The Role of the Androgen Receptor in Prostate Cancer-induced Platelet Aggregation and Invasion

by

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Abstract

Background: Prostate cancer (PCa) is the most common visceral malignancy and third leading cause of cancer death among men in Canada. The 2018 Canadian Cancer Society estimates suggest that 21,300 men were diagnosed and 4,100 patients died of this disease. Metastatic prostate cancer (mPCa) is incurable. The 5-year overall survival of hormone sensitive androgen receptor expressing phenotype (AR+) is 30%. Upon disease progression, mPCa becomes refractory to AR signalling, assuming the AR independent subtype (AR-). In this hormone refractory phase, mPCa has a 3-year overall survival rate of 30%. Hence, there is a strong need to better understand and identify novel targets to halt progression to metastasis. Human platelets have been shown to contribute to epithelial cancer metastasis by the process of tumor cell induced platelet aggregation (TCIPA). In the circulation, TCIPA aids in the ability of the tumor cells to evade the immune system, arrest, extravasate, then grow and disseminate in the secondary sites. Given more aggressive nature of hormone insensitive tumors, my hypothesis is that PCa cell lines lacking AR expression will exhibit higher platelet aggregation potency compared to cell lines with intact AR signaling.

Objective: To characterize the role of AR in PCa induced TCIPA and platelet-induced invasion.

Methods: TCIPA experiments were performed with benign prostate cells (RWPE, AR+) and PCa cell lines: LNCaP (AR+), DU145 (AR-), and PC3 (AR-). A subset of LNCaP cells were exposed to the androgen receptor inhibitor bicalutamide for 24 hours (LNCaP+bic). Prostacyclin-washed platelets isolated from healthy human donors were used for TCIPA experiments. TCIPA ($0.25x10^6$ – $2.0x10^6$ cells/mL) was investigated using light-transmittance aggregometry (Chrono-log Dual

Channel Lumi-Aggregator). Western blot was performed to measure PCa prothrombin/thrombin in cellular lysates. Boyden chamber invasion assay and gelatin zymography were performed to assess the effects of platelets on PCa invasion and matrix metalloproteinase (MMP) expression, respectively. One-way, two-way ANOVA, and two tailed t-test were used to evaluate differences between groups.

Results: Both AR+ cell lines, benign RWPE and LNCaPs, failed to induce platelet aggregation. However, AR- cell lines DU145 and PC3, and AR-inhibited LNCaP (LNCaP+bic) all induced platelet aggregation (64.06$\pm$0.88% vs. 26.34$\pm$11.78% vs. 43.73$\pm$8.62%, respectively). The direct thrombin inhibitor, dabigatran (0.8μM), completely abolished DU145, PC3 and LNCaP+bic induced TCIPA. Western blot analysis of PCa lysates demonstrated approximately three-fold greater expression of prothrombin/thrombin in AR- DU145, PC3, and AR-inhibited LNCaP (LNCaP+bic) compared to AR+ RWPE and LNCaPs, $p<0.05$. Platelets enhanced DU145 and PC3, and AR-inhibited LNCaP (LNCaP+bic) invasion and MMP-2 and -9 expression, but not that of LNCaP.

Conclusions: Inhibition or loss of AR signaling within PCa results in increased thrombogenicity due to upregulation of prothrombin/thrombin expression. Reciprocally, platelets enhance invasion of AR-negative PCa cells via increased MMP expression.

Preface

This thesis is an original work by Jan K Rudzinski. No part of this thesis has been previously published. The human platelet isolation protocol was approved by the University of Alberta Human Research Ethics Board for Dr. Paul Jurasz's laboratory; Project name "NOS-based platelet subpopulations", No. Pro00029836, March 8, 2012.

Dedication

I would like to dedicate this thesis to my beautiful family; my wife Kristian Melissa Rambaransingh, my beautiful kids; Samira Lucretia and Kai Krishendath, as well as, my mother Jolanta Rudzinska and my father Wladyslaw Jacek Rudzinski. Without their unconditional love and support I would not have been successful in this journey. My father unfortunately passed away in June 2018, but his passion for research and science was a big source of inspiration for me.

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1.1.1 Platelet structure

Within the human circulation, platelets are small anucleate discoid shaped cellular fragments derived from megakaryocytes. Platelets are approximately 2-4μm in diameter, 0.5 μm in thickness, with a volume of 7 μm^3 (Home S *et al.,* 1988; Jurasz P *et al.,* 2004; Quach ME *et al.,* 2018; Cimmino G *et al.,* 2013). The physiologic concentration of human platelets ranges from 150-400 platelets per μl which have a lifespan of 7-10 days (Schmitt A *et al.,* 2001, Larson & Watson 2006; Quach ME *et al.,* 2018). The primary physiologic function of platelets is to survey for vascular integrity and maintain hemostasis at the site endothelial barrier disruption. The secondary roles of platelets have been implicated in wound healing, angiogenesis, and inflammation (Ginsberg MH *et al*., 1988; Bazzoni G *et al.,* 1991; Celi A *et a.,* 1997; Radomski MW & Radomnski AS 2000; Schwarz UR *et al.,* 2001; Alonso D & Radomski, MW 2003). In conditions such as myocardial infarction, ischemic stroke, and cancer metastasis platelets have been implicated in pathologic thrombosis (Cangemi R *et al.,* 2014; Jurasz P *et al.,* 2004; Klinger MH & Jelkman W 2002; Patzelt J & Langer HF 2012). The platelet ultrastructure is composed of surface glycocalyx, plasma membrane, spectrin based membrane skeleton, peripheral microtubule band, actin-based cytoskeletal network, open canalicular system (OCS), dense tubular system (DTS), and α and δ (dense) granules, lysosomes, and mitochondria (White JG 1967, 1968, 1972). In the absence of activating stimuli, platelets circulate in quiescent discoid shape (White JG 1988; Jurasz P *et al.,* 2004). Upon exposure to activating stimuli, platelets undergo a rapid shape change,

transforming them into a sphere with long and thin filopodium extensions (Yan M & Jurasz P *et al.,* 2016). Figure 1 demonstrates electron micrograph images of resting and activated human platelets.

The platelet plasma membrane is a phospholipid bilayer embedded with cholesterol, glycolipids, glycoproteins, and surface receptors (Heemskerk JW *et al.,* 2002). To regulate intracellular ionic concentrations, the platelet plasma membrane contains sodium and calcium adenosine triphosphate (ATP) pumps (Heemskerk JW *et al.,* 2002). The composition of phospholipids on the platelet plasma membrane demonstrates asymmetrical distribution to regulate coagulation (Heemskerk JW *et al.,* 2002; Solum NO 1999). In their resting state, negatively charged phospholipids involved in coagulation, such as phosphatidylserine and phosphatidylethanolamine, are confined into the inner leaflet of the plasma membrane facing the platelet cytosol (Bevers EM *et al.,* 1983; Heemskerk JW *et al.,* 2002). This asymmetry is maintained by ATP dependent aminophospholipid translocase and the interaction of negatively charged phospholipids with cytoskeleton (Comfurius P *et al.,* 1985; Bevers EM *et al.,* 1989; Heemskerk JW *et al.,* 2002). The platelet plasma membrane is enriched in lipids rafts which are composed of cholesterol and sphingolipids which are involved in platelet signalling and intracellular trafficking (Bodin S *et al.,* 2003). In addition, the plasma membrane is also decorated with transmembrane receptor classes such as integrins ($\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$), leucine-rich receptors (glycoprotein (GP) Ib-IX-V), G-coupled receptors (PAR1, PAR4, $P2Y_1$, $P2Y_{12}$, TPα and TPβ), proteins in the immunoglobulin superfamily (GP VI, FcγIIA), and C-type lectin receptors (P-selectin) (Rivera J *et a.,* 2009). The most abundant and critical surface integrin is $\alpha_{IIb}\beta_3$ (GP IIb-IIIa) with approximately 40,000 – 80,000 copies per platelet with additional pool recruited following platelet activation (Woods VL *et al.,* 1986; Wagner CL *et al.,* 1996; Phillips DR *et al.,*

1988; Bennett JS *et al.,* 2009; Bledzka K *et al.,* 2013). The shift of GP IIb-IIIa from low to high affinity conformation is required for platelet aggregate formation (Peerschke EI *et al.,* 1985; Plow EF *et al.,* 1989). In its high affinity state, GP IIb-IIIa adheres to fibrinogen to form stable connections between adjacent platelets during platelet plug formation (Peerschke EI *et al.,* 1985; Plow EF *et al.,* 1989; Xiong *et al.,* 2003). Mutations in GP IIb-IIIa receptor result in Glanzmann's thrombasthenia where platelets are unable to form stable aggregates (Seligsohn U 2002; Nurden AT 2006). Furthermore, pharmacological agents such as eptifibatide and abciximab directly inhibit GP IIb-IIIa (Madan M *et al.,* 1998; Kleiman NS *et al.,* 1999).

The platelet cytoskeleton is composed of membrane-associated spectrin cytoskeleton, marginal microtubule coil, and cytoplasmic actin network (Kenney DM *et al.,* 1985; Kowit JD *et al.,* 1988; Fox JE 1993; Italiano JE *et al.,* 2007; Thon JN *et al.,* 2010; Italiano JE 2013). These cytoskeletal components provide support to the platelet plasma membrane and give shape to the resting and activated platelets (Fox JE 1993; Kenney DM *et al.,* 1985).

The spectrin cytoskeletal network is composed of; spectrin, filamin, and talin, which laminate the cytoplasmic surface of the platelet plasma membrane and the OCS (Fox JE 1993; Hartwig JH & DeSisto M 1991). The α and β subunits of spectrin are fortified by filamin A which closely associates with cytoplasmic actin filaments and the GP Ib-IX-V receptor (Fox JE 1993). During the resting state, filamin also associates with cytoplasmic domains of the β_3 subunits of integrin receptor GP IIb-IIIa keeping it in a low affinity state (Calderwood DA *et al.,* 2001; Kimea T *et al.,* 2006; Ithychanda SS *et al.,* 2009). Conversely, actin–myosin contraction during platelet activation induce conformational changes to GP IIb-IIIa converting it to high affinity state (Fox JE 1993).

The marginal microtubule band consists of 8-12 microtubule coils 24 nm in diameter that run along the perimeter of the platelet to maintain its discoid shape (White 1968; Italiano JE Jr *et al.,* 2003; Patel-Hett S *et al.,* 2008). The α and *β* tubulin subunits are in a dynamic equilibrium with assembled microtubules (White 1968; Italiano JE Jr *et al.,* 2003; Patel-Hett S *et al.,* 2008). RAN binding protein 10 (RanBP10) is a small guanosine triphosphatase (GTPase) involved in regulation of platelet microtubule dynamics (Kenney DM *et al.,* 1985; Kowit JD *et al.,* 1988; Patel-Hett S *et al.,* 2008; Kunert S *et al.,* 2009). Activation of platelets leads to disassembly of microtubule band which allows for platelets to assume a spherical morphology (Patel-Hett S *et al.,* 2008).

Within the resting platelets, 40% of cytoplasmic actin is present as filamentous (F-actin), whereas, the remainder is stored as globular monomeric (G-actin) complexed with β_4-thymosin (Safer D *et al.,* 1994). The actin filaments form a lattice within the interior of the platelet and are interconnected at various points by acting binding protein filamin and α-actinin (Rosenberg *et al.,* 1981, 1981; Rosenberg *et al.,* 1988). To increase the proportion of cytoplasmic F-actin during platelet activation, actin filaments within the resting platelets are severed and the smaller fragments serve as a nexus for new actin filament assembly (Barkalow KL *et al.,* 2003). In brief, platelet shape change begins with increase in intra-platelet calcium leading to activation of gelsolin which is responsible for actin filament fragmentation (Machesky *et al.,* 1997; Mahoney NM *et al.,* 1997). The assembly of new actin filaments begins with dissociation of capping proteins from barbed ends of the filament fragments and activation actin-related protein (ARP) 2/3 complex to nucleate de novo filaments (Machesky *et al.,* 1997; Mahoney NM *et al.,* 1997). During polymerization, actin monomers are transferred from β_4-thymosin to the barbed ends of actin filaments by profilin (Barkalow K *et al.,* 1996; Nachmias VT *et al.,* 1996; Barkalow KL *et al.,* 2003). Once the assembly

of new long actin filaments is complete, capZ stabilizes the end of the filaments (Barkalow K *et al.,* 1996; Nachmias VT *et al.,* 1996). In addition to fillipodium formation, phosphorylation of cytoplasmic myosin light chains during platelet activation leads to contraction of actin to further contribute to platelet shape change and granule exocytosis.

The plasma membrane is connected to OCS which are invaginations in plasma membrane that tunnel into the interior of the platelet (Benke O, 1970). OCS is important for entry of external elements into the interior of the platelet, it is a route of granule content release, and extensive storage of plasma membrane which is important during platelet shape change and formation of filipodia (White JG, 1974). It has also been suggested to store GP IIb-IIIa, GP Ib-IX, GPVI and $P2Y_1$, and TXA_2 receptors (Michelson AD, 1992; Nurden P *et al.*, 1994; Rendu F *et al.,* 2001; Suzuki H *et al.,* 2003; Nurden P *et al.,* 2003). The DTS is a closed channel of residual rough endoplasmic reticulum of megakaryocytes closely located to the OCS (Rendu F *et al.,* 2001). The primary role of DTS within the platelet is to store ionized calcium which is sequestered by calcium binding protein calreticulin (White JG, 1972; Michalak M *et al.,* 1998; Varga-Szabo D *et al.,* 2009). Binding of inositol 1,4,5 triphosphate (IP3) to IP3 type II receptor stimulates calcium release from DTS, whereas, sarcoplasmic reticulum calcium ATPase (SERCA) translocates cytosolic calcium back into the DTS. The DTS calcium release is associated with redistribution and activation of GP IIb-IIIa, cytoskeletal reorganization, and granule content release (Brwonlow SL & Sage SO 2003; Van Gorp RM *et al.,* 2002; Teijero RG *et al.,* 1999; Varga-Szabo D *et al.,* 2009). In addition, DTS is also a site of prostaglandin and TXA_2 synthesis (Gerrard JM *et al.,* 1978).

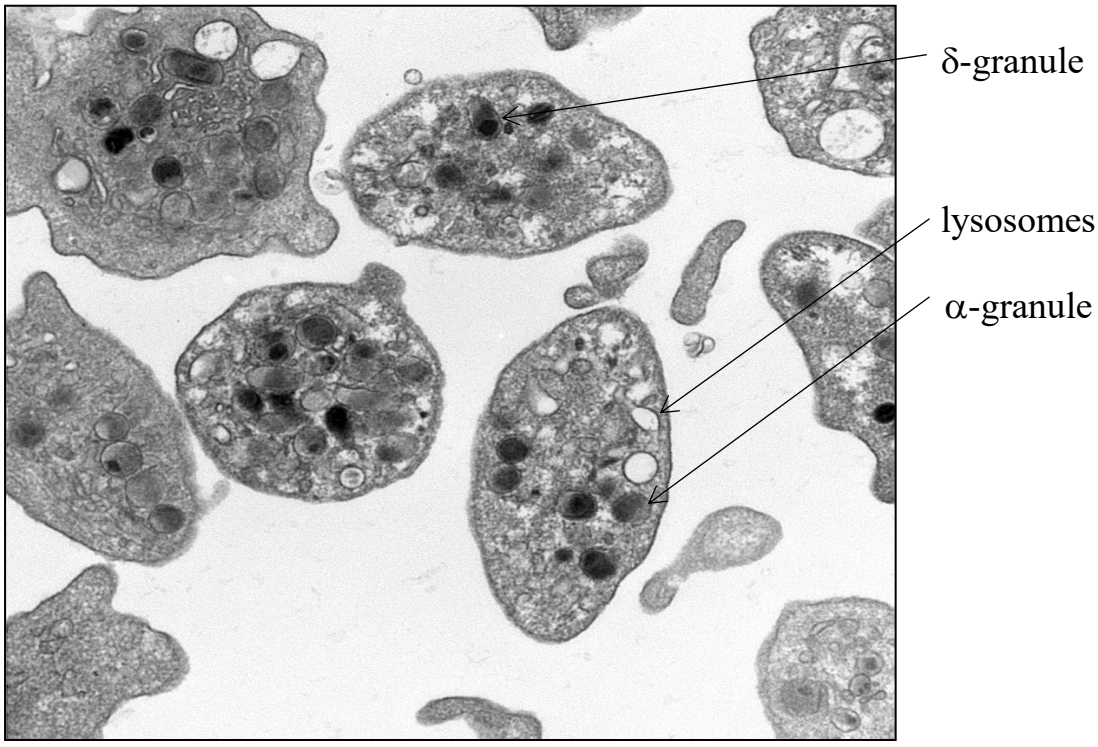
Platelet contain mitochondria located near the plasma membrane which are involved in oxidative energy metabolism and fatty acid oxidation (Rendu F *et al.,* 2001). Platelet activation

leads to an increase in glycolytic activity and oxidative ATP production (Akkerman JW *et al.,* 1979). The ATP within the platelets is devoted to maintaining ionic and osmotic homeostasis, supporting polymerization and depolymerisation of actin, continuous phosphorylation and dephosphorylation of phosphatidylinositols involved in signal transductions (Daniel JL *et al.,* 1986; Verhoeven AJ *et al.,* 1987; Dean WL, 1989). Platelets can also store glycogen and take up glucose from its surrounding environment (Karpatkin S & Langer RM, 1968). In addition to metabolism, regulation of mitochondrial Bcl-2 such as Bcl-x and Bak affects platelet apoptosis, which is responsible for determining platelet lifespan (Mason KD *et al.,* 2008).

Platelet contain three distinct granules; α, δ, and lysosomes. α granules are 200-400 nm in size and are the most abundant granules containing over 300 biologically active molecules (Hayward CP *et al.,* 1995; Harrison P & Cramer EM *et al.,* 1993; Flaumenhaft R, 2003; Coopinger JA *et al.,* 2004; Maynard DM *et al.,* 2007; Thon JN & Italiano JE, 2010). In brief, α granules contain; adhesion molecules, pro-coagulants, pro-angiogenic and anti-angiogenic factors, matrix metalloproteinases, and pro-inflammatory cytokines (Folkman J *et al.,* 2001; Maynard DM *et al.,* 2007; Santos-Martinez MJ *et al.,* 2008; Gleissner CA *et al.,* 2008; Blair P & Flaumenhaft R, 2009). δ granules are 150 nm in size and contain both protein and non-protein molecules such as ions, nucleotides, membrane proteins, transmitters, and protease inhibitors (Fukami MH *et al.,* 1977; McNicol A & Israels SJ *et al.,* 1999). There are approximately 3-8 δ granules per platelet. Lysosomes are 175-250 nm in size and contain digestive enzymes active under acidic conditions such as phospholipase A, glycosidases, glycohydrolazes, and proteases (Gordon JL, 1975; Waite M & Griffin HD *et al.,* 1976; Metzelaar MJ *et al.,* 1992; Emiliani C *et al.,* 1995; Ciferri S *et al.,* 2000). Table 1 provides a summary for platelet granule contents.

