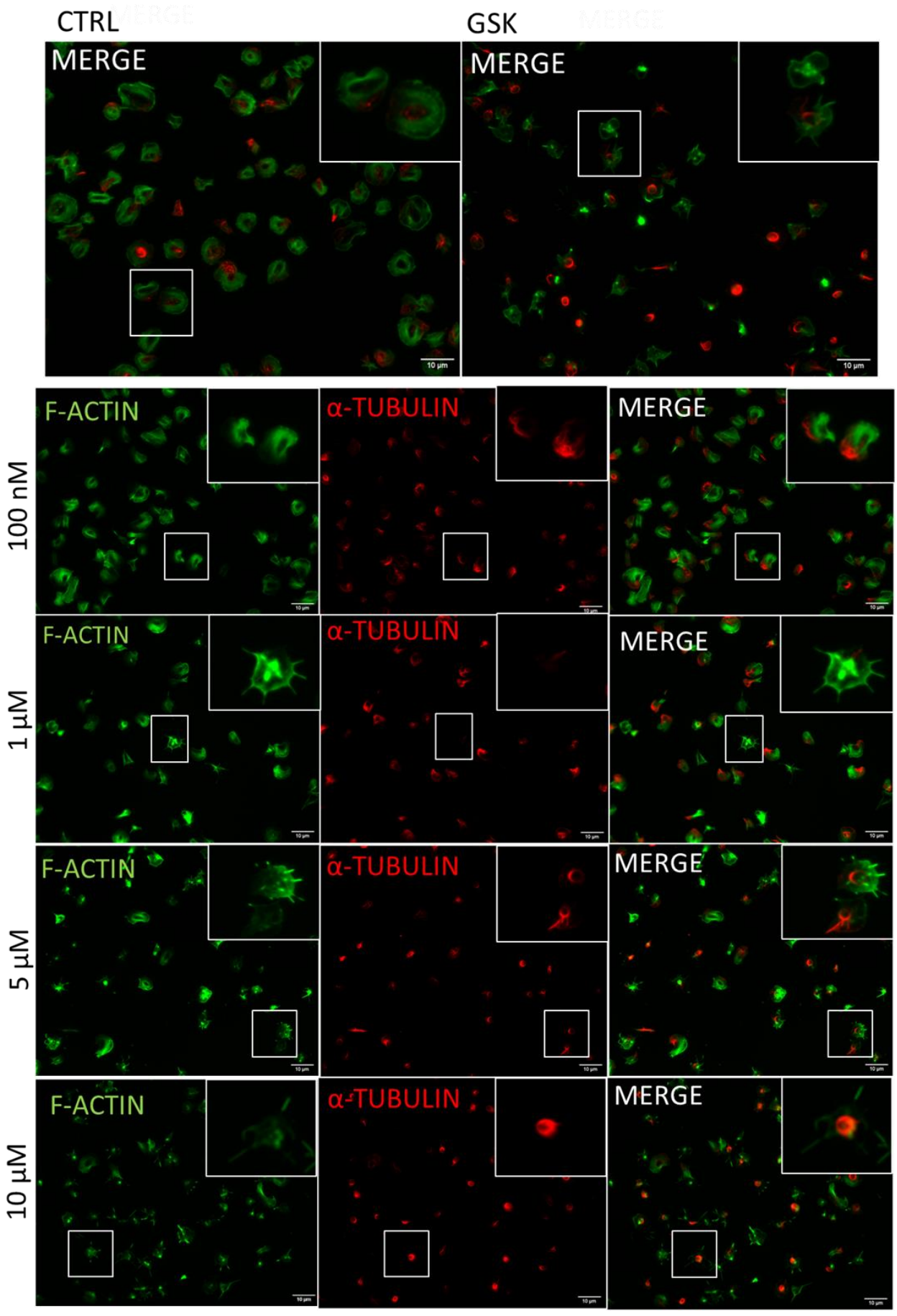
CTRL
GSK
MERGE
MERGE
F-ACTIN
α-TUBULIN
MERGE
100 nM
F-ACTIN
α-TUBULIN
MERGE
1 µM
F-ACTIN
α-TUBULIN
MERGE
5 µM
F-ACTIN
α-TUBULIN
MERGE
10 µM

Figure 16. Positive and negative control in activated PLTs and PI4KIIIβ inhibition with IN-10 inhibitor in activated platelets. PLTs were isolated from human peripheral blood and stained for α-tubulin and F-actin. The scale bar of the images is 10μm. PLTs were inhibited with 100 nM, 1 μM, 5 μM and 10 μM PI4KIIIβ inhibitor IN-10.

4.3.1 Spreading area of platelets and percentage of filopodia and lamellipodia

Visual examination of immunofluorescently stained PLTs treated with PI4KIIIβ inhibitor IN-10 indicated a few changes when PLTs were treated with higher inhibitor concentrations. Again, to objectively evaluate different visual fields of the slides and independent experiments, we measured spreading area of PLTs by ImageJ program and presented quantified data as graphs.

By comparing spreading area of untreated fully activated and spread PLTs with PI4KIIIβ inhibited PLTs, we gained confirmation that there is no significant difference in the spreading area of PLTs treated with 100 nM and 1 μM of IN-10 inhibitor. There is a significant difference between CTRL PLTs and PLTs inhibited with 5 and 10 μM of IN-10 (****P<0.0001, ANOVA, GraphPadPrism. As expected, there was a significant difference in the spreading area of CTRL PLTs and GSK-A1 inhibited PLTs (****P<0.0001, ANOVA, GraphPadPrism). The size of the graph columns decreases as the concentration gets higher which means that there exists an influence of inhibitor on spreading area of PLTs.