Platelet exocytosis and secretion requires fusion of intracellular granules with plasma membrane which is regulated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) (Lemons PP *et al.,* 1997; Weber T *et al.,* 1998*;* Polgar J *et al.,* 1999; Sudhof TC & Rothman JE, 2009). The granules are decorated with v-SNARE such as; vesicle-associated membrane protein (VAMP), whereas, plasma membrane is embedded with t-SNAREs such as; synaptosome associated protein (SNAP) and syntaxin. Human platelets contain SNAP-23,25,29, as well as, syntaxin 2,4,7, and 11 (Flaumenhaft R *et al.,* 1999; Polgar J *et al.,* 2003). VAMP-8 is required for secretion of all three types of platelet granules (Ren Q *et al.,* 2007). Interaction between SNAREs result in membrane fusion and is regulated by small GTPases such as Rab27 (Hampson A *et al.,* 2013). The phosphorylation of syntaxin 4 regulates α granule release, whereas, phosphorylation of syntaxin 2 leads to δ granule secretion (Chen D *et al.,* 2000; Chen D *et al.,* 2000; Lemmons PP *et al.,* 2000). The platelet release reaction can be measured by surveying expression of granule markers on the platelet surface such as; P selection (α granule), granulophysin (δ granule), and CD63 (lysosomes) (Rendu F & Brohard-Bohn B, 2001).

A.



B.

i. ***ii.*** ***iii.***

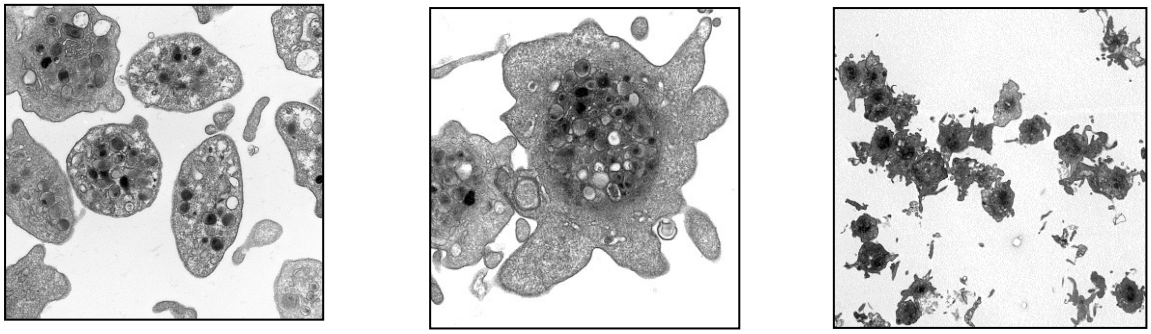


Figure 1: Platelet electron microscopy. (A) Resting platelets. Arrows point to select structures. **(B)** ***(i)*** Resting platelets in discoid shape ***(ii)*** Activated platelets during shape change ***(iii)*** Platelet aggregate.

α – granule	δ – granule	Lysosomes
Adhesion molecules: vWF, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, P-selectin, thrombospondin, fibrinogen, fibronectin	**Ions:** calcium, magnesium, phosphate, pyrophosphate	Phospholipase A
Coagulation factors: fibrinogen, factor V, factor VIII	**Nucleotides:** ATP, ADP, GTP, GDP	Glycohydrolase
Fibrinolytic factors: α2 macroglobulin, plasminogen, PAI-1, SERPINE1, urokinase plasminogen activator (uPA)	**Membrane proteins:** tetraspanin, LAMP2	
Growth factors: PDGF, EGF, HGF, IGF1, TGFβ, VEGF	**Transmitters:** 5-HT, epinephrine, histamine	
Pro- and anti- angiogenic factors: angiopoietin – 1, angiostatin, S1P	**Protease inhibitors:** TFPI	
Matrix metalloproteinases: MMP-1, 2, 3, 9, 14, **Inhibitor of metalloproteinases**: TIMP-1,2,4		
Pro-inflammatory mediators: CXC-motif chemokines; CXCL1,4,5,7,8, and 12 C-C motif ligands; CCL-2,3,5,7,17 IL-1β, PAF, and LPA		
Immunologic molecules: C1 inhibitor and IgG		
Other: albumin, α1 antitrypsin, Gas6, HMWK		

Table 1: Platelet granule content

1.1.2 Platelet generation, maturation, and clearance

Platelets originate from megakaryocytes which are derived from pluripotent hematopoietic stem cells (HSC) (Avecilla *et al.,* 2004). HSC give rise to early progenitors which undergo erythrocyte or megakaryocyte differentiation within the bone marrow (Avecilla *et al.,* 2004). To assemble and release platelets, megakaryocytes undergo endomitosis which results in polyploidy without cellular division by allowing the cells to proceed through G1, S, and G2 bypassing the M phase (Ravid *et al.,* 2002). Overall, the process of endomitosis leads to amplification of nuclear material and increase in cytoplasmic volume to support platelet synthesis (Bluteau *et al.,* 2009). Megakaryocyte differentiation and endomitosis is regulated by liver synthesized thrombopoietin (TPO) which signals through the megakaryocyte c-Mpl receptor (Kaushansky 2006; Tucker *et al.,* 2010). Other growth factors which stimulate thrombopoiesis include interleukin (IL)-3, stem cell factor (SCF), IL-6, and IL-11 (Kaushansky 2006). Once mature, megakaryocytes migrate to a vascular niche, disassemble at one pole, and extend long branching pro-platelets into the lumen of bone marrow sinusoidal blood vessels. This process is regulated by stromal derived growth factor - 1 (SDF-1), fibroblast growth factor - 4 (FGF-4), and intact sinusoids within the bone marrow (Avecilla *et al.,* 2004). The pro-platelets shaft becomes filled with microtubule bundles which elongate and loop around to re-enter the pro-platelet and serves as tracks for granules and mitochondria movement from megakaryocyte cytoplasm to the pro-platelet tip (Hartwig & Italiano 2006). Following further maturation, the pro-platelet fragments shear off into the vascular lumen where they continue to release individual platelets from their tips under shear stress conditions within the vasculature (Italiano *et al.,* 1999, 2007). Single megakaryocycates have the capacity to generate 1,000-3,000 platelets and its residual nuclear material is phagocytosed by macrophages (Stenberg & Levin 1989).

Within the circulation, mature platelets circulate for 7-10 days and become cleared by the hepatocytes in the liver (Leeksma CH *et al.,* 1955; Hartwig J & Italiano JR, 2003). There are two major proposed mechanisms of platelet clearance senescence induced signalling by glycan degradation and the apoptotic mechanism. The convergence of these pathways and their relative contribution is not well elucidated (Grozovsky R *et al.,* 2015). The removal of senescent sialic acid deprived platelets is mediated by the hepatocytes in an Ashwell-Morell receptor (AMR) dependent pathway (Grewal PL 2010; Grozovsky R *et al.,* 2015). In brief, the GP Ibα subunit (part of GP Ib-X-V complex), is heavily decorated with sialic acid which is cleaved by lysosome derived neuraminidases leading to exposure of galactose and β-N-acetyl-D-glucosamine (βGN) residues (Soslau G & Giles J, *et al.,* 1982; Sorensen AL *et al.,* 2009; Jansen AJ *et al.,* 2012; Grozovsky R *et al.,* 2015). Exposure of these residues on platelet surface leads to its recognition by the AMR on the surface of hepatocytes and macrophages (Rumjantseva V *et al.,* 2009). Furthermore, uptake of senescent platelets leads to TPO expression by hepatocytes which acts as a positive feedback stimulus to initiate platelet synthesis from megakaryocytes (Grozovsky R *et al.,* 2015). Platelet survival is also dependent on the balance between pro-survival Bcl-2, Bcl-XL, MCL-1 and pro-apoptotic Bak and Bax which are regulators of intrinsic apoptotic pathway within the platelets (Mason K *et al.,* 2007; Mason KD *et al.,* 2008; Josefsson EC *et al.,* 2011; Debrinacat MA *et al.,* 2012). The BH-3 initiator of apoptosis such as Bad inhibits Bcl-XL leads to activation of Bax and Bak resulting in mitochondrial permeabilization, cytochrome C release, and activation of caspase 9 (Van der Wal DE *et al.,* 2010; Zhang XH *et al.,* 2015). Furthermore, redistribution of phosphatidylserine from inner to outer leaflet of the platelet plasma membrane serves as a signal for clearance by phagocytosis (McArthur K *et al.,* 2018).

1.1.3 Hemostatic role of platelets

Overall, hemostasis at the site of vascular injury is divided into four distinct phases; vasoconstriction, platelet plug formation, reinforcement of platelet plug with fibrin mesh, and clearance of the thrombus by the fibrinolytic system. Within the microvasculature, laminar flow of blood laterally displaces platelets towards the vessel wall to allow for their surveillance of vascular integrity. Under normal physiologic conditions, to prevent platelet activation in uninjured vessels intact endothelial cells release of nitric oxide (NO), prostacyclin (PGI_2), and express ectonucleotidase CD39, as well as, thrombomodulin to limit platelet reactivity (Radomski MW *et al.,* 1987; Radomski MW & Moncada S, 1993; Michelson AD, 2003; Watson SP, 2009). In addition, platelets generate or release NO, matrix metalloproteinase 9 (MMP-9), and tissue inhibitor of matrix metalloproteinase 4 (TIMP-4) to contribute to the negative feedback system (Radomski MW *et al.,* 1990b; Santos-Martínez MJ *et al.,* 2008). However, upon disruption of endothelial barrier, the hemostatic function of platelets is separated into five major steps; rolling, adhesion, activation, granule secretion, and aggregation (Ruggeri ZM, 2002).

With exposure of sub-endothelial collagen, soluble circulating vWF becomes immobilized by its interaction with exposed type I and III collagen (Wu YP *et al.,* 2000; Brass LF, 2003; Davi G *et al.,* 2007; Varga-Szabo D *et al.,* 2008). The collagen immobilized vWF is recognized by platelet surface GP Ib-IX-V resulting in formation of transient tethering bonds leading to slowing and rolling of platelet under high shear stress conditions (Wu YP *et al.,* 2000; Kumar RA *et al.,* 2003; Brass LF, 2003; Davi G *et al.,* 2007; Varga-Szabo D *et al.,* 2008). The reduction in platelet velocity allows for intimate interactions between collagen and platelet surface receptor GP VI and GP Ia-IIa ($\alpha_2\beta_1$) (Savage B *et al.,* 1998; Jackson SP *et al.,* 2003). Firm attachment of collagen to GP VI triggers "inside-out" platelet activation leading to shift in conformation of integrin GP Ia-

IIa and GP IIb-IIIa to a high affinity state, prompt platelet shape change, and granule content secretion (Clemetson KJ 1995; Moroi M *et al.,* 1989; Nieswandt B & Watson SP 2003). At this stage, signaling events are mediated by small G-protein regulators (SGRs), Src-family kinases (SFKs), and serine/threonine-protein kinases (STKs) (Versteeg HH *et al.,* 2013; Bye AP *et al.,* 2016). The subsequent release of α and δ granule content is followed by amplification of secondary platelet response by epinephrine, ADP, and thromboxane A_2 (TXA_2) (Coller BS *et al.,* 2008; Kim C *et al.,* 2009; Shattil SJ *et al.,* 2009; Kim C *et al.,* 2013). These mediators act in an autocrine positive feedback manner to induce "outside-in" platelet activation through stimulation of phosphoinositide 3-kinases (PI3Ks) and phospholipase Cβ (PLCβ), calcium release into the platelet cytoplasm, activation of protein kinase C (PKC), and Rho-associated protein kinase (ROCK), which are all implicated in platelet shape change, further GP IIb-IIIa activation, and formation of filipodia (Coller BS *et al.,* 2008; Kim C *et al.,* 2009; Shattil SJ *et al.,* 2009; Kim C *et al.,* 2013; Versteeg HH *et al.,* 2013; Bye AP *et al.,* 2016). The growing platelet aggregate is stabilized by interaction with adjacent platelets through GP IIb-IIIa bound to fibrinogen (Coller BS *et al.,* 2008; Kim C *et al.,* 2009; Shattil SJ *et al.,* 2009; Kim C *et al.,* 2013). Lastly, to repair the endothelial barrier defect, activated platelets secrete angiogenic and growth factors to attract fibroblasts and leukocytes to the site of injury (Gawaz M *et al.,* 2013). Table 2 summarizes the major platelet receptors involved in recruitment, adhesion, aggregation, amplification, and negative regulation.

Following adhesion and aggregation, platelets stimulate the coagulation cascade to consolidate the platelet plug with a fibrin mesh (Luchtman-Jones L *et al.,* 1995; Hoffman M 2003; Cox K *et al.,* 2011). The pro-coagulant activity of platelets is mediated by binding to tissue factor (TF), releasing stored coagulation factors, providing surface for amplification of thrombin

generation, and exposure of negatively charged phospholipids on the plasma membrane (Luchtman-Jones L *et al.,* 1995; Hoffman M 2003; Cox K *et al.,* 2011). The coagulation cascade involves two pathways; the contact activation pathway (intrinsic pathway) and the tissue factor pathway (extrinsic pathway) both of which lead to generation active factor X (FXa) to convert prothrombin (FII) to thrombin (FIIa) (final common pathway) (Gailani D *et al.,* 2007; Mackman N 2009). Active thrombin converts circulating fibrinogen to insoluble fibrin monomers which trap additional platelets, erythrocytes, and leukocytes to form a stable thrombus at the site of vascular injury (Wolberg AS *et al.,* 2008; Palta S *et al.,* 2014). Overall, the intrinsic pathway is initiated by formation of a complex on exposed collagen by high-molecular-weight kininogen (HMWK), prekallikrein, and FXII (Luchtman-Jones L *et al.,* 1995; Hoffman M 2003; Cox K *et al.,* 2011). The conversion of prekallikrein to kallikrein leads to activation of FXII which ultimately leads to activation of FX. The primary pathway for initiation of coagulation *in vivo* is the tissue factor pathway in which the coagulation cascade is initiated by release of TF from injured endothelial cells and its association with circulating FVII (Furie B & Furie BC 1988; Furie B & Furie BC 2008; Luchtman-Jones L *et al.,* 1995). The TF-FVII complex activates both FIX and X to generate small amount of thrombin which exerts a positive feedback stimulus to further activate FV, FVIII, FXI, as well as, circulating platelets (Furie B & Furie BC 1988; Furie B & Furie BC 2008; Hoffman M 2003; Cox K *et al.,* 2011). During the propagation of the coagulation system, TF-FVII complex activates FIX, which along with FVIII forms a tenase complex activating more FX (Hoffman M 2003; Cox K *et al.,* 2011). The resultant active FX, in association with FV, calcium, and phospholipids on the surface of activated platelets forms pro-thrombinase complex converting prothrombin to active thrombin (Furie B & Furie BC 1988; Furie B & Furie BC 2008; Hoffman M 2003; Cox K *et al.,* 2011). The clotting cascade is summarized in Figure 2. To limit the extent

of thrombus formation to the site of vascular injury, the coagulation cascade is regulated by inhibition of thrombin generation and the fibrinolytic system, whereas, platelet aggregation is inhibited by endothelial-derived prostacylicn (PGI_2) (Cesarman-Maus G & Hajjar KA 2005). The three major regulators of thrombin synthesis are; protein C, anti-thrombin, and tissue factor pathway inhibitor (TFPI) (Furie B & Furie BC 2008; Luchtman-Jones L *et al.,* 1995). Thrombin binds to endothelial membrane receptor thrombomodulin and activates serine protease protein C to activated protein C (APC) (Sadler JE 1997). APC combines with a cofactor protein S and degrades active FV and FVIII (Sadler JE 1997). Anti-thrombin is a serine protease inhibitor which directly degrades thrombin and active FIX, FX, FXI, and FXII (Luchtman-Jones L *et al.,* 1995). Lastly, TFPI directly inhibits excess TF (Luchtman-Jones L *et al.,* 1995). Following repair of endothelial injury, the resultant thrombus is degraded by the process of fibrinolysis (Cesarman-Maus G & Hajjar KA 2005; Furie B & Furie BC 2008). The fibrinolytic system is primarily composed of plasmin which cleaves fibrin into fibrin degradation product (FDP) (Luchtman-Jones L *et al.,* 1995; Hoffman M 2003; Furie B & Furie BC 2008; Cox K *et al.,* 2011). Plasmin is derived from circulating plasminogen by endothelial cell derived tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) (Wolberg, AS & Campbell RA 2008). To regulate this system, t-PA and u-PA are both inhibited by plasminogen activator inhibitor (PAI) 1 and 2, whereas, α-2 macroglobulin, α-2 anti-plasmin inactivates plasmin (Wolberg, AS & Campbell RA 2008). In addition, thrombin activatable fibrinolysis inhibitor (TAFI) prevents formation of plasmin (Luchtman-Jones L *et al.,* 1995; Furie B & Furie BC 2008; Cox K *et al.,* 2011). On the other hand, the fibrinolytic system is amplified by APC which inactivates inhibitors of tissue plasminogen activator and leads to increased plasmin generation (Luchtman-Jones L *et al.,* 1995; Hoffman M 2003; Cox K *et al.,* 2011). The fibrinolytic system is summarized in Figure 3.

Recruitment	Adhesion & Aggregation	Amplification	Negative regulation
GPIb-IX-V (vWF)	**GPVI** (collagen)	**$P2Y_1$** (ADP)	**PGI_2** (Prostacyclin)
	GPIa-IIa (collagen)	**$P2Y_{12}$** (ADP)	
	GPIIb-IIIa (Fibrinogen)	**$P2X_1$** (ATP)	
		PAR1 (Thrombin)	
		PAR4 (Thrombin)	
		TPα (Thromboxane)	
		β-2 (Epinephrine)	
		5HT (Serotonin)	

Table 2: Major platelet receptors and agonists involved in platelet recruitment, adhesion, aggregation, signal amplification, and negative regulation. The platelet agonists that stimulate these receptors are denoted in the brackets.