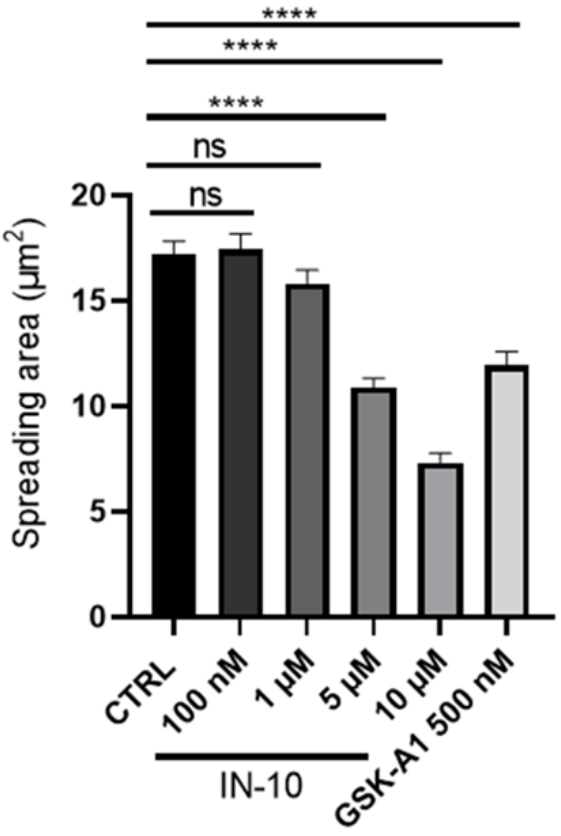

Figure 17. PI4KIIIβ inhibition does affect platelet spreading area at higher concentrations. Spreading area (μm2) was measured of untreated (CTRL), GSK-A1 (500 nM) or YM201636 (100 nM, 1 μM, 5 μM, 10 μM) inhibited PLTs. Results are presented as a column graph of 4 independent experiments. The statistical analysis was performed using the Ordinary One-way ANOVA test. Data are presented as mean ± standard error of the mean (SEM). ns, non-significant; ****P<0.0001.

As expected, there was a significant difference in number of lamellipodia and filopodia between CTRL and GSK-A1 PLTs (***P<0.001, ANOVA).

It was shown in the immunofluorescence experiments that exposing PLTs to four different concentrations of IN-10 inhibitor has modest results on the spreading area of PLTs. Next, I wanted to objectively evaluate and quantify the percentage of PLTs that are fully spread and show lamellipodia state as

well as incompletely spread PLTs holding filopodia, in order to see if there is any subtle difference in treated PLTs.

As expected, there was a significant difference in number of lamellipodia and filopodia between CTRL and GSK-A1 PLTs (***P<0.001, ANOVA). The number of lamellipodia is increased in CTRL conditions (8%) than in GSK-A1 treated PLTs (16%) while the number of filopodia is decreased in CTRL conditions (17%) and increased in GSK-A1 treated PLTs (84 %). As it was observed with immunostaining, there was no significant difference in number of lamellipodia and filopodia between 100 nM and 1 μM IN-10 inhibition. PLTs treated with 100 nM IN-10 form 78% of lamellipodia and only 22% of filopodia. Similarly, there is 80% of lamellipodia structures of PLTs treated with 1 μM IN-10 and 20% of filopodia. However, statistical analysis of the spreading area showed that there is a significance in 5 μM and 10 μM concentrations. Interestingly, the statistical analysis of the number of lamellipodia shows that there is no significant difference between CTRL and exposed PLTs. PLTs treated with 5 μM and 10 μM IN-10 form 71% and 72% of lamellipodia and only 29% and 28% of filopodia. The lowest number of lamellipodia, as expected, have PLTs treated with inhibitor GSK-A1 (***P<0.001, ANOVA). By looking at the size of columns and comparing it with CTRL, 5 μM concentration of IN-10 has the lowest number of lamellipodia. The same results came from counting a number of filopodia.

There is not present a decline in percentage of PLTs presenting lamellipodia as the concentration of inhibitor increased. There also is not an increase in number of filopodia as the concentration of inhibitor increased. Altogether, there is no significant difference in number of lamellipodia between CTRL and three groups of PLTs exposed to IN-10 inhibitor. This correlates with the immunofluorescence images above (Figure 13.) where most of PLTs show lamellipodia in untreated CTRL as well as inhibitor exposed PLTs.