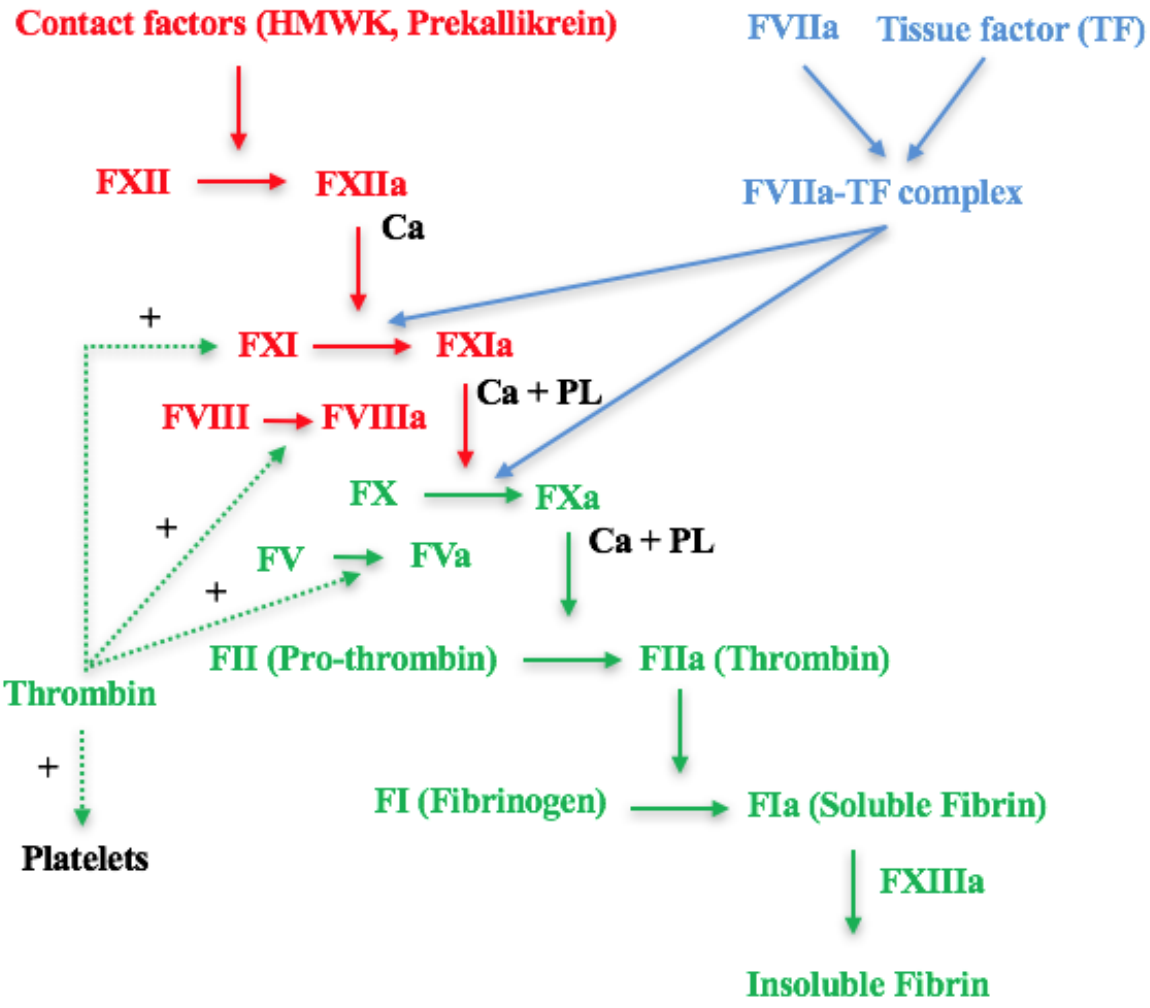


Figure 2: Coagulation cascade.

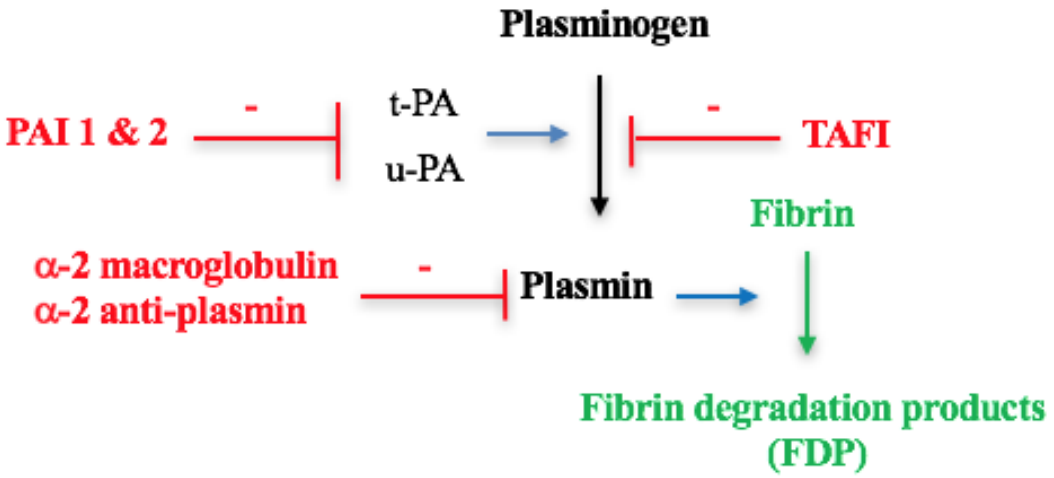


Figure 3: Fibrinolytic system. t-PA - tissue plasminogen activator and u-PA - urokinase plasminogen activator. TAFI - thrombin activatable fibrinolysis inhibitor. PAI - plasminogen activator inhibitor.

1.1.4. Platelet receptor signaling and second messengers

The platelet receptor signalling pathways can be categorized into adhesion receptor mediated and G-protein coupled receptor (GPCR) signalling. Both pathways lead to amplification of critical second massagers within platelets such as phospholipase C (PLC), protein kinase C (PKC), calcium, and Rho-family of GTPases (Rho/Rac).

1.1.4.1 vWF and GP Ib-IX-V

The GP Ib-IX-V complex is composed of leucine rich glycoproteins GP Ibα and GP Ibβ linked by disulfide bonds and non-covalently associated with GP IX and GP V (Lopez JA & Dong JF 1996). Under conditions of high shear stress, platelet deceleration in the vasculature is dependent on interaction between collagen immobilized vWF and platelet GP Ibα-IX (Savage B *et al.,* 1996; Du X 2007; Yago T *et al.,* 2008; Kim J *et al.,* 2010). The resultant tethering allows for platelets to form a more intimate bond with collagen by high affinity GP Ia-IIa and low affinity GP VI receptors (Savage B *et al.,* 1998; Jackson SP *et al.,* 2003). The GP Ib-IX-V complex is also intimately associated with GP VI, FcRγ, and GP Ia-IIa within lipid rafts on the plasma membrane (Sullam PM *et al.,* 1998; Wu Y *et al.,* 2001). Upon binding to collagen, GP VI associated Src family kinases (SFK) Lyn activates PI3K and its downstream effector AKT, leading to activation of mitogen activated protein kinase (MAPK) (p38) and extracellular signal–regulated kinase (ERK) (Li Z *et al.,* 2001; Yap CL *et al.,* 2002; Mazzucato M *et al.,* 2002; Li Z *et al.,* 2003; Kasirer-Friede A *et al.,* 2004; Garcia A *et al.,* 2005; Liu J *et al.,* 2005; Liu J *et al.,* 2006; Li Z *et al.,* 2006; Yin H *et al.,* 2008; Yin H *et al.,* 2008; Mu FT *et al.,* 2010). Overall, these events lead to PLCγ2 phosphorylation, TXA_2 synthesis, granule secretion, as well as, integrin GP IIb-IIIa and GP Ia-IIa activation (Du X 2007; Li Z *et al.,* 2010).

1.1.4.2. Collagen and GP VI

GP VI is a low-affinity collagen receptor required for collagen induced platelet activation (Li Z *et al.,* 2010). It is non-covalently associated with FcRγ homodimer adapter protein containing an immune-receptor tyrosine-based activation motif (ITAM) as the signal-transducing subunit (Enzumi Y *et al.,* 1998; Watson SP *et al.,* 2001; Bergmeier W *et al.,* 2013). Upon collagen binding to GP VI, the ITAM becomes phosphorylated by SFK (Lyn and Fyn) at its tyrosine residues (Enzumi Y *et al.,* 1998; Quek LS *et al.,* 2000). ITAM phosphorylation leads to activation of tyrosine kinase Syk, which phosphorylates scaffold proteins linker for activation of T cells (LAT) and Src homology 2 domain-containing leukocyte protein (SLP-76) (Zahid M *et al.,* 2012; Ozaki Y *et al.,* 2013). Overall, this leads to assembly of signaling complex composed of LAT, SLP-76, Vav1/3, Bruton's tyrosine kinase (BTk), PI3K, and PLCγ2, leading to PLCγ2 activation (Zahid M *et al.,* 2012; Ozaki Y *et al.,* 2013). Active PLCγ2 causes elevation in intra-platelet calcium, activation of calcium and diacylglycerol regulated guanine exchange factor I (CalDAG-GEFI), cleavage of PIP-2 into DAG and IP3, DAG-mediated activation of PKC, and converge on the small GTPase Rap1 that initiate TXA_2 synthesis, GP IIb-IIIa and GP Ia-IIa activation, and granule secretion (Zahid M *et al.,* 2012; Ozaki Y *et al.,* 2013).

1.1.4.3. G-protein coupled signalling

G-protein–coupled receptors (GPCRs) are a family of seven transmembrane domain receptors that transmit signals through heterotrimeric G proteins (Audet M *et al., 2012*; Katritch V *et al.,* 2013; Venkatakrishnan AJ *et al.,* 2013). Heterodimeric G proteins are composed of α, β, and γ subunits (Katritch V *et al.,* 2013). Overall, upon binding of an agonist to the GPCR, it leads to receptor conformational change leading to Gα conversion from GDP bound to GTP bound form

and dissociation from Gβ-Gγ complex (Audet M *et al., 2012*; Katritch V *et al.,* 2013; Venkatakrishnan AJ *et al.,* 2013). Once free, the Gα subunit interacts with guanine exchange factor (GEF) proteins which exchanges GTP for GDP on its downstream target (Audet M *et al., 2012*; Katritch V *et al.,* 2013; Venkatakrishnan AJ *et al.,* 2013). Human platelets express activating Gq, G13, Gi, and inhibitory Gs (Offermanns S *et al.,* 2006).

The Gq receptor activation leads to phosphoinositide hydrolysis by activation of phospholipase C isoforms PLCγ2 or PLCβ which catalyze hydrolysis of PIP-2 releasing DAG and IP3 (Offermanns S *et al.,* 1997; Zucker MB & Nachmias VT 1985; Stalker TJ *et al.,* 2012). The resultant DAG stimulates PKC to induce granule secretion and the IP3 signals the DTS to release calcium into the platelet cytoplasm (Lee HS *et al.,* 2009). Elevation in platelet calcium levels leads to activation of myosin light chain kinase (MLCK) for platelet shape change, PLA_2 for TXA_2 synthesis, and CalDAG-GEFI to trigger GP IIb-IIIa activation via CalDAG-GEFI/Rap1/RIAM pathway (Vogt S *et al.,* 2003; Shattil SJ *et al.,* 2010; Signarvic RS *et al.,* 2010). Combined activation of G13 with Gq results in reorganization of actin skeleton (Klages B *et al.,* 1999; Offermanns S 2001). The G13α activates Rho A converting it into its active GTP bound form (Moers A *et al.,* 2003).

The resultant Rho A activates Rho-activated kinase (p160ROCK) which phosphorylates and inhibits myosin light chain phosphatase, activates LIM-kinase thus enhancing myosin light chain phosphorylation and MLC dependent contraction leading to platelet shape change and granule secretion (Kozasa T *et al.,* 1998; Moers A *et al.,* 2003). Lastly, Gi receptor activation results in Giα subunit induced inhibition of adenylyl cyclase leading to suppression of cAMP synthesis (Yang J *et al.,* 2002; Noe L *et al.,* 2010). On the other hand, the Gβγ subunit activates

PI3K leading to AKT activation and Rap1B resulting in integrin activation (Dorsam RT *et al.,* 2002; Lova P *et al.,* 2002; Woulfe D *et al.,* 2002, 2004). Stimulation of the Gs receptor causes adenylyl cyclase to stimulate cAMP synthesis and resultant activation of protein kinase A (PKA) (Noe L *et al.,* 2010; Smolenski A 2012).

Thrombin-induced platelet activation is mediated by a dual system of G-protein–coupled protease-activated receptors (PARs); PAR1 and PAR4 coupled to Gq, G13, and Gi (Kahn ML *et al.,* 1998; Coughlin SR 1999, 2005; Kim S *et al.,* 2002). TXA_2 activates platelets by the Gq and G13 (Knezevic I *et al.,* 1993; Djellas Y *et al.,* 1999), whereas, ADP induces platelet activation by $P2Y_1$ (Gq coupled) and $P2Y_{12}$ (Gi coupled) (Ohlmann P *et al.,* 1995). Prostacylin exerts its inhibitory effect on platelet aggregation acts by stimulating the Gs (Yang J *et al.,* 2002).

1.1.4.4. Signal amplifications and second messengers

Platelet activation by surface receptors leads to an array of intracellular signaling cascades leading to cytoskeletal reorganization, integrin activation, and granule content secretion. These critical functions are carried out by second messengers such as PLC, PKC, cAMP, calcium, and Rho/Rac.

Within human platelets, PLC is present in the β and γ isoforms. The β form is activated by G proteins, whereas, the γ form is regulated by tyrosine phosphorylation (Brass LF *et al.,* 1991). Once activated, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Rittenhouse SE & Sasson JP 1985; Lian L *et al.,* 2009). The DAG activates PKC, which in turn phosphorylates platelet and leukocyte C kinase (pleckstrin) critical for granule secretion (Lian L *et al.,* 2009). On the other hand, IP3 binds to receptors in the DTS and raises cytoplasmic calcium concentration (Brass LF & Joseph SK 2009;

Kaibuchi K *et al.,* 1983). The PKC isozymes are a family of kinases that play an essential role in the signal transduction mechanisms by mediating phosphorylation of multiple cellular proteins on their serine and threonine residues (Harper MT & Poole AW 2010). PKCα stimulates granule content secretion and has been shown to regulate Rap1, as well as, integrin signaling. PKCδ and θ promote dense granule secretion in response to thrombin receptor agonists (Harper MT & Poole AW 2010). Furthermore, pleckstrin is also a major PKC substrate (Lian L *et al.,* 2009; Harper MT & Poole AW 2010).

Phosphatidylinositol 3-kinases (PI3K) are a group of enzymes that phosphorylate the D-3 position of the inositol ring of phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI 3-P), phosphatidylinositol 3,4-bisphosphates (PI 3,4-P2), and phosphatidylinositol 3,4,5-triphosphates (PI 3,4,5-P2 or PIP3) (Cantley LC 2002). PI3K is activated following stimulation of Gi or phosphotyrosine dependent pathway downstream of collagen receptor leading to stimulation of protein kinase AKT involved in activation of GP IIb-IIIa (Dorsam RT *et al.,* 2002; Lova P *et al.,* 2002; Woulfe D *et al.,* 2002, 2004; Zahid M *et al.,* 2012; Ozaki Y *et al.,* 2013).

Cytoplasmic calcium elevation triggered by calcium release from the DTS by 1,4,5-IP3, and the mediators of calcium influx from the extracellular fluid are store-operated calcium channels (SOCC) (Varga-Szabo D *et al.,* 2009). The rise in cytosolic calcium contributes to platelet activation by actin-myosin interaction by activation of MLC, stimulation of PLA_2 and PLC, as well as, activation of PKC, gesolin, and positive regulation of SFK, PI3K/AKT pathway (Varga-Szabo D *et al.,* 2009).

The raise in the cAMP concentration in platelets inhibits platelet activation and its effect is mediated by cAMP-dependent protein kinase A (PKA) (Schwarz UR *et al.,* 2001). Overall, raising cAMP levels results in impaired phosphoinositide hydrolysis and an accelerated uptake of

calcium into the DTS (Smolenski A 2012). Platelet substrates for PKA enzyme include the 24 kDa β chain of GP Ib, actin-binding protein and MLC, VASP and Rap1B (Siess W *et al.,* 1990; Smolenski A 2012). The PGI_2 released from endothelial cells elevates platelet cAMP levels by stimulating receptors on the platelet surface that are coupled to adenylyl cyclase by the Gs proteins Murata T *et al.,* 1997). Pharmacological agents such as dipyridamole, sildefanil, cilostazol exert their effect by all raising intra-platelet cAMP as well as cGMP (Beebe HG *et al.,* 1999; Schwarz UR *et al.,* 2001; Li Z *et al.,* 2003).

Reorganization of platelet cytoskeleton and subsequent morphological changes to form filipodia are mediated by the Rho family of GTPases (Ridley AJ & Hall A 1992; Ridley AJ *et al.,* 1992; Goggs R *et al.,* 2012; Collins C *et al.,* 2013). Once GTP bound, Gα13 activates p115RhoGEF, leading to activation of RhoA, which in turn activates Rho-associated protein kinase (ROCK) (Fujita A *et al.,* 1991; Huang JS *et al.,* 2001). ROCK then phosphorylates and inactivates MLC phosphatase, increases phosphorylation at MLCK, and stimulates LIM-kinase (LIMK) to phosphorylate cofilin to regulate actin polymerization and cytoskeletal reorganization (Calaminus SD *et al.,* 2001; Pandey D *et al.,* 2001; Ueda K *et al.,* 2003; Leisner TM *et al.,* 2005; Pandey D *et al.,* 2006; Getz TM *et al.,* 2010).

1.1.5. Major platelet agonists, negative regulators, and pharmacological reagents

Exposed sub-endothelial collagen can bind to four platelet receptors; directly to GP Ia-IIa, GP VI, and indirectly to GP IIb-IIIa, and GP Ibα. GP VI is the most potent collagen receptor involved in signaling through ITAM, whereas, GP Ia-IIa is necessary for firm adhesion to collagen. The details of intracellular signaling cascade initiated by platelet interaction with collagen are described in section 1.1.3.1 and 1.1.3.2.