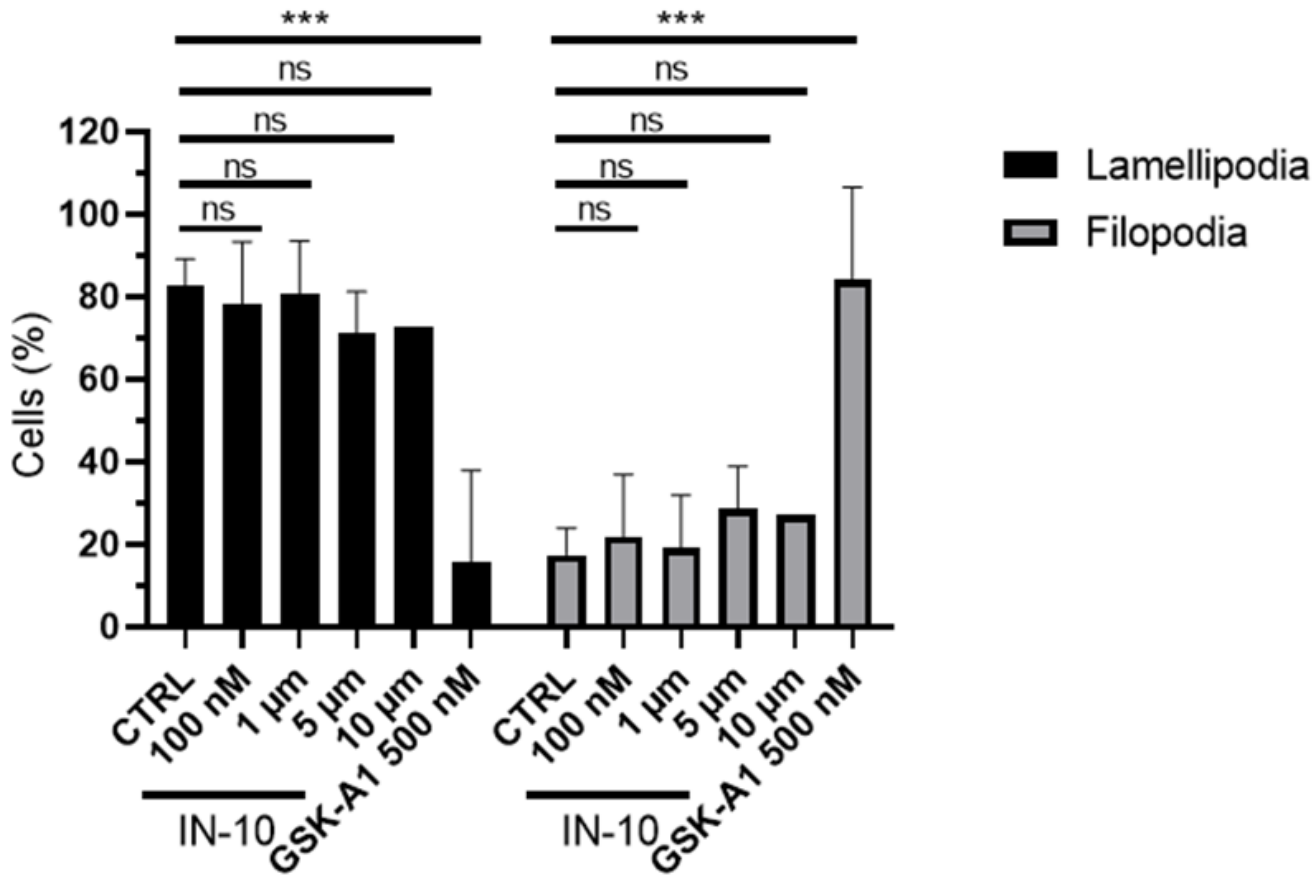

Figure 18. PI4KIIIβ inhibition does not affect lamellipodia formation. Graph shows comparison of percentage (%) of PLTs with lamellipodia or filipodia in untreated (CTRL), GSK-A1 (500 nM) or YM201636 (100 nM, 1 µM, 5 µM, 10 µM) inhibited PLTs. Results are presented as a column graph of 4 independent experiments. Data are presented as mean ± standard error of the mean (SEM). ns, non-significant; ***P<0.001. The statistical analysis was performed using the Two-way ANOVA test.

4.4. Western blot analysis of Syk kinase phosphorylation and tubulin acetylation in PIKfyve and PI4Kβ inhibited platelets

After observing with immunofluorescence that PIKfyve inhibition in PLTs has no effect and PI4KIIIβ inhibition has a slight effect on activation and spreading of PLTs, we performed Western blot analysis to further confirm inhibition of YM201636 and IN-10 on PIKfyve and PI4KIIIβ. The goal was to

analyze signaling pathways by Western blot to see if some of them were affected.

Untreated resting PLTs were used as control (CTRL). Rest of the PLTs were incubated with different concentrations of inhibitors and activated with 1 µg/mL collagen-related peptide (CRP). CRP is a synthetic peptide that mimics collagen activation of PLTs. TRAP-6 was used as a negative control for acetylation of tubulin [24]. GAPDH was used as a control for the amount of p-Syk and acetylated tubulin per sample. Activation was stopped after 2 minutes by lysing PLTs with 3% SDS-STOP/β-mercaptoethanol.

GPVI is a platelet collagen receptor associated with the FcRγ. When GPVI activates, spleen tyrosine kinase (Syk) binds to the ITAM through two SH2 domains and becomes phosphorylated. Phosphorylation of Syk leads to phosphorylation of several adaptor proteins as well as activation of PIKs [30]. We wanted to determine the influence of the inhibition of PIKfyve and PI4KIIIβ on this signaling pathway. As expected, in CTRL, untreated PLTs, p-Syk is not present since in resting state signaling pathways are not activated. PLTs that were treated with inhibitors and CRP in suspension do not show change in the level of p-Syk and they have similar levels as PLTs that were treated only with CRP. Some changes in p-Syk levels could be observed in PLTs treated only with IN-10 or YM201636 inhibitor.

The microtubules are highly acetylated and during the activation of PLTs and depolymerization of the microtubules they deacetylate [31]. As expected, resting PLTs had high level of acetylated tubulin but activation of PLTs with CRP in suspension did not change the level of tubulin acetylation (Figure 19). Only upon TRAP-6 activation levels of acetylated tubulin significantly dropped. PI4KIIIβ inhibited PLTs (IN-10 inhibitor) and PIKfyve inhibited PLTs (YM210636) also preserved acetylated tubulin.