ADP is a weak platelet agonist stored within the dense granules (Fukami MH *et al.,* 1977; McNicol A & Israels SJ *et al.,* 1999). Upon its secretion, it interacts with two types of purinergic GPCRs $P2Y_1$ and $P2Y_{12}$ which are coupled to Gq and Gi, respectively (Le´on C *et al.,* 1997; Hollopeter G *et al.,* 2001; Burnstock G 2007). Stimulation of $P2Y_1$ initiates mobilization of intra-platelet calcium, initiation of platelet shape change, and transient platelet aggregation, whereas, $P2Y_{12}$ is coupled to the inhibition of adenylyl cyclase activity, potentiates activity of TXA_2, thrombin, and contributes to expression of P selectin (Hechler B & Leon C *et al.,* 1998; Gachet C *et al.,* 2008). Activation of both $P2Y_1$ and $P2Y_{12}$ is required for optimal ADP induced increase in intra-platelet calcium concentration, TXA_2 synthesis, shape change, granule secretion, and activation of GP IIb-IIIa leading to platelet aggregation (Jantzen HM *et al.,* 1999; Gachet C *et al.,* 2008; Rivera J *et al.,* 2009). ADP also interacts with ligand gated $P2X_1$ which allows rapid change in membrane permeability to calcium, sodium, and potassium (Murugappa S & Kunapuli SP 2006). Stimulation of $P2X_1$ receptors triggers transient shape change and amplifies platelet responses to low concentrations of other agonists (Murugappa S & Kunapuli SP 2006). Inhibition of $P2Y_{12}$ plays an important role in prevention of thrombotic vascular events in patients with acute coronary syndrome (ACS) and in patients undergoing percutaneous coronary intervention (Smith SC Jr *et al.,* 2006; Hamm CW *et al.,* 2011). Clinically, thienopyridine pro-drugs such as ticlopidine, clopidogrel or prasugrel require hepatic activation for irreversible inhibition of $P2Y_{12}$ *in vivo* (Barret NE *et al.,* 2008). For *in vitro* studies, ADP hydrolyzing apyrase is commonly utilized (Jurasz P *et al.,* 2001; Medina C *et al.,* 2006).

Eicosanoids are derived from arachidonic acid metabolism and are involved in multiple biological functions such as pain, inflammation, as well as, stimulation and inhibition of platelet activation. Arachidonic acid is derived from platelet membrane phospholipids by phospholipase

A2 which is oxidized by COX-1 or 2 to synthesize PGH_2 (Dennis EA *et al.,* 2011). Thromboxane is derived from PGH_2 by TXA_2 synthase and it readily diffuses across platelet plasma membrane to aggregate adjacent platelets through stimulation of Gq and G13 coupled TPα receptor (Samuelsson B 1987; FitzGerald GA *et al.,* 1991; Knezevic I *et al.,* 1993; Ushikubi F *et al.,* 1994; Djellas Y *et al.,* 1999; Offermanns S 2006). Furthermore, since TXA_2 has a very short half-life, its potent agonist activity is contained to the area of endothelial injury. On the other hand, prostacyclin (PGI_2) is synthesized from PGH_2 by the action of the PGI_2 synthase and it is released by endothelial cells (Samuelsson B 1987). Prostacyclin exerts its anti-platelet effect through the IP receptor coupled to Gs which stimulates adenylyl cyclase to increase intra-platelet cAMP resulting in PKA activation, reduction in intracellular calcium concentration, decreased actin polymerization and myosin light chain kinase activity (Kawata M *et al.,* 1989). Acetylsalicylic acid (ASA) irreversibly inhibits COX 1 within platelets causing reduction in TXA_2 synthesis and its inhibitory effect persists for the entire lifespan of platelets for 7-10 days (Davi G *et al.,* 2007).

Thrombin is a serine protease which is one of the most potent platelet agonists. It is derived from 72kDa prothrombin activated by FXa, FVa, calcium prothrombinase complex assembled on the membrane of activated platelets to 37 KDa active thrombin (McNicol A *et al.,* 1989; Brummel KE *et al.,* 2002; Mann KG 2003). Active thrombin is the effector of the coagulation cascade through its cleavage of fibrinogen to fibrin and direct platelet agonist effect through GPCR protease-activated receptors PAR-1 and PAR-4 (Coughlin SR 2005). The PAR-1 receptor is activated by cleavage of N-terminal exodomain at a thrombin cleavage site which then unmasks a new tethered N terminus domain (SFLLRN) allowing for its association with the body of the receptor (Vu TKH *et al.,* 1991; Brass LF 2003). Overall, this results in stimulation of transmembrane signaling through activation Gα13, Gαq, and Gαi with consequent activation of

PLCβ, PI3-K, Rho, Rac, rise in intra-cellular Ca^{2+}, inhibition of cAMP formation, TXA_2 production, and ADP release (Andersen H 1999; Brass LF 2003). Consequently, all these events lead to platelet shape change, integrin activation, and subsequent platelet aggregate formation. PAR-1 is activated at lower thrombin concentration, whereas, PAR-4 requires higher concentration of thrombin (Andersen H *et al.,* 1999; Covic L *et al.,* 2000; Shapiro MJ *et al.,* 2000). Despite the lower affinity of thrombin to PAR-4, both PAR-1 and PAR-4 contributes to platelet activation (Kahn ML *et al.,* 1998; Kahn ML *et al.,* 1999; Covic L *et al.,* 2000; Shapiro MJ *et al.,* 2000). In addition to PARs, it has also been shown that GIbα contains a high affinity binding site for thrombin at its N-terminal globular domain to facilitate cleavage of PARs (De Marco L *et al.,* 1991; Mazzucato M *et al.,* 1998; De Candia E *et al.,* 2001). To activate PAR 1 *in vitro,* synthetic thrombin receptor activating peptide (TRAP) or TFLLR-amides are commonly used (Vu TKH *et al.,* 1991; Kawabata A *et al.,* 1999). There are currently two competitive PAR-1 inhibitors in clinical trials for prevention of arterial thrombosis E-5555 and SCH530348 (Kogushi M *et al.,* 2007; Chackalamannil S *et al.,* 2008). Alternatively, dabigatran which is a direct thrombin inhibitor, is currently approved for use in atrial fibrillation and venous thromboembolism (Sarah S 2013).

Matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent endopeptidases responsible for remodeling extra-cellular matrix. Under physiological conditions, MMPs are involved in embryonic development and would healing (Sternlicht MD & Werb Z 2001; Visse R & Nagase H 2003). However, MMPs have also been implicated in several pathologic processes such as inflammation, atherosclerosis, and cancer metastasis (Sternlicht MD & Werb Z 2001; Visse R & Nagase H 2003). MMP structure is divided into pre (N-terminal domain), a pro-peptide, a zinc-containing catalytic domain, and a hemopexin-like C-terminal domain linked to the

catalytic domain by a flexible hinge region (Sternlicht MD & Werb Z 2001). To tightly regulate their activity *in vivo,* MMPs are expressed in an enzymatically inactive state where a cysteine residues of the pro-domain interact with the zinc ion of the catalytic site (Van Wart HE & Birkedal-Hansen H 1990). Disruption of this interaction by a process of "cysteine switch" leads to proteolytic removal of the pro-domain resulting in activation of MMP activity (Van Wart HE & Birkedal-Hansen H 1990). The MMP family is divided into several subgroups depending on their substrate specificity and structure. Collagenases such as MMP-1, 8, 13, and 18 tend to digest type I, II, and III collagens (Santos-Martinez MJ *et al.,* 2008). Gelatinases such as MMP-2 and MMP-9, have been shown to degrade type IV collagen and gelatin *in vitro* (Santos-Martinez MJ *et al.,* 2008). Stromeolysins (MMP-3, 7, 10, and 11) have a broader substrate specificity and degrade substrates such as; proteoglycans, type IV collagen, fibronectin, and laminin (Santos-Martinez MJ *et al.,* 2008). There is a subtype of membrane bound MMPs such as MT1-MMP (MMP-14) which are not secreted and contain an additional transmembrane domain (Rundhaug J 2005; Malemud C 2006; Fragai M & Luchinat, C 2015). Activity of MMPs is regulated by two major types of endogenous inhibitors; α2-macroglobulin and tissue inhibitors of metalloproteinases TIMP-1, 2, 3, 4 (Sottrup-Jensen L & Birkedal-Hansen H 1989; Baker AH *et al.,* 2002). The TIMPs bind reversibly to the catalytic domain of MMPs and its interaction is not selective for specific subtype of MMP (Gomez DE *et al.,* 1997; Baker AH *et al.,* 2002). Overall, several MMPs have been identified within platelets such as pro-MMP-1, 2, 3, 9, MT1-MMP (MMP-14), and their endogenous inhibitors α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMP-1, 2, and 4) (Murate T *et al.,* 1997; Sawicki G *et al.,* 1997; Fernandez-Patron C *et al.,* 1999; Kazes I *et al.,* 2000; Colciaghi F *et al.,* 2002; Galt SW *et al.,* 2002; Radomski A *et al.,* 2002; Bergmeier W *et al.,* 2004). MMP-2 is stored in the platelet cytoplasm, whereas, MMP-9 has been detected in the

platelet granules (Sawicki G *et al.,* 1998; Sheu JR *et al.,* 2004). There is evidence to suggest that MMP-2 is complexed with TIMP-4 within the platelet cytoplasm and upon platelet activation, it dissociates from TIMP-4, resulting in platelet surface translocation together with MMP-9 (Sawicki G *et al.,* 1998; Radomski A *et al.,* 2004; Sheu JR *et al.,* 2004). MMP-1 can activates platelets by cleavage of PAR-1 at a site distinct from active thrombin and enhances phosphorylation of p38 MAPK which is involved in platelet cytoskeletal reorganization (Galt S.W *et al.,* 2002; Trivedi V *et al.,* 2009). MMP-3 has not been shown to have a role in platelet function (Galt S.W *et al.,* 2002). MMP-14 participates in the activation of pro-MMP-2 on the platelet surface membrane through formation of trimolecular MT1-MMP-TIMP-2-MMP-2 complex, which cleaves 72kDa pro-MMP-2 into active 64 kDa MMP-2 (Kazes I *et al.,* 2000). Once activated, MMP-2 facilitates modification of platelet glycoprotein GPIIb-IIIa, GPIb-V-IX, and increase GPIb level during adherence to vWF (Radomski A *et al.,* 2001; Santos-Martinez MJ *et al.,* 2008). Furthermore, active MMP-2 can amplify platelet aggregation response to various agonists such as; thrombin, TXA_2, and ADP through activation of PLC, PKC, intra-platelet calcium mobilization, and stimulation of PI3K (Falcinelli E *et al.,* 2005). On the other hand, active MMP-9 counteracts the platelet-potentiating effects of MMP-2 and inhibits platelet aggregation through inhibition of PLC, calcium mobilization and TXA_2 synthesis. In addition, MMP-9 can also increase intra-platelet nitric oxide (NO) and cyclic GMP (cGMP) levels resulting in net inhibition of platelet aggregation (Sheu JR *et al.,* 2004; Lee YM *et al.,* 2006). MMP inhibitors are either broad spectrum or they target individual MMPs. O-phenantroline is a broad spectrum MMP inhibitor commonly utilized for *in vitro* experiments (Sawicki G *et al.,* 1997; Jurasz P *et al.,* 2001; Martinez A *et al.,* 2001). Furthermore, compounds such as barmastat, GM6001, GW280264X and TAPI-2 demonstrate inhibition of platelet adhesion, aggregation, and GPIb shedding *in vitro* (Kazes I *et al.,* 2000;

Bergmeier W *et al.,* 2004; Rabie T *et al.,* 2005). Unfortunately, randomized clinical trials evaluating the role of MMP inhibitors on advanced cancer progression and prevention of hemorrhagic transformation in ischemic stroke demonstrate no significant therapeutic benefit (Coussens LM *et al.,* 2002; Fingleton B 2003; Zucker S *et al.,* 2004; Overall CM *et al.,* 2006; Zhao BQ *et al.,* 2006).

Nitric oxide (NO) is a potent inhibitor of platelet activation with a very short half-life synthesized and released by both endothelial cells and platelets to exert multiple paracrine effects (Radomski MW *et al.,* 1987; Radomski MW *et al.,* 1990a; Radomski MW *et al* 1990b). Nitric oxide is generated from L-arginine and oxygen by endothelial (eNOS), cytokine induced (iNOS), or neuronal NO synthase (Radomski MW *et al.,* 1990a). Upon endothelial NO synthesis, it diffuses into vascular smooth muscle cells and the lumen. Within the smooth muscle cells, NO activates soluble guanylyl cyclase, which dephosphorylates guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) leading to downstream smooth muscle relaxation and resultant vasodilation (Garg UC & Hassid A 1989). Similarly, within platelets, NO increases intra-platelet cGMP levels leading to inhibition of PKC causing downstream inhibition of both IP3 formation and intracellular calcium mobilization (Nakashima SF *et al.,* 1986; Ramdomski MW *et al.,* 1987d; Durante W *et al.,* 1992). Furthermore, NO has also been suggested to be indirectly involved in phosphorylation and inhibition of TXA_2 receptor in platelets (Wang GR *et al.,* 1998).

1.1.6. Light transmission aggregometry to measure platelet function

In 1962**,** Born and O'Brien developed a simple method for investigating platelet aggregation by light transmission aggregometry (LTA) (Born GV 1962; O'Brien JR 1962). LTA is considered a gold standard for platelet function testing as and it records changes in light transmission of stirred platelet suspension exposed to agonists as the platelet aggregates are

forming (Born GV 1962; O'Brien JR 1962). Platelet function disorders are commonly evaluated with LTA using agonists such as collagen, epinephrine, ADP, arachidonic acid, and ristocetin on platelet rich plasma (PRP), platelet poor plasma (PPP), or isolated platelets (Moffat KA *et al.,* 2005; Hayward CP *et al.,* 2007). The specific parameters recorded by LTA include; rate of platelet aggregation (% platelet aggregation per minute) and % platelet aggregation at a fixed point in time (Hayward CP *et al.,* 2009; Hofer CK *et al.,* 2010; Picker SM 2011; Harrison P 2013; Kehrel BE & Brodde MF 2013). The major drawbacks of LTA are requirement of large sample volume, non-physiologic low and high shear experimental conditions, and poor sensitivity for detecting micro aggregates comprised of less than 100 platelets (Born GV & Hume M 1967; Thompson NT *et al.,* 1986).

1.2. Platelets and cancer metastasis

1.2.1. Thrombocytosis and thrombosis in cancer

Cancer induced thrombocytosis is observed in nearly every type of solid tumor and its extent is associated with poor clinical outcome, as well as, poor response to chemotherapy or surgery (Gucer F *et al.,* 1998; Monreal M *et al.,* 1998; Symbas NP *et al.,* 2000; Ikeda M *et al.,* 2002; Taucher S *et al.,* 2003; Brown KM *et al.,* 2005; Lin RJ *et al.,* 2014; Long Y *et al.,* 2016; Gao L *et al.,* 2017). In 1872, Reiss and colleagues discovered that patients with malignancies demonstrated elevated platelet counts (Tranum BL & Haut A 1974). This was further corroborated by Levin and Conley in 1960, who found that that thrombocytosis was present in up to 38% of patients with inoperable solid tumors (Levin J & Conley CL 1964). Thrombocytosis in cancer patients is also associated with higher risk of venous thromboembolism (VTE) (Simanek R *et al.,* 2010). High levels of serum TPO was reported among patients with cancer associated thrombocytosis (Uppenkamp M *et al*., 1998). Tumor cells have the capability to generate TPO and

secrete interleukins such as interleukin (IL)-1, IL-3, IL-11, IL-6 to stimulate platelet generation from megakaryocytes (Sasaki, Y *et al.,* 1999; Baj-Krzyworzecka *et al.,* 2002; Stone RL *et al.,* 2012; Lin RJ *et al.,* 2014). In a mouse model of ovarian cancer, tumor secreted IL-6 stimulates hepatic TPO synthesis (Stone RL *et al.,* 2012).

Patients diagnosed with cancer frequently present with pathologic thrombosis, which is more pronounced at a metastatic stage (Wojtukiewicz MZ *et al.,* 2006; Sierko & Wojtukiewicz MZ 2007; Khorana AA *et al.,* 2007; Menter, DG *et al.,* 2014). Thromboembolic disease can be an early diagnostic feature and a major cause of death among patients diagnosed with cancer (Khorana AA *et al.,* 2007; Riedl J *et al.,* 2017). Severe forms of thrombosis in this patient population include; disseminated intravascular coagulation, migratory thrombophlebitis and pulmonary embolism secondary to aberrant platelet activation and aggregation (Simanek R *et al.,* 2010). Overall, cancer patients have 4-7-fold higher risk of developing pulmonary embolism or deep vein thrombosis (DVT) compared to healthy individuals (Simanek R *et al.,* 2010; Timp JF *et al.,* 2013). There are several proposed mechanisms by which platelets are involved in cancer associated VTE exposure of negatively charged phospholipids necessary for thrombin generation, platelet-leukocyte interaction leading to platelet-rich micro-thrombi generation, adhesion to endothelial cells, and formation of neutrophil extracellular traps (Wahrenbrock M *et al.,* 2003; Vanschoonbeek K *et al.,* 2004; Brill A *et al.,* 2005; Clark JH *et al.,* 2010).

The initial link between cancer and the coagulation system was proposed by Jean-Baptiste Bouillard who reported a case of DVT associated with cancer (Bouillaud S *et al.,* 1823). In 1865, the relationship between abnormalities of the coagulation system and cancer was described by Armand Trousseau who described migratory thrombophlebitis in patients with occult malignancies

suggesting that thrombosis could be a sign of malignancy (Trousseau A 1865). In 1878, Theodor Billroth hypothesized that platelets can form a thrombus around the tumor and promote its metastasis by providing protection and enhancing endothelial adhesion within the vasculature (Billroth T 1878).

The experimental link between platelets and cancer metastasis was first explored by Gasic and colleagues who induced thrombocytopenia in mice with neurominidase or antiplatelet serum and demonstrated reduction in experimental metastasis (Gasic GJ *et al.,* 1973). On the other hand, infusion of platelet-rich plasma reversed this effect, suggesting that platelets may play an active role in cancer progression (Gasic GJ *et al.,* 1973). Furthermore, mouse studies disrupting platelet function with knockout of GP VI or P-selectin demonstrated 50% reduction in distant metastasis, whereas, depletion of platelet α-granules showed 80% reduction in metastatic dissemination (Kim YJ *et al.,* 1998; Camerer E *et al.,*2004; Jain S *et al.,* 2007, 2009; Guerrero JA *et al.,* 2014). Metastatic behavior of tumors is influenced by their capacity to activate and aggregate platelets (Karpatkin S *et al.,* 1988). Taken together, this data suggests that there is a bidirectional relationship between platelets and cancer; tumor cells influence platelet count and activation status which then contributes to cancer progression and metastasis.

1.2.2. Alteration of platelets in cancer patients

Platelets isolated from patients with solid tumors demonstrate higher level of baseline activation and reactivity compared to healthy donors (Ferriere JP *et al.,* 1985; Yazaki T *et al.,* 1987; Prisco D *et al.,* 1995; Abbasciano V *et al.,* 1995). This phenomenon is further pronounced in patients with metastatic dissemination (Yazaki T *et al.,* 1987; Prisco D *et al.,* 1995; Abbasciano V *et al.,* 1995). For example, when stimulated with same concentration of collagen or thrombin,

platelets from metastatic prostate cancer patients demonstrated higher thrombogenicity compared to platelets from patients with non-metastatic disease (Jurasz P *et al.,* 2003). In addition, platelets from cancer patients express higher level of platelet activation markers such as; P-selectin, CD40, and β-thromboglobulin which correlate with higher risk of VTE, cancer progression, and poor prognosis (Ay C *et al.,* 2008; Riedl J *et al.,* 2014).

In cancer patients, platelet content can also be altered. This phenomenon is either due to direct effect of tumor cells on platelet transcriptome or secondary to platelet sequestration (Zimmerman GA *et al.,* 2008; Best MG *et al.,* 2015). Compared to healthy donors, platelets from patients with cancer contain larger number of α-granules with a higher concentration of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), platelet factor – 4 (PF-4), angiopoietin-1, MMP-2, and IL-6 (Mohle R *et al.,* 1997; Battinelli EM *et al.,* 2011; Battinelli EM *et al.,* 2014; Farooqi AA *et al.,* 2015; Johnson KE *et al.,* 2017). There is also emerging evidence that tumors can also modulate platelet ribonucleic acid (RNA) content by generation of tumor educated platelets (TEP) (Best MG *et al.,* 2015; Joosse SA *et al.,* 2015). In glioma and prostate cancer, tumor associated RNA such as EGFRvIII and PCA3 have been detected within platelets, respectively (Nilsson RJ *et al.,* 2011). Furthermore, messenger RNA (mRNA) sequencing of TEP has been investigated for detection and staging of non-small cell lung cancer, glioblastoma, colorectal, pancreatic, hepatobiliary, and breast cancers (Calverley DC *et al.,* 2010; Best MG *et al.,* 2015; Joosse SA *et al.,* 2015). With further development, TEP may become a platform for liquid biopsy for variety of solid tumors.

1.2.3. Tumor cell induced platelet aggregation and degranulation

During rapid tumor cell proliferation, aberrant and leaky vascular network supplies the growing tumor mass (McDonald DM *et al.,* 2002; Kuznetsov HS *et al.,* 2012). Tumor associated vasculature tends to have exposed sub-endothelial connective tissue allowing for platelet recruitment, activation, and secretion of metastasis promoting factors. Infiltration of platelets into the tumor mass can also prevent development of intra-tumoral hemorrhage (Kisucka J *et al.,* 2006; Ho-Tin-Noe B *et al.,* 2008). Continued tumor proliferation and basement membrane degradation leads to cancer cell intravasation into the circulation and platelet aggregate formation through a process known as tumor cell induced platelet aggregation (TCIPA) (Pacchiarini L *et al.,* 1991; Boukerche H *et al.,* 1994; Alonso-Escolano D *et al.,* 2004). The ability and extent of a cancer cell induced platelet aggregation tends to correlate with its metastatic potential (Pearlstein E *et al.,* 1980; Honn KV *et al,* 1992; Green DL *et al.,* 2009). The functional consequence of TCIPA is cancer cell survival under high shear stress conditions within the vasculature, immuno-evasion by platelet mediated shielding of tumor cells from natural killer cells and tumor necrosis factor (TNF)-α mediated cytotoxicity, embolization and extravasation at a secondary site, as well as, tumor angiogenesis and growth (Pearlstein E *et al.,* 1980; Rickles FR *et al.,* 2001; Honn KV & Tang D 1992; Philippe C *et al.,* 1993; Nieswandt M *et al.,* 2001; Alonso-Escolano D *et al.,* 2006). The reciprocal platelet induced tumor signaling can further enhance the invasive potential of cancer cells (Alonso-Escolano D *et al.,* 2006).

In brief, there are several mechanisms by which tumor cells activate and attract circulating platelets. Tumor cells release soluble platelet activating mediators such as ADP, TXA_2 and proteases such as; MMP-2, thrombin, cathepsin B, and cancer pro-coagulant (Honn KV *et al.,* 1982; Rickles FR *et al.,* 2001; Jurasz P *et al.,* 2004; Yan M & Jurasz P 2016). Tumors cells also

secrete TF to initiate activation of coagulation cascade (Thomas GM *et al.,* 2009; van den Berg YW *et al.,* 2012; Geddings JE *et al.,* 2013). Once activated, platelets also release ADP, MMP-2, and generate TXA_2 to potentiate the tumor cell-platelet aggregate formation (Sawicki G *et al.,* 1997; Jurasz P *et al.,* 2004). Direct receptor mediated interactions between platelets and cancer cells are predominantly reported to be through GP Ib-IX-V, GP IIb-IIIa, P-selectin, and CLEC-2 receptors (Stone JP *et al.,* 1993; Ludwig RJ *et al.,* 2004; Bertozzi CC *et al.,* 2007). Fibrinogen forms a bridge between platelet GP IIb-IIIa and tumor cell integrin $\alpha_V\beta_3$, P-selectin adheres to tumor cell mucins, whereas, platelet surface CLEC-2 interacts with cancer cell podoplanin (Honn KV *et al.,* 1992; Borsig L *et al.,* 2001; Felding-Habermann B *et al.,* 2001; Alonso-Escolano D *et al.,* 2004; Bertozzi CC *et al.,* 2007). More recently, cancer cells have been shown to secrete granulocyte colony stimulating factor (G-CSF) which acts on neutrophils to promote release of platelet activating neutrophil extracellular DNA traps (Fuchs TA *et al.,* 2010; Demers M *et al.,* 2012). Overall, the release of platelet α-granule derived vWF, fibronectin, as well as, increased expression of GP IIb-IIIa and P-selectin on activated platelet surface strengthens adhesive interactions between tumor cells and platelets.

ADP secreted by tumor cells interacts with platelet surface $P2Y_1$ and $P2Y_{12}$ leading to platelet shape change, aggregation, and TXA_2 release. In return, activated platelets release ADP from δ granules to further potentiate platelet aggregation. Cancer cell derived ADP has been shown to play a role in TCIPA induced by SKNMC neuroblastoma, A549 small cell lung cancer, MCF-7 breast adenocarcinoma, and HT-1080 fibrosarcoma cells (Bastida E *et al.,* 1986; Heinmoller E *et al.,* 1996; Jurasz P *et al.,* 2001; Alonso-Escolano D *et al.,* 2004). In MCF-7 cell lines, ADP was shown to activate platelets through stimulation of $P2Y_{12}$ (Alonso-Escolano D *et al.,* 2004). Scavenging ADP with apyrase or pharmacologic inhibition of $P2Y_{12}$ *in vitro* demonstrates

significant reduction in the extent of TCIPA in ovarian, breast, hepatocellular, and melanoma cell lines (Grignani G *et al.,* 1989; Boukerche H *et al.,* 1994; Jurasz P *et al.,* 2001; Alonso-Escolano D *et al.,* 2004; Cooke N *et al.,* 2015; Holmes CE *et al.,* 2016). Furthermore, soluble apyrase/ADPase, APT102, given in combination with ASA shows inhibition of TCIPA and subsequent reduction in breast cancer and melanoma metastasis in mice models *in vivo* (Uluçkan O *et al.,* 2008). In an *in vivo* murine ovarian, pancreatic, and melanoma cancer cell model, pharmacologic inhibition of $P2Y_{12}$ with ticagrelor or clopidogrel demonstrated reduction in tumor growth, metastatic dissemination, and improvement in survival (Gebremeskel S *et al.,* 2015; Mezouar S *et al.,* 2015; Cho MS *et al.,* 2017). Unfortunately, in a Phase II clinical trial investigating the combined effect of clopidogrel and ASA on number of circulating tumor cells (CTCs) in breast cancer patients, there was no significant reduction in detectable CTCs (Roop RP *et al.,* 2013).

The release of TXA_2 into tumor microenvironment initiates TCIPA through stimulation of TP receptors on platelets. Colorectal adenocarcinoma cell lines such as; HT-29 and T-84 express thromboxane synthase (TS) and secrete high levels of TXA_2 (Guillem-Llobat, P *et al.,* 2016; Wojtukiewicz MA *et al.,* 2015; 2016). Furthermore, injection of platelet-exposed HT-29 cells into immune-deficient mice results in higher metastatic dissemination to lungs which is attenuated by ASA treatment *in vivo* (Sierko E & Wojtukiewicz MZ 2007; Shiao J *et al.,* 2017). On the other hand, ASA does not demonstrate significant reduction in TCIPA induced by Caco-2 colonic adenocarcinoma cells *in vitro* (Medina C *et al.,* 2006). Overexpression of TS has also been reported in prostate, breast, bladder, colon, brain, and non-small cell lung cancers (Ekambaram P *et al.,* 2011). ASA is a weak inhibitor of TCIPA *in vitro,* but it has been shown to inhibit experimental metastasis of hepatocellular and Lewis lung carcinoma models *in vivo*. In addition, ASA inhibits proliferation of SKOV-3 ovarian, SW480 colon, and PANC-1 pancreatic cancer cell lines (Cooke

NM *et al.,* 2015; Mitrugno, A *et al.,* 2017). Selective TS antagonist, OKY-046, has not shown a significant effect on osteogenic sarcoma induced TCIPA, whereas, TP specific antagonist, SQ-29, 548 and a mixed TS inhibitor, BM-567, both effectively attenuate TCIPA *in vitro* (Mehta P *et al.,* 1986; De Leval X *et al.,* 2003). Clinically, randomized trials demonstrate that low dose ASA can reduce the incidence, distant recurrence rate, and mortality associated with colorectal carcinoma (CRC) (Cooper K *et al.,* 2010; Rothwell P *et al.,* 2010). In addition, COX-2 selective inhibitors and ASA have demonstrated effectiveness in prevention of colorectal adenoma and development of colon cancer in patients with familial adenomatous polyposis (Thun MJ *et al.,* 2002). Epidemiological data from prospective observational studies and multi-institutional registries suggest that ASA can also lower the incidence, decrease distant recurrence, and lower cancer specific mortality associated with breast and prostate cancer, respectively (Choe K *et al.,* 2012; Holmes C *et al.,* 2016).

Overexpression of MMP-2 by cancer cells is responsible for extracellular matrix degradation and invasion. In addition, it has been demonstrated that TCIPA induced by A549, HT-1080, Caco-2, and MCF-7 cell lines is in part mediated by cancer cell and platelet released MMP-2 (Jurasz *et al.,* 2001; Radomski *et al.,* 2002; Alonso- Escolano *et al.,* 2004; Medina C *et al.,* 2006). Inhibition of MMP-2 with phenanthroline, MMP-2 selective antibodies, or addition of recombinant TIMP-4 demonstrates attenuation of MCF-7 and HT-1080 induced TCIPA *in vitro* (Jurasz *et al.,* 2001; Radomski *et al.,* 2002; Alonso- Escolano *et al.,* 2004). MMP-2 may also contribute to increased platelet reactivity in patients with metastatic prostate adenocarcinoma (Jurasz P *et al.,* 2003). However, clinical trials investigating the role of MMP inhibition on progression of advanced malignancies demonstrate no significant therapeutic benefit (Overall CM & Lopez-Otin C 2002).

Thrombin is the most potent platelet agonist exerting its effect through PARs and has been shown to be secreted by glioblastoma (U87MG), neuroblastoma, pancreatic, prostate cancer (PC3), non-small cell and small cell lung cancer cell lines to induce TCIPA (Bastida E *et al.,* 1986; Esumi N *et al.,* 1987; Heinmöller E *et al.,* 1995; 1996; Swaim M *et al.,* 1996). Thrombin induced platelet activation results in generation and release of TXA_2, increased surface expression of P-selectin, as well as, release of vWF and fibronectin facilitating platelet cancer cell adhesion (Wojtukiewicz, MZ *et al.,* 2015; Di Vito C *et al.,* 2016). Indirectly, thrombin can also upregulate TF and VEGF release by MDA-231 breast cancer cell line to enhance its metastatic potential (Asanuma K *et al.,* 2013). Thrombin also promotes tumor cell invasion through stimulation of PI3K pathway and induction of MMP-9 expression within the cancer cells(Radjabi AR *et al.,* 2008). Inhibition of thrombin with dansylarginine N-(3-ethyl-1,5-pentanediyl) amide demonstrated attenuation of colon cancer cell induced TCIPA *in vitro*, whereas, thrombin inhibition with hirudin decreases TCIPA induced by non-small cell and small cell lung carcinoma cell lines (Pearlstein E *et al.,* 1982; Heinmöller E *et al.,* 1996). Direct thrombin inhibitors such as argatobran and dabigaran have shown to decrease tumor migration and distant metastatic burden in breast cancer models *in vivo* (Asanuma K *et al.,* 2004; DeFeo K *et al.,* 2010; Asanuma K *et al.,* 2013; Alexander ET *et al.,* 2015). Thrombin generation can be indirectly targeted by inhibition of factor Xa with rivaroxaban. There is an ongoing clinical trial evaluating the efficacy of preoperative thrombin inhibition with rivaroxaban on proliferation of estrogen receptor negative breast cancer (Kirwan CC *et al.,* 2016). On the other hand, PAR targeted agents such as vorapaxar implemented in cardiovascular disease have not yet been tested for treatment of cancer (Cunningham M *et al.,* 2016).

Tissue factor is a transmembrane glycoprotein and the main initiator of coagulation cascade through the extrinsic pathway. Once released into the circulation, TF associates with activated

FVII to form the TF-FVII complex responsible for proteolytic activation of FX leading to thrombin generation and fibrin mesh deposition. It has been shown that TF expressed by cancer cells plays an important role in promoting tumor growth and angiogenesis (Liu Y *et al.,* 2011; Ruf W *et al.,* 2013). Breast cancer cell lines were noted to express and release TF or TF bearing microparticles into the circulation (Ruf W *et al.,* 2013). Inhibition of TF-FVII *in vivo* has been shown to result in tumor growth arrest in mice models of breast cancer (Liu Y *et al.,* 2011).

The GP Ib-IX-V receptor complex and GP VI physically interact with vWF and collagen, respectively. GP Ib-IX-V expression has been demonstrated on the surface of platelets and MCF-7 breast cancer cells during TCIPA *in vitro* (Oleksowicz L *et al.,* 1995; Oleksowicz L & Dutcher JP 1995; Jurasz *et al.,* 2001; Alonso- Escolano *et al.,* 2004). Furthermore, addition of purified vWF potentiates HT-1080 induced TCIPA whereas, inhibition of GP Ib-IX-V attenuates platelet cancer interactions (Karpatkin S *et al.,* 1988; Clezardin P *et al.,* 1993; Jurasz *et al.,* 2001). In an *in vivo* experimental mouse model, inhibition of tumor cell GP Ib-IX-V or GP VI demonstrated reduction in TCIPA and distant metastasis. Furthermore, mice deficient in platelet GP VI demonstrate significant reduction in lung metastasis after injection with Lewis Lung carcinoma or B16-F10 mouse melanoma cell lines (Nieswandt B & Watson SP 2003; Jain S *et al.,* 2007; Jain S *et al.,* 2009; Bambace NM & Holmes CE 2011). In addition to adhesion, platelets have the capability to upregulate the expression of GP Ib-IX and GP IIb-IIIa on the surface of MCF-7 breast cancer cells (Alonso-Escolano *et al.,* 2004).

GP IIb-IIIa is a key platelet integrin involved in establishing firm interactions between other platelets and the extracellular matrix. Antagonism of GP IIb-IIIa results in inhibition of final pathway of platelet aggregation. In experimental models *in vitro,* GP IIb-IIIa antagonists are noted to be the most effective inhibitors of TCIPA (Karpatkin S *et al.,* 1988). Initial experiments

performed in 1988 demonstrated that blockade of GP IIb-IIIa with antibodies resulted in attenuation of colorectal and melanoma cancer cell line platelet interactions *in vitro* and further reduced their metastatic potential *in vivo* (Karpatkin S *et al.,* 1988). Pharmacologic inhibitors of GP IIb-IIIa such as tirofiban and eptifibatide or monoclonal antibody against GP IIb-IIIa; abciximab, are currently approved for use in cardiovascular disease (Hagemeyer C Peter K 2010). The blockade of GP IIb-IIIa results in disruption of TCIPA in MCF-7 breast cancer, HeLA cervical carcinoma, LL2 Lewis Lung carcinoma, B16-F10 mouse melanoma, Sos-2 osteosarcoma, and PC3 prostate cancer cell lines (Amirkhosravi A *et al.,* 1990; Sheu JR *et al.,* 1993; Nierodzik ML *et al.,* 1995; Chiang HS *et al.,* 1995; Swaim MW *et al.,* 1996; Lonsdorf AS *et al.,* 2012). Currently GP IIb-IIIa antagonists are not approved for use in cancer therapy (Wujtkiewicz MZ *et al.,* 2017). Furthermore, novel selective β3 integrin blockers are not yet supported by strong clinical data for approval in use for treatment of cancer (Wujtkiewicz MZ *et al.,* 2017).