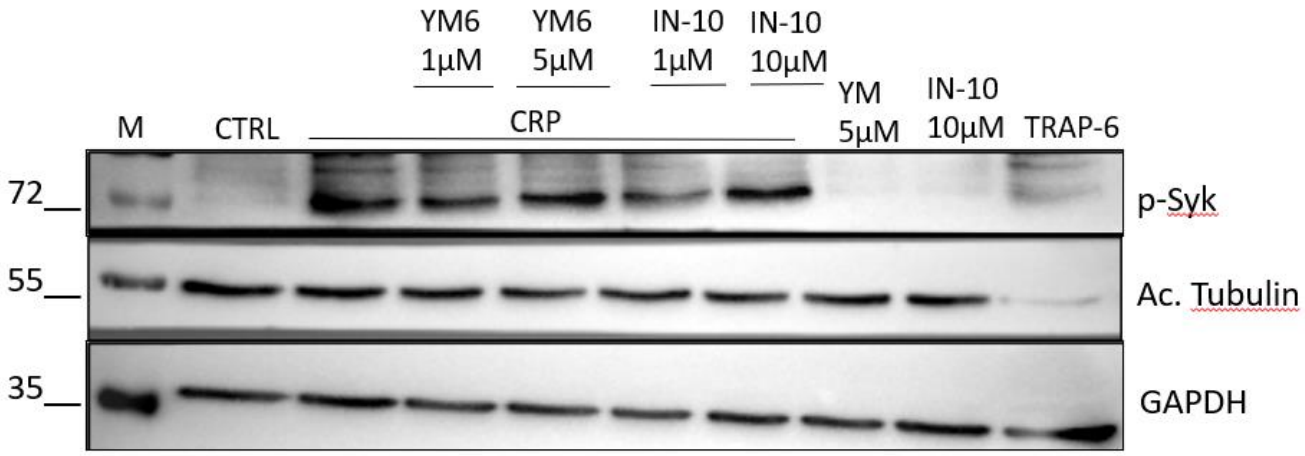

Figure 19. Western blot analysis of Syk kinase phosphorylation and tubulin acetylation in PIKfyve PI4KIIIβ. PLTs were isolated from human peripheral blood, inhibited with different concentrations of IN-10 or YM201636 inhibitors for 15 minutes, activated with 1 μg/mL collagen-related peptide (CRP). Concentration of TRA-6 is 10 μM. First band is marker (M).

4.5. Optimisation of PI4KIIβ immunostaining in human platelets

Previously, PI4KIIβ transcripts were found in both mouse and PLTs and its protein expression was observed in PLTs by Western blot (unpublished data, Laboratory of hematopoiesis, Sara Čabrijan). Therefore, next we wanted to determine the localization of PI4KIIβ in resting and activated PLTs by immunofluorescence. For this reason, we analyzed which permeabilization reagent is optimal for staining. Both resting and activated PLTs were permeabilized with either 0.1% Triton X-100 or 0.5% Tween 20 in PBS, in the same way as described above with PIKfyve.

Interestingly, staining of F-actin was equally successful and clear in Triton X-100 permeabilized cells as in Tween 20 permeabilized cells (Figure 7). Permeabilization with Tween 20 showed to be more efficient as immunostaining of PI4KIIβ was stronger than in the case of Triton X-100 (Figure 20). F-actin filaments in resting PLTs appear as a sparse fibrous network [3] while PI4KIIβ stained uniformly within resting PLTs without

forming any specific structures (Figure 20). Colocalization of PI4KIIβ and F-actin is visible throughout the whole platelet.

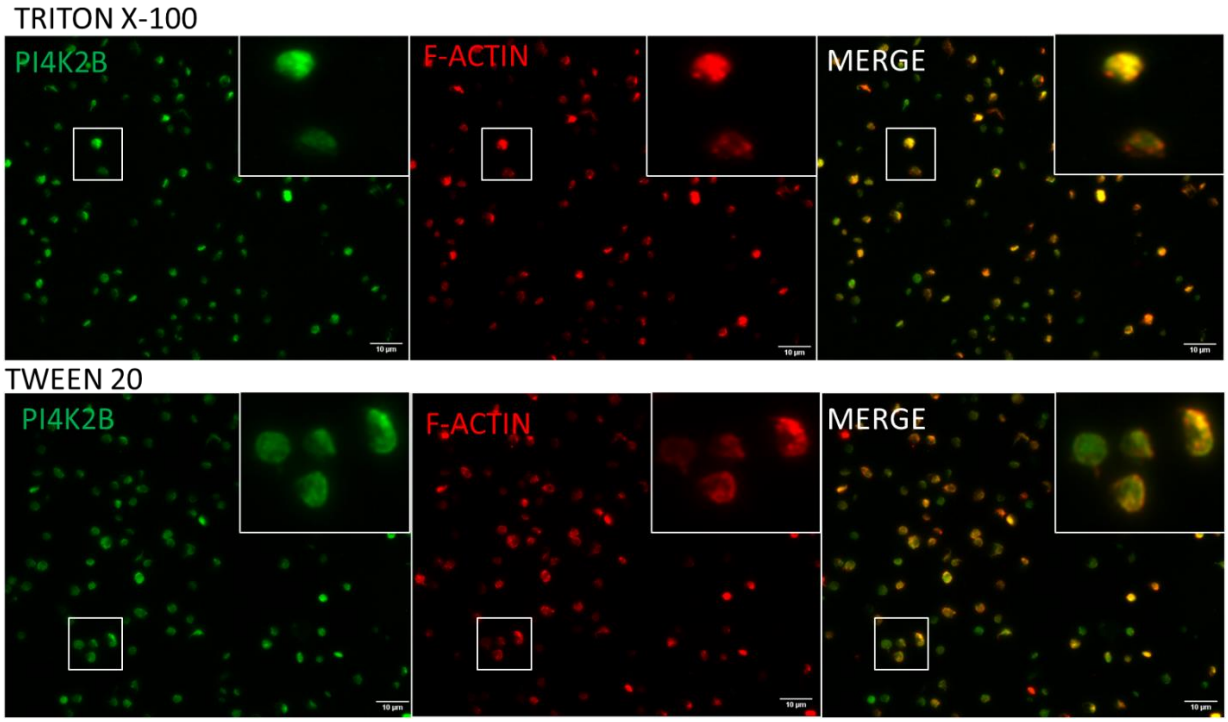

Figure 20. Optimisation of PI4KIIβ immunostaining in resting PLTs. PLTs were permeabilized with 0.1% Triton X-100 or 0.5% Tween 20 in PBS and stained for PI4KIIβ (green) and F-actin (red). The scale bar images is 10 μm.