Under physiological conditions, expression of platelet P selectin plays an important role in mediating interactions between activated platelets and leukocytes. However, in experimental cancer models, P selectin has been suggested to mediate interactions between activated platelets and colorectal, lung, breast, gastric, and melanoma cancer cell lines (Plantureux L *et al.,* 2018). Deficiency in platelet P selectin expression results in reduction in the number of platelet-cancer aggregates, attenuation of tumor growth, and reduction in lung metastasis *in vivo* (Kim YJ *et al.,* 1998; Qi C *et al.,* 2015). C-type lecithin-like receptor 2 (CLEC-2) is a newly discovered platelet surface receptor which binds to podoplanin. Physiologically, podoplanin is involved in the development of lymphatic vessels and formation of a thrombus (Kato Y *et al.,* 2008; Suzuki-Inouse K *et al.,* 2011; Shirai T *et al.,* 2017). However, podoplanin has also been shown to be expressed on colorectal, bladder, and lung carcinomas leading to its interaction with platelet CLEC-2

resulting in increased tumor growth and metastasis. Monoclonal antibodies which disrupt podoplanin-CLEL-2 interaction results in reduction of tumor emboli formation and distant metastasis *in vivo* (Kato Y *et al.,* 2003; Kato Y *et al.,* 2005; Nakazawa Y *et al.,* 2011; Fujita N *et al.,* 2012; Takagi S *et al.,* 2013; Takagi S *et al.,* 2014; Miyata K *et al.,* 2014; Sekiguchi T *et al.,* 2016).

Following activation and recruitment of platelets by cancer cells, numerous growth factors are released into the tumor microenvironment. Growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor – 1 (IGF-1), transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), angiopoietin-1, stromal cell derived factor – 1 (SDF-1) are released from platelet α-granules to stimulate tumor growth and angiogenesis (Menter DG *et al.,* 2014). In addition, platelet α-granules also release MMP-1, 2, 3, 9 and 14 to promote vascular remodeling and invasion (Santos-Martinez MJ *et al.,* 2008). The release of δ granule content such as; calcium, magnesium, and ADP stimulate platelet activation and aggregation, whereas, serotonin, epinephrine, and histamine potentiate the effect of ADP on TCIPA (Vanags DM *et al.,* 1992).

1.2.4. The effect of platelets on tumor microenvironment and metastasis

Once recruited to the tumor microenvironment (TME), platelets can influence tumor cell proliferation, migration, and invasion. It has been demonstrated that platelet derived PDGF, TXA_2, and platelet activating factor (PAF) promote cancer cell proliferation *in vitro* (Di Stefano JF *et al;* 1990; Cathcart MC *et al.,* 2011; Kim HA *et al.,* 2011). Furthermore, within the TME, platelets enhance tumor cell invasion through induction of epithelial to mesenchymal transformation (EMT), release of MMPs into the peritumoral space, and facilitating local inflammation leading to

increased vascular permeability (Menashi S et al., 1991; Belloc C *et al.,* 1995; Holmes CE *et al.,* 2009; Alonso-Escolano D *et al.,* 2006; Labelle M *et al.,* 2011). The process of EMT transforms neoplastic epithelial cells to attain an invasive mesenchymal phenotype leading to loss of cell to cell adhesion, loss of cellular polarity, and increases in cellular motility (Thiery JP *et al.,* 2006; Scheel C *et al.,* 2007). Platelets have been shown to induce expression of EMT regulators within cancer cells such as twist, smad, slug, and nuclear factor - κB (NF-κB) (Labelle M *et al.,* 2011). Platelet derived TGF-β is required for initiation of EMT through NF-κB and to a lesser extent, platelet released HGF and PDGF also contribute to EMT (Gotzmann J *et al.,* 2006; Labelle M *et al.,* 2011). Overall, EMT leads to upregulation of vimentin, fibronectin, MMP-9 and downregulation of E-cadherin (Thiery JP *et al.,* 2006; Labelle M *et al.,* 2011). The release of platelet-derived MMP into peritumoral space further contribute to ECM breakdown, whereas, of release of ADP, TGF-β, and VEGF also facilitates tumor intravasation through disruption of endothelial barrier (Anderberg C *et al.,* 2013; Schumacher D *et al.,* 2013; Leblanc R *et al.,* 2014).

Platelets have a significant contribution to tumor angiogenesis as they contain various pro-angiogenic factors such as VEGF, PDGF, TGF, EGF, IGF-1, sphingosine-1-phosphate, MMPs and anti-angiogenic factors such as PF-4, thrombospondin-1, endostatin, serotonin, plasminogen activator inhibitor-1, and angiostatin (Jurasz P *et al.,* 2003; Battinelli EM *et al.,* 2011; Menter DG *et al.,* 2014; Yan M *et al.,* 2014). It has been shown that stimulation of platelet PAR-1 leads to preferential release of pro-angiogenic factors, whereas, PAR-4 activation results in secretion of anti-angiogenic factors (Ma L *et al.,* 2005; Italiano JE Jr *et al.,* 2008). Furthermore, experimental data demonstrate that platelets stimulate endothelial cell proliferation and migration *in vitro* and *in vivo* (Langer H *et al.,* 2006). Overall, the net effect of platelets on TME is to promote angiogenesis (Jurasz P *et al.,* 2003). The newly synthesized tumor neovasculature tends to be leaky

and platelets preferentially adhere, stabilize, and contribute to maturation of these vessels through release of angiopoietin-1 and serotonin (Ho-Tin-Noe *et al.,* 2008; Pietramaggiori *et al.,* 2008). Furthermore, the angiogenic vessels provide a potential route for tumor cells to escape the primary site. Platelets can also sequester VEGF and deliver it to distant metastatic sites (Lakka Klement G *et al.,* 2009). This finding is corroborated by clinical data demonstrating that platelet content from cancer patients is enriched with VEGF compared to healthy controls (Holmes CE *et al.,* 2008; Peterson JE *et al.,* 2010).

Metastasis is a complex biological process involving detachment of tumor cells form a primary site, vascular intravsation, arrest in distal capillaries, and extravsation at a secondary site. Platelets enhance metastatic potential of cancer cells at each stage of metastatic cascade. Upon entry into the circulation, cancer cells induce TCIPA to withstand high shear stress conditions and gain protection from natural killer (NK) cells. In fact, only about 0.1% of cancer cells survive the hostile environment within the circulation (Fidler IJ 1970). Platelets contribute to cancer immune-evasion by expression of NK inhibitory ligand glucocorticoid-induced tumor necrosis factor ligand (GITRL), downregulation of NK cell natural killer group 2D (NKG2D) immune-receptor, downregulation of NK cell derived IFN-γ by TGF-β, and transfer of MHC class I onto cancer cells (Palumbo JS *et al.,* 2005; Kopp HG *et al.,* 2009; Placke T *et al.,* 2012; Lee YL *et al.,* 2013). Tumor cell platelet aggregates arrest and adhere to endothelial cells through interaction with P-selectin on activated platelets (Läubli H & Borsig L 2010). Furthermore, surface expression of GPIbα, GPIIb-IIIa, and $\alpha_v\beta_3$ on tumor cells augments their adherence to endothelial cells (Desgrosellier JS & Cheresh DA 2010). Platelet derived MMP-1, TGF-β, and ADAM12 contribute to breakdown of endothelial junctions allowing for cancer cell transmigration and successful colonization at a metastatic site (Lewalle JM *et al.,* 1991; Reymond N *et al.,* 2013).

In addition to its granule content and surface receptors, platelets can also shed metastasis promoting platelet micro particles (PMPs). The PMPs are shed during platelet activation and are composed of membrane bound proteins and cytoplasmic contents (Barry OP *et al.,* 1998; Janowska-Wieczorek A *et al.,* 2006). High levels of PMPs were demonstrated in the circulation of patients with several different cancers (Kim HK *et al.,* 2003). Pre-incubation of prostate cancer cell line with PMPs demonstrated increased expression of MMP-2 within the cancer cells which then translated to increased invasiveness *in vitro* (Dashevsky O *et al.,* 2009). Furthermore, *in vivo* models of Lewis lung carcinoma demonstrate that co-incubation of cancer cells with PMPs resulted in greater distant metastatic burden and enhanced tumor angiogenesis (Brill A *et al.,* 2005; Janowska-Wieczorek A *et al.,* 2005).

1.3. Prostate cancer

1.3.1. Prostate physiology and androgen receptor signalling

Prostate gland is a tubulo-avelolar exocrine gland of the male reproductive system derived from the urogenital sinus and its development is driven by testosterone. It is anatomically located at the bladder neck and is composed of epithelial and stroma cells (Wang Y *et al.,* 2001). The prostatic epithelial compartment is composed of basal cells, intermediate cells, neuroendocrine cells, and luminal secretory cells (De Marzo AM *et al.,* 1998). The stromal compartment is comprised of connecting tissue, smooth muscle cells, and fibroblasts which provide support to the epithelium (De Marzo AM *et al.,* 1998). The luminal columnar secretory epithelial cells constitute most of the prostatic epithelium and express prostate specific antigen (PSA), acid phosphatases, androgen receptor (AR) leucine amino peptidases, 15-lipoxygenase-2, as well as cytokeratin CK 8 and 18 (Shappell SB *et al.,* 1995). Androgens such as testosterone, dihydroepiandrosterone (DHEA), androstenediol, and androstenedione influence growth and maintenance of the prostate

gland by affecting proliferation and differentiation of luminal epithelial cells (Isaacs JT *et al.,* 1994; Cunha GR *et al.,* 2004). Overall, the main function of the prostate is to secrete alkaline fluid into the semen to prolong life-span of sperm in the female reproductive system.

The prostate is stimulated in its growth, maintenance, and secretory function by continued presence of testosterone which is converted within the prostate into more potent dihydrotestosterone (DHT) by type II 5-α-reductase (5-AR) in conjunction with nicotinamide-adenine dinucleotide phosphate (NADP) (Radmayr C *et al.,* 2008). In brief, circulating testosterone in the plasma is delivered to the prostate by albumin and steroid binding globulins (Radmayr C *et al.,* 2008). Diffusion of free testosterone into the prostatic epithelium results in its rapid and irreversible conversion to DHT which binds to AR in the cytoplasm leading to dimerization and activation of the steroid receptor (Radmayr C *et al.,* 2008). Nuclear transportation of activated AR leads to chromatin remodelling though interaction with co-regulatory proteins (Trapman J, Cleutjens KB 1997). Binding of activated receptor-coactivator complex to DNA segments known as androgen response elements (ARE) results in activation or repression target genes by regulating production of their mRNA (Trapman J, Cleutjens KB 1997).

The AR gene in located on chromosome X (Xq11-12) and encodes for 110 kilo Dalton (kDa) protein (Gelmann EP *et al.,* 2002). The AR protein is composed of multiple functional domains such as; zinc finger containing DNA-binding and steroid-binding domain, as well as, nuclear localization motif and several coactivator-corepressor binding sites (Jenster G *et al.,* 1991; Gelmann EP *et al.,* 2002; Claessens F *et al.,* 2008). Androgen receptor is a member of nuclear receptor superfamily that also consists of mineralocorticoid, glucocorticoid, estrogen, and progesterone receptors (Detera-Wadleigh SD & Fanning TG 1994). Immediately after its production, AR forms a complex with chaperonins such as Hsp90, 70, Hip, p60, 23, FKB51, 52,

and Cyp 4 (8S complex) which sequesters and inactivates the AR in the cytoplasm (Gelmann EP *et al.,* 2002). The monomeric AR is in constant equilibrium with the chaperonins, however, upon ligand binding, AR undergoes conformational change which results in its inability to re-associate with the 8S complex leading to ligand-receptor nuclear translocation and dimerization (Gelmann EP *et al.,* 2002). Once the activated AR is localized within the nucleus, along with its co-activators such as; p160 family, pCAF, CREB-binding protein, and p300 it associates with ARE (Alen P *et al.,* 1999; Ma H *et al.,* 1999; Zhou HJ *et al.,* 2005). The AR binding to ARE facilitates further recruitment of co-activators with histone acetyltransferase activity such as; CBP, p300, CAF through p160, resulting in chromatic remodelling and enhanced DNA transcription (Alen P *et al.,* 1999; Ma H *et al.,* 1999; Zhou HJ *et al.,* 2005). Non-ligand bound AR tends to be transported out to the cytoplasm for further ligand binding or it is shuttled for degradation following ubiquitination by E3 ubiquitin ligase. In prostatic epithelial cells, AR regulates the expression of multiple genes which modulate cell growth, differentiation, and function such as NKX3.1, FOX family of transcription factors, IGF-1R, UBEC2C, UGT2B15, KLK3, TMRSS2, FKBP5, and PSA (Takayama K & Inoue S 2013). In addition, AR is also involved in non-genomic signalling at the plasma membrane and the cytoplasm regulating the activity of other nuclear receptors or transcription factors through triggering raise in intra-cellular calcium levels, as well as, activation of protein kinases such as; MAPK, PKA, Akt, and PKC (Baron S *et al.,* 2004; Foradori CD *et al.,* 2008).

1.3.2. Prostate cancer epidemiology, pathophysiology, diagnosis, and staging

Prostate cancer (PCa) is the most common visceral malignancy and third leading cause of cancer death among men in Canada (Izawa JI *et al.,* 2011). Majority of PCa is of adenocarcinoma subtype which arises from glandular epithelial cells. Globally, PCa is reported to be the second

most common cancer and sixth leading cause of cancer death worldwide (Center MM *et al.,* 2012). The 2017 Canadian Cancer Society estimates suggest that 21,300 men were diagnosed and 4,100 patients died of this disease in Canada. Since 1991, there is a steady decline in PCa mortality secondary to; early detection, widespread prostate specific antigen (PSA) screening, as well as, increased effectiveness of curative treatments for localized and advanced PCa (Etzioni R *et al.,* 1999; Walsh PC 2000; Etzioni R *et al.,* 2008; Welch HG & Albertson PC 2009).

The peak incidence of PCa is between 70 to 77 year of age and the risk factors for developing PCa include; age over 65, family history in first degree relative, African American race, chronic prostatic inflammation, smoking, and obesity (Izawa JI *et al.,* 2011). Prostate cancer is screening is achieved by periodic measurement of serum prostate specific antigen (PSA) and physical examination by digital rectal examination (DRE). Prostate specific antigen is a 33 kDa serine protease produced and released by luminal prostatic epithelial cells. Disruption of normal glandular architecture during proliferation of prostatic adenocarcinoma facilitates PSA access into the systemic circulation (Izawa JI *et al.,* 2011). Despite the controversy associated with PSA screening, appropriate use of PSA provides a diagnostic lead time of 5-10 years (Izawa JI *et al.,* 2011). Therefore, current North American guidelines recommend annual DRE and PSA screening for men 50-65 years of age with >10-year life expectancy (Izawa JI *et al.,* 2011).

The gold standard for PCa diagnosis is tissue sample obtained through trans-rectal ultrasound (TRUS) guided prostate biopsy. Dedicated genitourinary pathologists utilize the Gleason scoring system to analyze the biopsy specimen. The architectural patters of prostatic adenocarcinoma are assigned a grade from 1 to 5, with 1 being most differentiated and 5 being least differentiated (Epstein JI *et al.,* 2005). The final pathological report states the most prominent pattern, the second most prominent pattern, and the composite Gleason Grade Group (GG)

(Epstein JI *et al.,* 2005; Pierorazio PM *et a.,* 2013). Overall, the higher the GG, the greater the aggressiveness of PCa (Epstein JI *et al.,* 2005, 2006).

Prostate cancer is staged using the American Joint Committee on Cancer (AJCC) TNM Staging System (Carroll PH & Mohler JL 2018). In brief, T1 is clinically localized and non-palpable, T2 is palpable and confined to the prostate, T3 disease exhibits extra-prostatic extension with or without seminal vesicle invasion, whereas, T4 disease invades adjacent structures.

Following tissue diagnosis, PCa is then risk stratified based on clinical T stage, PSA level, and GG into; very low, low, favourable and unfavorable intermediate, as well as, high risk, and very high risk disease categories (Carroll PH & Mohler JL 2018). The risk stratification system predicts the risk of biochemical failure and cancer specific mortality following curative intent treatment (Epstein JI *et al* 2006; Carroll PH & Mohler JL 2018). Patients in the very low, low, and favourable intermediate risk category with >10-year life expectancy are usually offered; active surveillance (AS), radical prostatectomy (RP), external beam radiotherapy (EBRT) or brachytherapy (BT) (Carroll PH & Mohler JL 2018). In the unfavorable risk category, patients can be treated with RP with pelvic lymph node dissection or EBRT with 4-6 months of androgen deprivation therapy (ADT) (Carroll PH & Mohler JL 2018). Lastly, patient classified into high risk or very high risk are usually offered EBRT with ADT for 2-3 yeas, EBRT with brachytherapy and 1-3 years of ADT, or RP + PLND in experienced centers (Carroll PH & Mohler JL 2018). In most risk categories, if the life expectancy is <10-years, patients can be observed to spare the morbidity of PCa treatment. However, once the disease becomes metastatic, ADT becomes the mainstay of treatment. Upon further progression, in the metastatic castrate-resistant phase, second generation androgen antagonist or steroidogenesis inhibitors can further prolong overall survival (OS) (Scher HI *et al.,* 2012; Rathkopf DE *et al.,* 2017).

1.3.3. Prostate cancer metastasis

In patients with localized PCa, the 5-year OS is approximately 100%, whereas, in patients with hormone sensitive distant metastatic disease the 5-year survival drops to 30%. Furthermore, in the late stage castrate resistant phase, the 3-year OS is 30% (Ferlay, J *et al.,* 2010; Jemal A *et al.,* 2010). Prostatic adenocarcinoma has the propensity to metastasize to the pelvic lymph nodes, bone, liver, lungs, and brain (Varkarakis MJ *et al.,* 1974; Cho KR *et al.,* 1987; Hess KR *et al.,* 2006). The metastatic cascade of PCa involves local invasion and migration, intravasation, circulation, extravasation, and colonization at a secondary site.

Normal prostatic epithelium is joined together by E-cadherin and β-catenin complex to maintain cell to cell adhesion (Takeichi M *et al.,* 1991; van Roy F & Berx G 2008). In addition, interaction of prostatic epithelium with extracellular matrix (ECM) through expression of α5β1, α6β1, α6β4, αvβ3, α3β1 is essential for benign prostatic epithelial proliferation, survival, and migration (Mol AJ *et al.,* 2007). However, during initial malignant transformation, prostatic epithelial cells undergo epithelial to mesenchymal transformation (EMT) to obtain more migratory and invasive properties (Mol AJ *et al.,* 2007). *In vivo* experiments in nude mice have demonstrated that EMT is critical for the development of metastatic prostate adenocarcinoma (He H *et al.,* 2010). During EMT, cadherin switching leads to downregulation of E-cadherin and upregulation of N-cadherin, which is also accompanied by downregulation of β-catenin (Gravdal K *et al.,* 2007). Compared to low grade prostate cancer, high grade prostatic adenocarcinoma such as Gleason $\geq$8 disease demonstrated lower E-cadherin and β-catenin expression, but higher level of N-cadherin expression (Umbas R *et al.,* 1992, 1994; Jaggi M *et al.,* 2005). In addition, prostatic adenocarcinoma tissue samples demonstrate reduced expression of α6 integrins which promotes aberrant cellular migration (Schmelz M *et al.,* 2002). Disruption of cellular attachments is further

accompanied by increase in activity of focal adhesion kinases (FAK) and Src family of non-receptor protein kinases (SFKs) which leads to actin cytoskeleton rearrangement and increase in cellular migration (Tremblay L *et al.,* 1996; Mitra SK *et al.,* 2005, 2006). In prostate cancer cells, inhibition of SFK leads to reduction in proliferation, migration, and invasion (Slack JK *et al.,* 2001; Recchia I *et al.,* 2003; Nam S *et al.,* 2006).