For activation, PLTs were left to fully spread on collagen coated slides for 45 min. Activated PLTs were also permeabilized with two different permeabilizers in order to compare the efficiency of immunostaining between the two. In both, Triton X-100 and Tween 20 permeabilized cells, F-actin is visible. With both permeabilizers, staining of PI4KIIβ is very distinctive and it is present throughout the whole activated PLTs. Colocalization of the two is not obvious but it can be seen in few cells in the middle where α-tubulin is supposed to be.

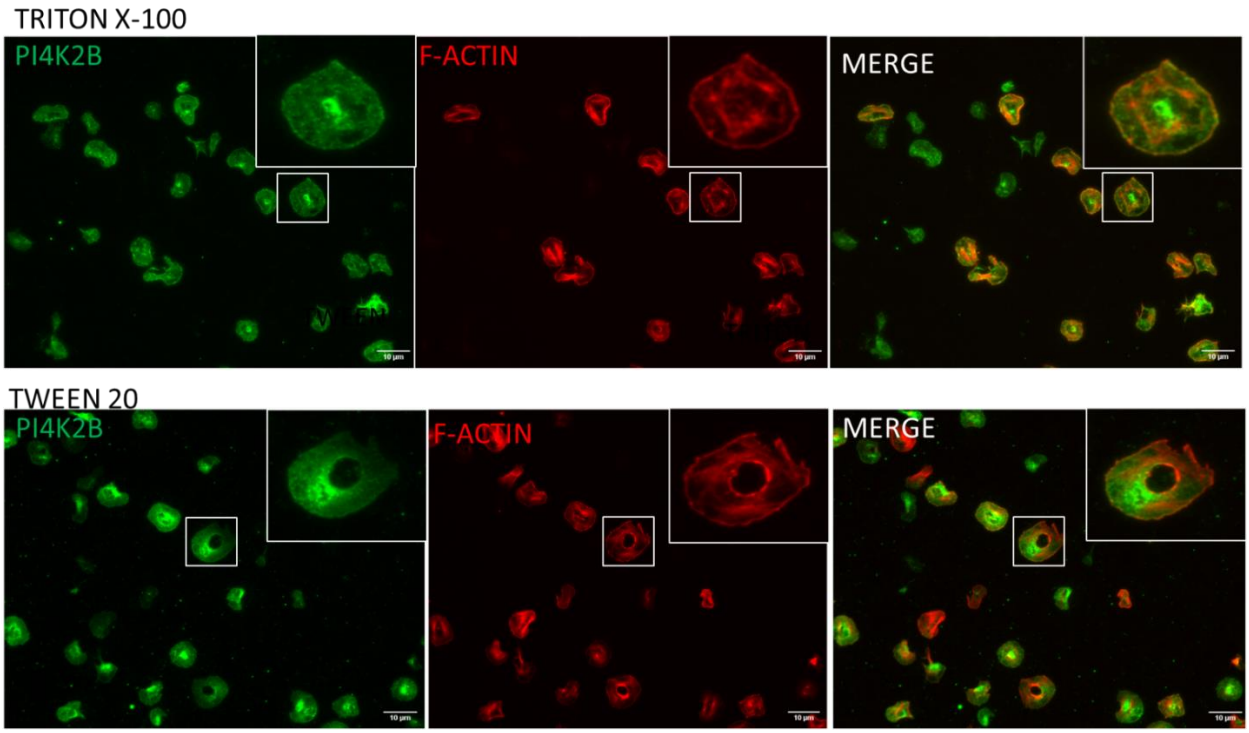

Figure 21. Optimization of PI4KIIβ immunostaining in PLTs activated on collagen. PLTs were isolated from human peripheral blood. PLTs were permeabilized with 0.1% Triton X-100 or 0.5% Tween 20 in PBS and stained for PI4KIIβ (green) and actin (red), colocalization is evident in merged pictures (yellow). Scale bar for images is 10 μm.

5. DISCUSSION

An important part of platelet activation that leads to their spreading have PI(4,5)P_2 and PI3-kinases that have been studied in platelets thus far. There is still not much literature on PI(3,5)P_2 and PI4P and related enzymes (PIKfyve and PI4Ks) that produce these PIs in platelets.

It was shown that PIKfyve kinase is expressed at low levels in platelets and that deficiency of PIKfyve does not influence thrombopoiesis [19]. As it was shown in our experiments, PIKfyve stained uniformly within resting platelets without specific colocalization with cytoskeletal components. In activated platelets, it colocalized with α-tubulin in less spread PLTs.