The alteration in migratory capacity in prostatic adenocarcinoma is associated with upregulation of ECM degrading proteinases such as MMP and serine protease - urokinase-type plasminogen activator (uPA). In prostatic adenocarcinoma tissue samples, MMP-2 and 9 expression is upregulated compared to TIMP-1 (Wood M *et al.,* 1997; Lichtinghagen R *et al.,* 2002; Trudel D *et al.,* 2003). In addition to MMPs, high expression of uPA is also involved in ECM breakdown, as well as, activation of MMPs and broad spectrum protease such as plasmin (Testa JE *et al.,* 1990; Sheng S 2001; Arya M *et al.,* 2006; Li Y & Cozzi PJ 2007). Elevated expression of uPA in prostate cancer is associated with higher grade tumors and presence of lymph node metastasis (Kumano M *et al.,* 2009). Prostate specific antigen (PSA) has also been suggested to play a role in prostate cancer cell invasion through degradation of fibronectin (Webber MM *et al.,* 1995). To support this finding, *in vitro* neutralization PSA in AR expressing prostate cancer cells results in reduction in cancer cell invasion (Webber MM *et al.,* 1995). Following successful ECM breakdown, prostatic adenocarcinoma intravsates into the circulation and attach to vascular endothelial cells at a secondary metastatic site. Prostatic adenocarcinoma has high affinity for bone marrow endothelial cells which is facilitated by; sialyl-Lews carbohydrate, $\alpha v\beta 3$, $\alpha 5\beta 1$, and $\alpha 3\beta 1$ (Martensson S *et al.,* 1995; Romanov VI *et al.,* 1999; Lehr JE & Pienta KJ *et al.,* 1998; Cooper CR *et al.,* 2000). Colonization within the bone is further mediated by $\alpha v\beta 3$ and $\alpha 2\beta 1$ which promotes tumor growth and attachment to type I collagen, respectively (Nesbitt S *et al.,* 1993).

The chemotactic factors which also attract prostatic adenocarcinoma preferentially to the bone include SDF-1, EGF, IGF, and HGF (Taichman RS *et al.,* 2002; Arya M *et al.,* 2006).

Androgen deprivation therapy (ADT) is the mainstay of treatment for metastatic castrate sensitive prostate cancer (CSPC). Continued androgen ablation with agents such as leuprolide (Eligard) result in cancer regression through increase in the proportion of cancer cells that undergo apoptosis (Dutt SS & Gao AC 2009). However, prolonged androgen deprivation leads to transformation of CSPC into a more aggressive castrate-resistant prostate cancer (CRPC), which continues to proliferate despite castrate levels of testosterone (van der Kwast *et al.,* 1991; Hobisch A *et al.,* 1995). There are several proposed mechanisms to explain the development of CRPC which include; hypersensitivity pathway, outlaw pathway, promiscuous pathway, coactivators and corepressors, bypass pathway, and loss of AR expression (Dutt SS & Gao AC 2009).

In the hypersensitivity pathway, AR becomes responsive to extremely low levels of androgens. This effect is achieved through mutation in AR, over amplification of AR, or increase in expression of type II 5-AR (Feldman BJ & Feldman D 2001; Arnold JT *et al.,* 2002; Pienta KJ, & Bradley D 2006). The outlaw pathway is due to AR activation by other growth factors such as IGF, KGF, IL-6, IL-4, and receptor tyrosine kinases HER-2/neu (Feldman BJ *et al.,* 2001; Dehm SM & Tindall DJ 2003; Lee SO *et al.,* 2003). The promiscuous pathway results in AR being receptive to ligands other than DHT such as non-androgenic steroids (Feldman BJ & Feldman D 2001). Elevated expression of coactivator and corepressor levels enhances sensitivity of AR to various ligands other than androgens. Coactivators such as ARA70, ARA55, SRC-1, P/CAF and TIF2 are overexpressed in CRPC (Feldman BJ *et al.,* 2001; Dehm SM & Tindall DJ 2003; Grossmann ME *et al.,* 2001). In the bypass pathway, the AR utilizes other pathways to stimulate proliferation (Grossmann ME *et al.,* 2001). In addition to aberrant AR signalling, there are also

studies which suggest that loss of AR expression as a contributor to CRPC development (Kinoshita, H *et al.,* 2000).

1.3.4. Prostate cancer cell lines

The most commonly used model to study pathologic characteristics of PCa is cancer cell lines in culture. There are various PCa lines commercially available through American Tissue Culture Collection (ATCC) which originate from metastatic deposits of PCa. As a benign control, RWPE cell line derived from peripheral zone of benign prostatic tissue with intact AR signalling is commonly used (Rhim JS *et al.,* 1994; Bello D *et al.,* 1997). Overall, LNCaP cells represent early stages of PCa based on their androgen sensitive cell growth (Horoszewicz JS *et al.,* 1983; Nunlist EH *et al.,* 2004). In contrast, DU145 and PC3 cell lines represent advanced disease based on their metastatic origin and androgen insensitive characteristics (Kaighn ME *et al.,* 1979). Androgen insensitivity (AI) with functional AR can characterize advanced prostate cancer in some patients, however, AI due to lack of AR expression results in a more aggressive metastatic phenotype (Stone KR *et al.,* 1978; Kinoshita H *et al.,* 2000; Culig Z & Bartsch G 2006; Bonaccorsi L *et al.,* 2008).

LNCaP cell line was isolated from a lymph node of a metastatic prostate adenocarcinoma deposit (Horoszewicz JS *et al.,* 1980). This cell line is AR responsive, expresses PSA, cytokeratin (CK) 8, 18, and has preserved expression prostate specific genes. LNCaPs are responsive to TGF-α, EGF, and IGF-1 (Horoszewicz JS *et al.,* 1980; Connolly JM & Rose DP 1990). DU145 cell line was isolated from a brain deposit of metastatic prostate adenocarcinoma (Stone KR *et al.,* 1978). This is a hormone independent cell line which does not express AR or PSA (Stone KR *et al.,* 1978). DU145 express CK 8, 18, and are responsive to TGF-α, TGF-β, IGF-1, and EGF (Stone KR *et al.,* 1978). PC3 cell line was originally isolated from vertebra of metastatic prostate adenocarcinoma

and also exhibit hormone independent characteristics by lacking expression of AR and PSA (Kaighn ME et al., 1979). PC3 have been shown to express CK 7, 8,18, 19, VEGF-C, TGF-α, IL-8, and MMP-9 (Kaighn ME et al., 1979; Ching KZ *et al.,* 1993; Aalinkeel R *et al.,* 2004; Araki S *et al.,* 2007).

2. Hypothesis and Objectives

2.1. Rationale

Despite successes in early detection of PCa, we continue to lack comprehensive knowledge on molecular mechanisms which contribute to cancer progression and its transformation into more aggressive metastatic phenotype. Metastatic prostate cancer (mPCa) is incurable, and the current therapeutic paradigm aims at controlling the disease burden to prolong overall survival. Despite androgen deprivation therapy (ADT), mPCa eventually develops into highly aggressive castrate-resistant phenotype. Given the limitations in prevention and treatment of mPCa, there is a need for identification of novel molecular pathways to better understand the mechanism that drive PCa metastasis.

As discussed in previous sections, circulating human platelets which are involved in hemostasis and thrombosis play an important role in in tumor growth and metastasis through TCIPA. There are numerous studies which characterize and investigate the mechanism of TCIPA in various of cancer cell lines. Highly invasive cancer cell lines such as fibrosarcoma and small cell lung cancer cell lines demonstrate high platelet aggregation potency (Jurasz P *et al.,* 2001). However, there is a paucity in the literature with regards to role of platelets in PCa metastasis and even less is known about interaction of platelets with PCa during its androgen dependent, as well as, more aggressive castrate resistant states. Delineating the pathway by which PCa induce TCIPA could pave the way for novel therapeutic approach with significant chance for impact.

In this study, the overarching question is whether platelets and their interaction with PCa cells contribute to PCa progression and development of metastasis. Furthermore, we also aim to understand if AR expression and signaling influences interaction of prostate cancer cell lines with platelets.

2.2. Hypotheses

My hypothesis is that PCa adenocarcinoma cell lines will activate and aggregate platelets which will enhance their invasive potential *in vitro*. Given more aggressive nature of hormone insensitive tumors, my hypothesis is that PCa cell lines lacking AR expression will exhibit higher platelet aggregation potency compared to cells with intact AR signaling.

2.3. Objectives of the Study

1) To delineate the TCIPA profile of benign prostate and PCa cell lines that express or lack AR.

2) To determine the effect of pharmacologic inhibition of AR on TCIPA profile in AR expressing PCa cells.

3) To determine the mechanism behind PCa cell induced TCIPA.

4) To determine the role of platelets on invasive potential of AR+ and AR- PCa cell lines.

3. Materials and Methods

3.1. Reagents

Reagents for whole blood and platelet isolation such as trisodium citrate, sodium chloride (NaCl), prostacyclin (PGI_2), and Tyrodes salt solution were obtained from Sigma (St. Louis, MO, USA). Tyrodes buffer is an isotonic solution pH adjusted to 7.4 containing $CaCl_2$, $MgCl_6$, KCl, $NaHCO_3$, NaCl, NaH_2PO_4, and D-glucose. Type I collagen was purchased from Chrono-log (Haverston, PA, USA).

Cell medium such as Dulbecco's Modified Eagle Medium (DMEM) supplemented with high glucose, DMEM: Nutrient Mixture F12 (DMEM-F12), Keratinocyte serum free medium with bovine pituitary extract and human epidermal growth factor, Roswell Park Memorial Institute (RPMI) 1640 medium were all obtained from Thermofisher Scientific (Waltham, MA, USA). The media were supplemented with fetal bovine serum (FBS), and antibiotic (penicillin 0.06mg/mL and streptomycin 0.01mg/mL) which were both supplied by Sigma (St. Louis, MO, USA). For detachment of cellular monolayers from tissue culture flasks, Gibco™ TrypLE Express Enzyme solution was obtained from Thermofisher Scientific (Waltham, MA, USA) and Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma (St. Louis, MO, USA). Components of phosphate buffered saline such as NaCl, KCl, Na_2HPO_4, KH_2PO_4 and dimethyl sulfoxide (DMSO) for cryopreservation were purchased from Sigma (St. Louis, MO, USA). Complete media was defined as media containing 10% FBS and 1% antibiotic, whereas, serum starved medium was defined as medium without any FBS supplementation.

For determination of total protein concentration within samples prior to polyacrylamide gel electrophoresis, bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA), and DC™ Protein assay reagent A, B, and S were purchased from Biorad (Hercules, CA, USA).

All reagents for western blot and gelatin zymography analysis such as, glycerol, sucrose, protease inhibitors, ammonium per sulfate, glycine, sodium dodecyl sulfate (SDS), bromophenol blue, Tris-base, Tris-hydrochloride (HCl), ammonium per sulfate, tetramethylethylenediamine (TEMED), coomassie blue, NaN_3, and Tween 20, were supplied by Sigma (St. Louis, MO, USA). 30% acrylamide/Bis solution was purchased from Biorad (Hercules, CA, USA), and 2-mercaptoethanol, β-mercaptoethanol, as well as, Triton X-100 was purchased from Thermofisher Scientific (Waltham, MA, USA). 0.45 μm polyvinylidene difluoride (PVDF) transfer membrane was purchased from Biorad (Hercules, CA, USA).

For cell viability assays, 0.4% trypan blue viability dye was obtained from Sigma (St. Louis, MO, USA). AlamarBlue Cell Viability Reagent was supplied by Thermofisher Scientific (Waltham, MA, USA). Pharmacological reagents such as ARP-100 was obtained from Santa-Cruz Biotechnology (Santa-Cruz, CA, USA), presurgrel active metabolite (PAM) was obtained from SiChem (Bremen, Germany). Acetosalicyclic acid (ASA), apyrase, integrilin, dabigatran, and bicalutamide, were all obtained from Sigma (St. Louis, MO, USA). Solvents for these reagents such as acetonitrile, ethanol, and methanol were purchased from Sigma (Santa-Cruz, CA, USA), Thermofisher Scientific (Waltham, MA, USA) respectively.

Primary antibodies for western blot analysis were obtained as follows: monoclonal androgen receptor (441) from Santa-Cruz Biotechnology (Santa-Cruz, CA, USA), polyclonal thrombin from abcam (Mississauga, ON, Canada), polyclonal β-tubulin from abcam (Mississauga, ON, Canada), monoclonal β-actin peroxidase from Sigma (St. Louis, MO, USA). Secondary antibodies conjugated to Alexaflour 680-750 and horseradish peroxidase was obtained from Thermofisher scientific (Waltham, MA, USA) and Sigma (St. Louis, MO, USA), respectively. All the antibodies were aliquoted and stored as per manufacturer specifications at -20°C.

For modified gelatin coated boyden chamber experiments, formaldehyde was obtained from Sigma (St. Louis, MO, USA), whereas, the Diff-Quick staining solutions were purchased from Siemens Healthcare Diagnostics Inc (Newark, DE, USA).

3.2. Cell Culture

The cells were cultured and handled as per manufacturer specifications. One benign and three prostate cancer cell lines were obtained from ATCC®. These cells were selected to represent the following; RWPE-1 (benign prostatic cells derived from peripheral zone of the prostate, ATCC® CRL-11609™), DU145 (metastatic prostate cancer cells derived from the brain, ATCC® HTB-81™), PC3 (metastatic prostate cancer cells derived from the bone, ATCC® CRL-1435™), and LNCaP (metastatic prostate cancer cell line derived from supraclavicular lymph node, ATCC® CRL-1740™). RWPE-1 and LNCaP cells express androgen receptor (Bellow D *et al*., 1997, Horoszewicz JS *et al*., 1980, Cunningham D *et al.,* 2015), whereas, DU145 and PC3 cell lines lack the expression of an androgen receptor (Stone KR *et* al., 1978, Kaighn ME *et al.,* 1979, Cunningham D *et al.,* 2015). Figure 6 demonstrates the representative light microscopy images of the prostate cancer cell lines at 10x magnification.

RWPE-1 cells were cultivated in Keratinocyte Serum Free Medium (K-SFM) supplemented with 25mg bovine pituitary extract, 2.5μg human recombinant epidermal growth factor, and 1% antibiotics. DU145 and LNCaP cells were grown in RPMI 1640 supplemented 10% fetal bovine serum (FBS) and 1% antibiotics. PC3 were grow in in DMEM:F12 supplemented with 10% FBS and 1% antibiotic.

HT-1080 epithelial fibrosarcoma cells were purchased from American Tissue Culture Collection (ATCC®, CCL-121™) (Manassas, VA, USA) and cultured in DMEM supplemented with high glucose, 10% FBS, and 1% antibiotic.

All cell culture procedures were performed under sterile conditions. The cell lines were cultured as monolayer in humidified cell incubator supplied with 5% CO_2 at 37°C.

Fresh medium was exchanged every second day and cells were passaged upon reaching 80-90% confluency. For subculturing, the cellular monolayer was rinsed with room temperature phosphate-buffered saline (PBS) then incubated with Gibco™ TripLE Express solution for 5-8 minutes. The cells were then inspected under Olympic CKX41 light microscope (Olympus America Inc. Melville, NY), and once fully detached, twice the volume of complete medium was added. The detached cells were transferred to a Falcon™ Conical Centrifuge Tube and centrifuged at 130 relative centrifugal force (RCF) for 7 minutes. The supernatant was discarded the cell pellet was re-suspended in appropriate volume of complete medium and distributed into tissue culture flasks supplied by Thermofisher Scientific (Waltham, MA, USA). Cellular passages between 5-20 were used for all experiments.

For long term cryopreservation, the cells were suspended in respective cell culture medium supplemented 5% DMSO and stored in liquid nitrogen.