A recent study showed that pharmacological inhibition of PIKfyve with its potent inhibitor YM201636 decreases the intracellular synthesis of PI(3,5)P_2 and leads to cell vacuolation. [9]. We wanted to see if using the same inhibitor would have any effect on platelet spreading. We show that neither low (500 nM) or high (5 μM) concentrations had no influence on resting platelets as well as on platelets spread on collagen. Modest effect was observed with 1 and 5 μM YM201636 inducing few filopodia structures in some platelets. By gathering all results, we conclude that PIKfyve inhibition does not have any significant effect on activation and spreading of platelets. This is in agreement with previous studies with another PIKfyve inhibitor, STA 5326, that did not affect actin cytoskeleton or platelet spreading [32]. PIKfyve expression increases with MK maturation and its products, PI5P and PI(3,5)P_2, were found in dense and alpha granule membrane. Following the inhibition of PIKfyve in MKs with this inhibitor, the residual levels of PI5P and PI(3,5)P_2 were found, suggesting that there exists an alternative pathway for their production in MK [32]. PIKfyve inhibition with STA 5326 significantly decreased levels of PI5P and to a lesser extent PI(3,5)P_2[32]. By defective granule biogenesis and identity they concluded how PIKfyve and its dependent mechanisms regulate both granules [32]. They also showed how there was no effect of this inhibitor on αIIbβ$_3$ integrin activation in response to CRP[32]. Another study showed that the platelet lysosome secretion of

enzymes could be required for the remodeling of extracellular matrix during healing of a wound so proper platelet lysosome secretion is required [33]. Their experiment with PIKfyve-null platelets showed excessive accumulation of lysosomal components in platelets which explains how PIKfyve is critical for lysosome homeostasis in platelets [33]. Interestingly, previous studies showed that pharmacological inhibition of Vps34 with SAR405 inhibitor decreases proplatelet formation from MKs [19]. Also, along endosome maturation and PI3P, PI(3,5) was confirmed to be needed for proplatelet formation [19].

We showed that pharmacological inhibition of PI4KIIIβ with IN10 inhibitor at higher concentrations (5 and 10 μM) reduced platelet spreading area, although without major effect on ratio of filopodia and lamellipodia. In order to confirm results, additional studies should be performed. Interestingly, one of the donors showed greater sensitivity to PI4KIIIβ inhibition, even with smaller concentrations of inhibitor. These results are somewhat in line with my colleague's data from the laboratory with another PI4KIIIβ inhibitor IN9, that had more consistent inhibitory effect between different experiments even at lower concentrations (already at 1 μM, Klaudia Liker, Laboratory of hematopoiesis; personal communication). It could be that IN10 is less specific or binds to lower extent to PI4KIIIβ than IN9. Previously, it was shown that PI4KIIIα inhibitor GSK-A1 reduces PI4P levels in resting platelets [21]. As can be observed in control experiments, when using GSK-A1 inhibitor, there is a consistent and strong inhibition of platelet spreading at low concentration (500 nM, Sara Čabrijan, Laboratory of hematopoiesis; unpublished data), indicating high importance of this kinase in platelet spreading.

OCRL is 5-phosphatase that dephosphorylates PI(4,5)P_2 into PI4P and it is localized in different cellular compartments [24]. In resting platelets, it was shown that OCRL inhibition increases PI(4,5)P_2 and decreases PI4P [21]. In a recent study, inhibiting OCRL with an inhibitor, showed reduced platelet spreading on fibrinogen [24]. In most of the OCRL-inhibited PLTs when spread

on fibrinogen, $PI(4,5)P_2$ localized in ring-like shape while there was an increased number of F-actin structures resembling nodules [24]. On glass, OCRL-inhibited PLTs did not preserve microtubules but the elevated levels of acetylated tubulin were present[24]. There was an increase of $PI(4,5)P_2$ in PLTs spread on collagen or fibrinogen while, interestingly there was no change when spread on glass [24]. Further studies need to be done when inhibiting PI4KIIIβ to investigate whether there is a difference between spreading on different matrices (glass, collagen or fibrinogen). PI4KIIIα is a kinase that produces PI4P from PI at the plasma membrane while PI4KIIIB also produces PI4P at the plasma membrane as well as at the Golgi apparatus [5]. Since inhibition of PI4KIIIα in platelets resulted in a modest decrease of the intracellular PI4P pools without changing $PI(4,5)P_2$ levels ([21]), further studies would be interesting to see the contribution of PI4KIIIβ an its inhibition on intracellular pools of PI4P and $PI(4,5)P_2$ in platelets.

Other possible sources of PI4P are PI4K type II kinases, of which PI4KIIβ is poorly studied. With immunofluorescence, we showed that PI4KIIβ stains uniformly within resting platelets where it colocalizes with F-actin. In activated platelets, its staining was very distinctive, but it did not colocalize with F-actin. PI4KIIβ is cytosolic PI4K that stimulates $PI(4,5)P_2$ syntheses when recruited to membrane and is an important for synthesis of PI4P pools between the TGN and endosomes [5]. Its crystal structure was only recently solved, and there are still no available inhibitors. A study in which PI4KIIβ was depleted with small interfering RNA showed remodeling of actin and induced invadopodia formation [34]. Loss of PI4KIIβ showed complete loss of actin stress fibers [34]. Further studies are warranted on platelets once new inhibitors of PI4KIIβ will be accessible.

6. Conclusion

In our research, we analyzed the effect of PIKfyve and PI4KIIIβ inhibitors on platelet spreading. We also stained PIKfyve and PI4KIIβ in PLTs. Our results show that there is no specific localization of PIKfyve in resting platelets with cytoskeletal components while in activated platelets, it colocalized with α-tubulin in less spread PLTs. Also, it was shown that PIKfyve inhibition does not have any significant effect on activation and spreading of human platelets. However, it needs to be further examined since PIKfyve and its products are associated with function of alpha granules. Our results also show that inhibition of PI4KIIIβ with IN-10 inhibitor at higher concentrations (5 and 10 μM) reduced platelet spreading area without major effect on ratio of filopodia and lamellipodia. Interestingly, PI4KIIβ stained uniformly within resting platelets and it colocalized with F-actin. Further studies are needed to decipher its role in PLT function.

7. Literature

1 York MJ. *Clinical Pathology*. Second Edi. Elsevier Inc., 2016 doi:10.1016/B978-0-12-803620-4.00014-1.

2 Thomas SG. The Structure of Resting and Activated Platelets. *Platelets* 2019; : 47–77.

3 Michelson AD. *Platelets*. Elsevier, 2013.

4 Mitchell WB, Biology P, York N, York N. Platelets in Blood Clotting. 2014; : 1–6.

5 Pip TS, Bura A, Cabrijan S, Đuri I. A Plethora of Functions Condensed into Tiny Phospholipids : 2023.

6 Min SH, Abrams CS. Regulation of platelet plug formation by phosphoinositide metabolism. *Blood* 2013; **122**: 1358–1365.

7 Li Y ping, Mikrani R, Hu Y fan, Faran Ashraf Baig MM, Abbas M, Akhtar F *et al.* Research progress of phosphatidylinositol 4-kinase and its inhibitors in inflammatory diseases. *Eur J Pharmacol* 2021; **907**: 174300.

8 Falkenburger BH, Jensen JB, Dickson EJ, Suh BC, Hille B. Phosphoinositides: Lipid regulators of membrane proteins. *J Physiol* 2010; **588**: 3179–3185.

9 Min SH. ScholarlyCommons Role of Pikfyve in Platelet Lysosomal Homeostasis Role of Pikfyve in Platelet Lysosomal Homeostasis. 2014.

10 Hasegawa J, Strunk BS, Weisman LS. PI5P and PI(3,5)P2: Minor, but essential phosphoinositides. *Cell Struct Funct* 2017; **42**: 49–60.

11 Jin N, Lang MJ, Weisman LS. Phosphatidylinositol 3,5-bisphosphate: Regulation of cellular events in space and time. *Biochem Soc Trans* 2016; **44**: 177–184.

12 Minogue S. The Many Roles of Type II Phosphatidylinositol 4-Kinases

in Membrane Trafficking: New Tricks for Old Dogs. *BioEssays* 2018; **40**: 1–10.

13 Sbrissa D, Ikonomov OC, Filios C, Delvecchio K, Shisheva A. Functional dissociation between PIKfyve-synthesized PtdIns5P and PtdIns(3,5)P 2 by means of the PIKfyve inhibitor YM201636. *Am J Physiol - Cell Physiol* 2012; **303**: 436–446.

14 Burke JE. Structural Basis for Regulation of Phosphoinositide Kinases and Their Involvement in Human Disease. *Mol Cell* 2018; **71**: 653–673.

15 Waugh MG. The Great Escape: How phosphatidylinositol 4-kinases and PI4P promote vesicle exit from the Golgi (and drive cancer). *Biochem J* 2019; **476**: 2321–2346.

16 Dornan GL, McPhail JA, Burke JE. Type III phosphatidylinositol 4 kinases: Structure, function, regulation, signalling and involvement in disease. *Biochem Soc Trans* 2016; **44**: 260–266.

17 Born G. Platelets: Past, present and future. *Handb Exp Pharmacol* 2012; **210**: 59–85.

18 Burke JE, Triscott J, Emerling BM, Hammond GRV. Beyond PI3Ks: targeting phosphoinositide kinases in disease. *Nat Rev Drug Discov* 2023; **22**: 357–386.

19 Bertović I, Kurelić R, Milošević I, Bender M, Krauss M, Haucke V *et al.* Vps34 derived phosphatidylinositol 3-monophosphate modulates megakaryocyte maturation and proplatelet production through late endosomes/lysosomes. *J Thromb Haemost* 2020; **18**: 1756–1772.

20 Du C, Guan X, Yan J. Two-pore channel blockade by phosphoinositide kinase inhibitors YM201636 and PI-103 determined by a histidine residue near pore-entrance. ; : 1–11.

21 Bura A, Begonja AJ. Imaging of Intracellular and Plasma Membrane Pools of PI(4,5)P2 and PI4P in Human Platelets. *Life* 2021; **11**.

doi:10.3390/life11121331.

22 Nebl T, Oh SW, Luna EJ. Membrane cytoskeleton: PIP2 pulls the strings. *Curr Biol* 2000; **10**: 351–354.

23 Insall RH, Weiner OD, Ave L, C- CBK. Similar Messages , Different Meanings ? *Oncogene* 2001; **1**: 743–747.

24 Bura A, de Matteis MA, Bender M, Swinkels M, Versluis J, Jansen AJG *et al.* Oculocerebrorenal syndrome of Lowe protein controls cytoskeletal reorganisation during human platelet spreading. *Br J Haematol* 2023; **200**: 87–99.

25 Jooss NJ, Simone I De, Provenzale I, Fernández DI, Brouns SLN, Farndale RW *et al.* Role of platelet glycoprotein VI and Tyrosine Kinase Syk in thrombus formation on collagen-like surfaces. *Int J Mol Sci* 2019; **20**. doi:10.3390/ijms20112788.

26 Mangin PH, Onselaer MB, Receveur N, Le Lay N, Hardy AT, Wilson C *et al.* Immobilized fibrinogen activates human platelets through glycoprotein VI. *Haematologica* 2018; **103**: 898–907.

27 Nowakowski AB, Wobig WJ, Petering DH. Native SDS-PAGE: High resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. *Metallomics* 2014; **6**: 1068–1078.

28 Chicanne G, Severin S, Boscheron C, Terrisse AD, Gratacap MP, Gaits-Iacovoni F *et al.* A novel mass assay to quantify the bioactive lipid PtdIns3P in various biological samples. *Biochem J* 2012; **447**: 17–23.

29 Grantor D. Platelets - cells with many functions. 2023.

30 Manne BK, Badolia R, Dangelmaier C, Eble JA, Ellmeier W, Kahn M *et al.* Distinct pathways regulate Syk protein activation downstream of immune tyrosine activation motif (ITAM) and hemITAM receptors in platelets. *J Biol Chem* 2015; **290**: 11557–11568.

31 Cuenca-Zamora EJ, Ferrer-Marín F, Rivera J, Teruel-Montoya R. Tubulin in Platelets: When the Shape Matters. *Int J Mol Sci* 2019; **20**: 1–13.

32 Manuella Caux RMJ-MXGCJVAVFBBPHT and SS. PIKfyve-Dependent Phosphoinositide Dynamics in Megakaryocyte/Platelet Granule Integrity and Platelet Functions. .

33 Min SH, Suzuki A, Stalker TJ, Zhao L, Wang Y, McKennan C *et al.* Loss of PIKfyve in platelets causes a lysosomal disease leading to inflammation and thrombosis in mice. *Nat Commun* 2014; **5**. doi:10.1038/ncomms5691.

34 Alli-Balogun GO, Gewinner CA, Jacobs R, Kriston-Vizi J, Waugh MG, Minogue S. Phosphatidylinositol 4-kinase IIβ negatively regulates invadopodia formation and suppresses an invasive cellular phenotype. *Mol Biol Cell* 2016; **27**: 4033–4042.

europass

Korina Švorinić

Datum rođenja: 11/07/1999 | **Državljanstvo:** hrvatsko | **Spol:** Žensko | **Telefonski broj:** (+385) 0 (Mobilni telefon) | **E-adresa:** korina409@gmail.com | **LinkedIn:** https://www.linkedin.com/in/korina-švorinić-723254223/

RADNO ISKUSTVO

06/2021 – 07/2021 Zadar, Hrvatska
STAŽIST HRVATSKI ZAVOD ZA JAVNO ZDRAVSTVO (HZJZ)

Studentsku praksu sam odradila mjesec dana na Zavodu za Javno Zdravstvo, OB Zadar na odjelu Zdravstvene ekologije i zaštite okoliša na poslovima ispitivanja kvalitete hrane i vode.

03/2023 – 04/2023 Rijeka, Hrvatska
LABORATORIJSKI DEMONSTRATOR ODJEL ZA BIOTEHNOLOGIJU

Asistirala sam pri pripremi i izvođenju laboratorijskih vježbi iz kolegija Opća fiziologija i patofizilogija.

12/2022 – TRENUTAČNO Rijeka, Hrvatska
PROMOTOR BIODERMA - NAOS

Promotor i savjetnik za Biodermine proizvode u ljekarnama u gradu Rijeci.

12/2022 – TRENUTAČNO Rijeka, Hrvatska
IZRADA DIPLOMSKOG RADA: UČINAK INHIBICIJE PIKFYVE I PI4KB NA ADHEZIJU TROMBOCITA
SVEUČILIŠTE U RIJECI, ODJEL ZA BIOTEHNOLOGIJU

Laboratorij za hematopoezu
Voditelj laboratorija: dr.sc. Antonija Jurak Begonja
Korištene metode istraživanja: metoda izolacije stanica krvi, Western blot, imunofluorescencija

OBRAZOVANJE I OSPOSOBLJAVANJE

2006 – 2014 Zadar, Hrvatska
OSNOVNOŠKOLSKO OBRAZOVANJE Osnovna škola Krune Krstića

2014 – 2018 Zadar, Hrvatska
SREDNJOŠKOLSKO OBRAZOVANJE Gimnazija Jurja Barakovića

2018 – 2021 Rijeka, Hrvatska
UNIV. BACC. BIOTECHN. ET PHARM. INV Odjel za biotehologiju

Završen preddiplomski studij Biotehnologija i istraživanje lijekova. Završni rad sam pisala na temu Hematološki poremećaji uzrokovani ribosomopatijama.

2021 – TRENUTAČNO Rijeka, Hrvatska
MAG. BIOTECH. IN MED. Odjel za biotehologiju

Diplomski studij Biotehnologija u medicini na Odjelu za Biotehnologiju. Eksperimentalni dio diplomskog rada radila sam u Laboratoriju za hematopoezu na Odjelu.

JEZIČNE VJEŠTINE

Materinski jezik/jezici: **HRVATSKI**

Drugi jezici: **ENGLESKI** | **TALIJANSKI**

DIGITALNE VJEŠTINE

MS Office (Word Excel PowerPoint) | Internet | UniProt | Rad u programima za molekularno modeliranje (VMD, Chimera, PyMol, Gamess, Avogadro) | Poznavanje rada proframa Avogdro, GAMESS, Chimera, Marvinsketch, PyMOL

DODATNE INFORMACIJE

POČASTI I NAGRADE

2023
Science arena STEM Games Osvojeno 2. mjesto na STEM Games Science Arena (Umag, 2023.)

VOLONTIRANJE

2020 – 2022 Rijeka
Udruga studenata biotehnologije Sveučilišta u Rijeci (USBRI) U projektu "Studenti mentori" – mentorstvo dvjema studentima 1. godine, pomoć u orijentaciji na Odjel za biotehnologiju