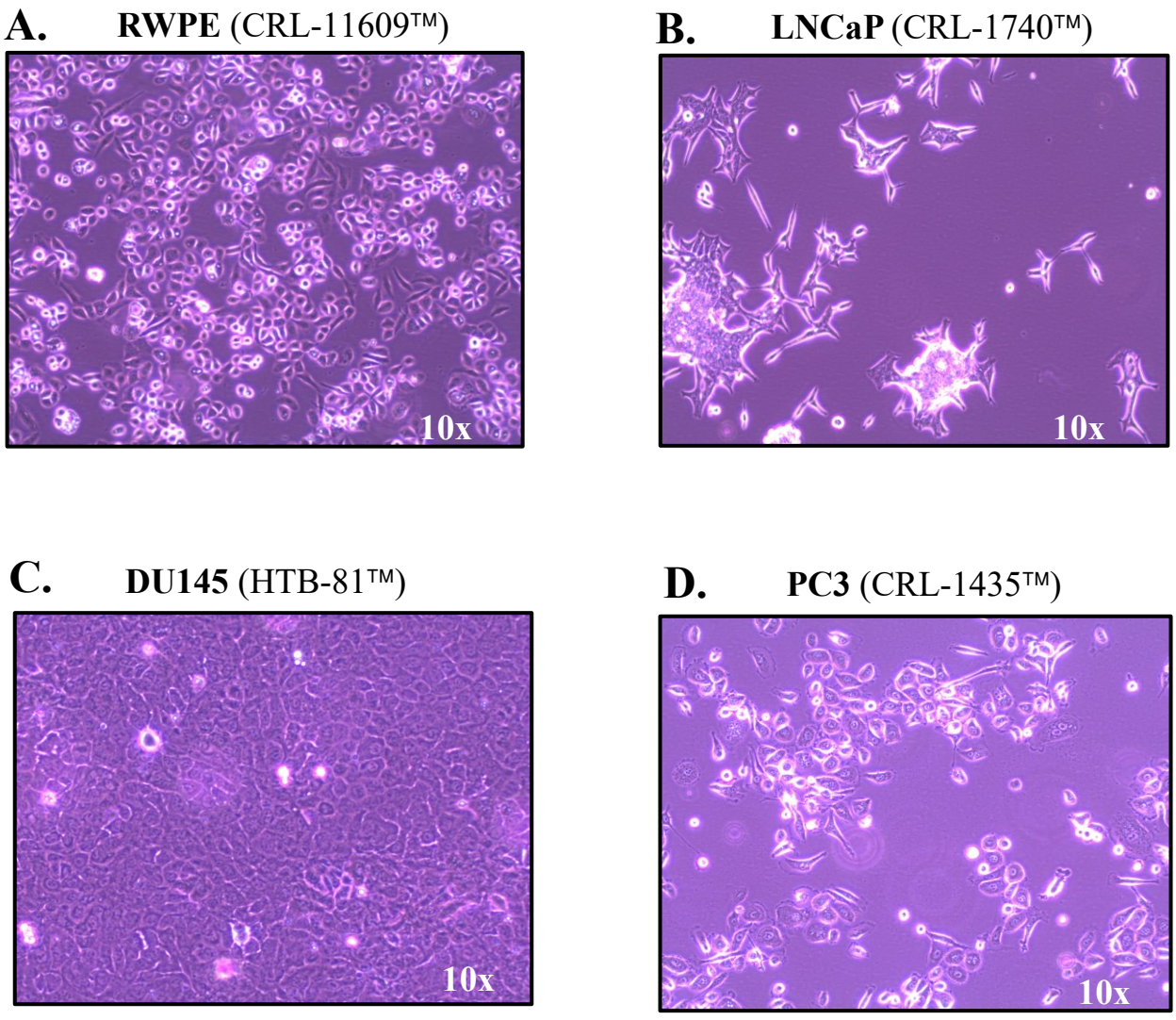


Figure 4: Representative light micrograph images of prostate cancer cell lines purchased from ATCC®. (A) benign prostate cells, **(B)** metastatic prostate cancer cells derived from a supraclavicular lymph node **(C)** metastatic prostate cancer cells derived from the brain, **(D)** metastatic prostate cancer cells derived from the bone. 10x magnification.

3.3. Treatment of LNCaP cell line with bicalutamide *in vitro*

Bicalutamide (Casodex) is a nonsteroidal antiandrogen, which is a competitive inhibitor for the binding of dihydrotestosterone or testosterone to AR (Lexicomp 1987-2018). The *in vivo* half-life of bicalutamide is approximately 6 days and it has an *in vitro* IC_{50} of 7μM (Lexicomp 1987-2018, Pignatta S *et al*., 2014). Bicalutamide is supplied as ≥98% (HPLC) power solubilized in sterile filtered DMSO (solubility >5mg/mL) and stored at 4°C as per manufacturer specifications. To determine the role of androgen receptor signalling in LNCaP induced TCIPA, the cells were exposed to 20μM of bicalutamide according to previously published protocol (Pignatta S *et al*., 2014).

The LNCaP cell monolayer was grown in $25cm^2$ tissue culture flask until it reached 80% confluency. The cells were incubated with 2mL of RPMI 1640 medium supplemented with 20μM of bicalutamide, 10% FBS, and 1% antibiotic for 24-hours in humidified cell incubator supplied with 5% CO_2 at 37°C. Alternatively, for control cells, equal volume of sterile filtered DMSO was added to RPMI 1640 with 10% FBS and 1% antibiotic. Following this incubation, the bicalutamide and DMSO treated cells were either utilized for TCIPA experiments, collected for Alamar blue cell viability assay, or lifted, centrifuged, and lysed for western blot analysis. Post centrifugation, cell pellets were stored at -80°C.

3.4. Isolation of human platelets

Prostacyclin (PGI_2) was dissolved in 1M Tris (containing Tris base and Tris HCl dissolved in double distilled H_2O) adjusted to pH of 9.0. For long term storage, the stock solution of PGI_2 was aliquoted into 15µl and stored at -80°C. During whole blood isolation, PGI_2 was kept on ice and discarded after 30 minutes.

The current platelet isolation protocol was approved by the Human Research Ethics Board at the University of Alberta. Following informed consent, whole blood was obtained from healthy individuals who have not taken any anti-platelet agents for minimum 14 days. PGI_2 washed platelets were prepared according to previously established protocol (Radomnski MW *et al*., 1983). In brief, 40mL of whole blood was drawn by venous puncture and mixed with 4 mL of anticoagulant trisodium citrate (9:1,v:v). 0.06µg/mL of PGI_2 was then added to prevent platelet activation during centrifugation. Whole blood was then centrifuged at 250 RCFs for 20 minutes in Eppendorf 5810R Centrifuge to separate hematocrit from platelet rich plasma (PRP). The resultant PRP was then isolated and mixed with 0.3µg/mL PGI_2 and further centrifuged at 900 RCF for 10 minutes. The resultant platelet pellet was then resuspended in 5 mL of Tyrodes buffer at room temperature, counted using a hemocytometer, and diluted to a final physiologic concentration of $2.5x10^8$ platelets/mL in Tyrodes buffer. Functional platelet aggregation experiments were commenced 1hr after platelet resuspension in appropriate volume of Tyrodes buffer to allow for the effect of PGI_2 to wear off.

3.5. Tumor cell induced platelet aggregation (TCIPA)

To study the effect of tumor cells on platelet activation and aggregation, Chrono-log Dual Channel Lumi-Aggregator (Model 560, Chrono-Log, Harveston, PA, USA) was utilized. In this assay, platelets suspended in Tyrodes buffer are aliquoted into a transparent cuvette containing a magnetic stir bar. As homogenously dispersed platelets aggregate in response to stimulation with various agonists, they form aggregates which allow for increased light transmittance through the cuvette. The extent of light transmittance through the sample is directly correlated with % total platelet aggregation over the specified time.

Throughout the assay platelets were incubated in the Chrono-log Dual Channel Lumi-Aggregator at 37°C with the magnetic stir bar spinning at 1200 revolution per minute (rpm). Platelet aggregation was initiated with addition of type I collagen (0.3-10μg/mL) or either benign prostate cells or prostate cancer cell lines (0.001-2.0x10^6 cells/mL). The % platelet aggregation was monitored by Aggro-Link software. Following addition of platelet activating agonists at 2 minutes, two parameters were collected; time to initiation of platelet aggregation, defined as the first time point at which platelets begin to aggregate and % total aggregation (Figure 7). For collagen activated platelets, the reaction was monitored for 4 minutes, whereas, for tumor cell activated platelets the reaction was monitored for 30 minutes.

For experiments examining the effect the of tumor cells on platelet aggregation, benign prostate and prostate cancer cell lines were grown in a monolayer and detached with complete medium containing 7mM EDTA to minimize damage to surface proteins. Once lifted, the cells were centrifuged at 130 RCF for 7 minutes and re-suspended in sterile PBS. This cycle was repeated three times to ensure removal of excess ETDA. The final stock solution containing the tumor cells was suspended in 1mL PBS and counted using the hemocytometer. Once the cellular

stock concentration was determined, the cells were stored on ice and gently vortexed every 30 minutes throughout the duration of the assay. While on ice, the cellular viability was measured using 0.4% trypan blue every one hour to ensure the cells maintained adequate viability throughout the assay. The protocol for investigating prostate cancer TCIPA profile was derived from previously published experiments by Jurasz P *et al*., 2001, Alonso-Escolano D *et al.,* 2004, and Medina C *et al.,* 2006.

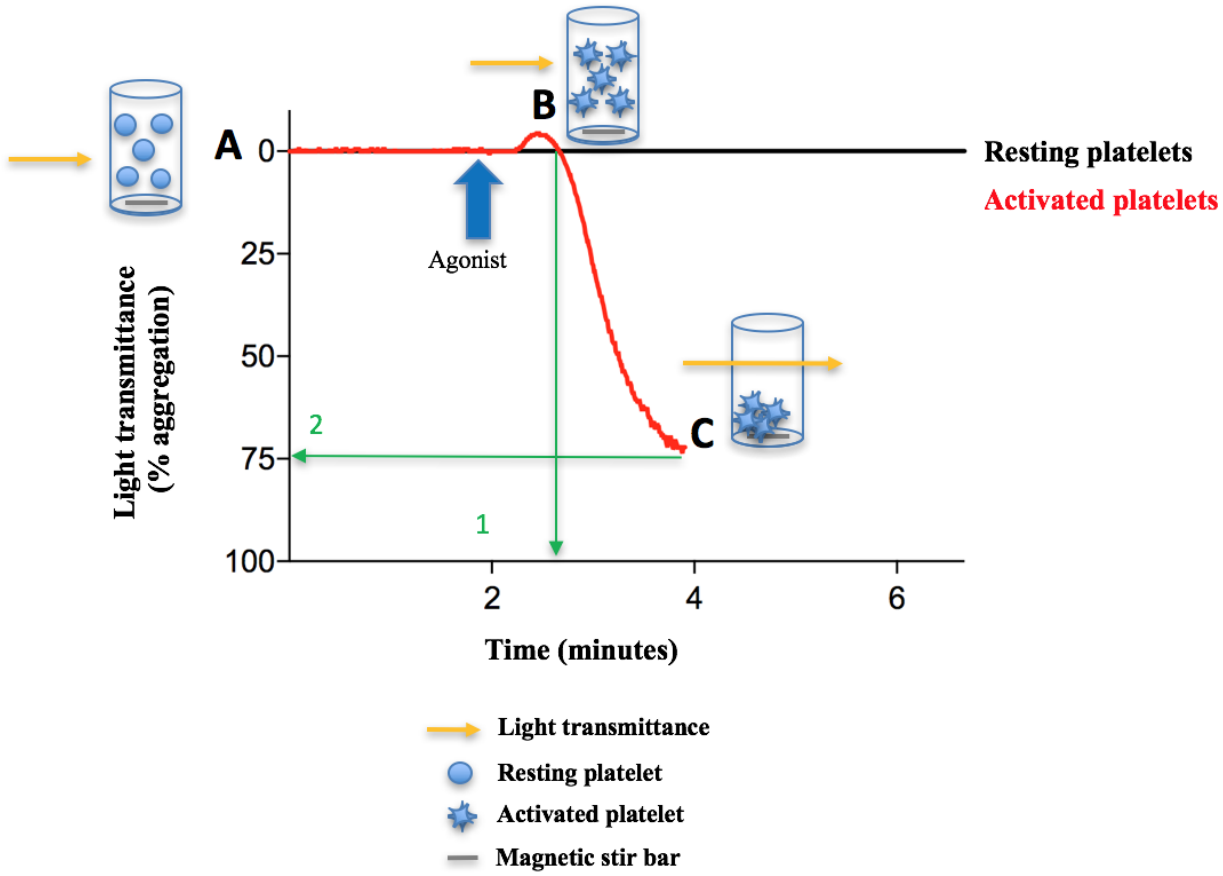


Figure 5: Representative in vitro platelet aggregometry trace obtained from Chrono-log Dual Channel Lumi-Aggregator (Model 560, Chrono-Log, Harveston, PA, USA). (A) Resting platelets are suspended in Tyrodes buffer inside a transparent cuvette containing a magnetic stir bar. In response to stimulation with an agonist such as type I collagen or tumor cells, the homogenously dispersed platelets undergo shape change **(B)** and aggregate **(C)**. As the platelets aggregate, there is increased light transmittance through the transparent cuvette (orange arrow). The extent of light transmittance through the sample is directly correlated with % total platelet aggregation over the specified time. The two parameters collected in this assay (as depicted by the green arrows) include, time to initiation of platelet activation **(1)** – defined as the time at which initiation of platelet aggregation was observed, as well as, **(2)** % total aggregation.

3.6. Platelet releasate collection following TCIPA

Following platelet aggregation with type I collagen or prostate cancer cells, the tumor-platelet pellet and platelet releasates were separated by centrifugation, as demonstrated by Figure 8 A-B. To minimize further platelet activation and degranulation during centrifugation, 1µl of PGI_2 was added, and the sample was centrifuged at 900 RCF for 10 minutes to separate the sample into tumor cell-platelet aggregate rich pellet and supernatant containing the platelet releasates. The platelet releasates were stored at -80°C until they were assayed for the presence of pro-MMP-2/MMP-2 and pro-MMP-9/MMP-9 by gelatin zymography (Jurasz P *et al*., 2001).

To determine the passive release of pro-MMP-2/MMP-2 and pro-MMP-9/MMP-9 from non-activated platelets, the platelets were incubated at 37°C and 1200 rpm in the Chrono-log Dual Channel Lumi-Aggregator for 30 minutes without exposure to collagen or tumor cells. Following addition of 1µl of PGI_2 the sample was centrifuged at 900 RCF for 10 minutes and the resultant platelet releasate was stored at -80°C. Alternatively, to determine the passive release of pro-MMP2/MMP-2 and pro-MMP-9/MMP-9 from prostate cancer cells during these experimental conditions, the cells were suspended in Tyrodes buffer without platelets at 37°C and 1200 rpm in the Chrono-log Dual Channel Lumi-Aggregator for 30 minutes without exposure to tumor cells. The sample was centrifuged at 900 RCF for 10 minutes and the resultant tumor cell pellet and supernatant was stored at -80°C until they were assayed for the presence of pro-MMP-2/MMP-2 and pro-MMP-9/MMP-9 by gelatin zymography.

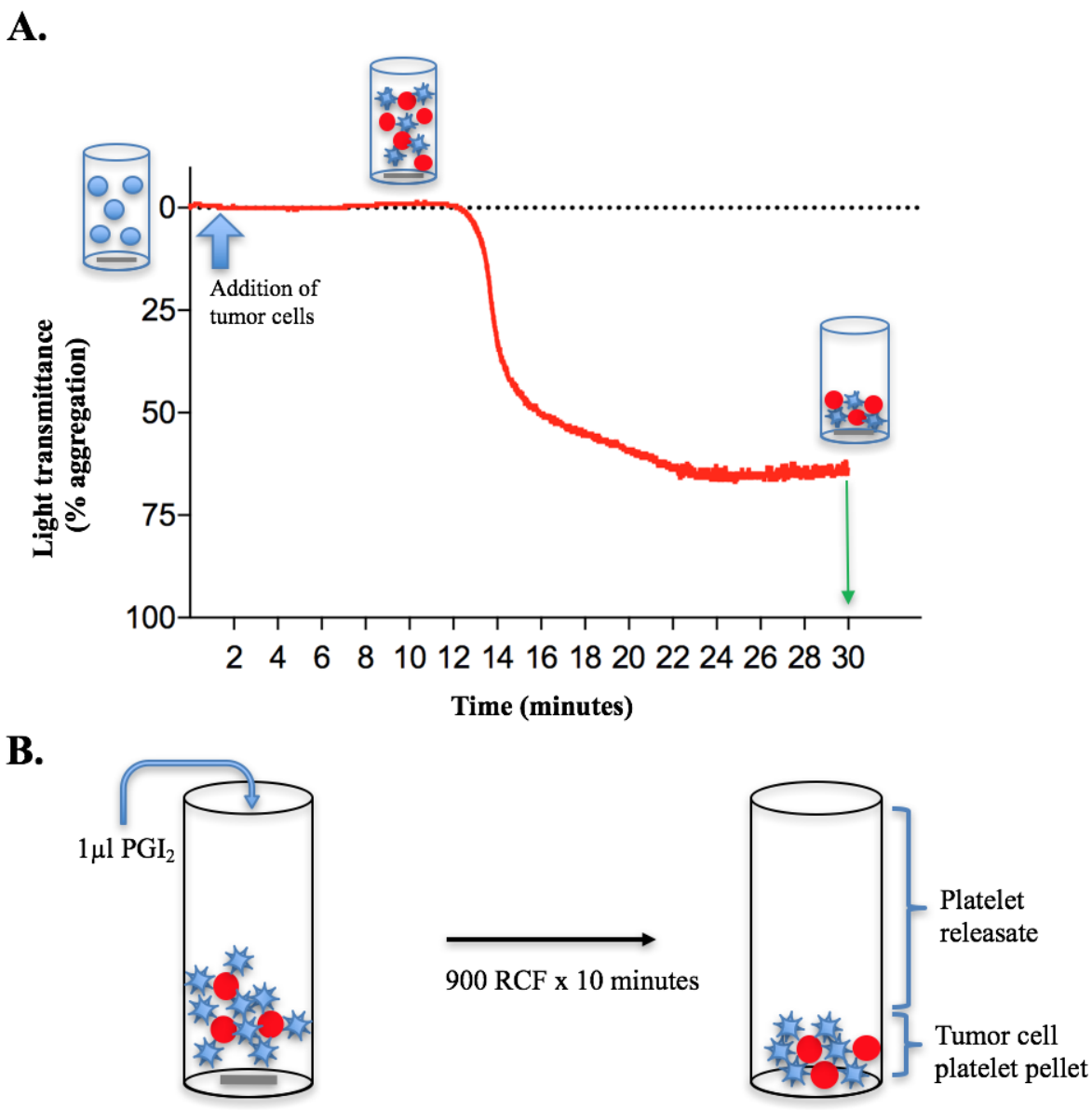


Figure 6: Collection of platelet releasates following tumor cell induced platelet aggregation. Following completion of platelet aggregation with prostate cancer cell lines at 30 minutes **(A)**, platelet-tumor cell pellet and releasates were separated by centrifugation **(B)** – in order to minimize further platelet activation and degranulation during centrifugation, 1µl of PGI_2 was added, and the sample was centrifuged at 900 RCF for 10 minutes to separate the sample into tumor cell-platelet aggregate rich pellet and supernatant containing the platelet releasates.

3.7. Cellular viability assays

3.7.1. Trypan blue

To ensure that the effect of tumor cells on platelet aggregation is not due to loss of cellular viability secondary to experimental conditions, trypan blue exclusion test was performed. Trypan blue is an azo dye not absorbed by viable cells. First, cells were grown in three separate tissue culture flasks. The cellular monolayer was detached with 7mM EDTA and centrifuged at 130 RCF for 7 minutes. The resultant cell pellet was re-suspended in sterile PBS. This cycle was repeated three times to ensure removal of excess EDTA. Three separate cell pellets were re-suspended in 1mL of Tyrodes buffer, PBS, and complete media separately. With the use of sterile pipette, cells were stained with 0.4% trypan blue and counted using the hemocytometer (Figure 9B). In brief, blue stained non-viable cells and unstained cells were counted. The total % viable cell each of the three media was calculated by determining the ratio of unstained viable cells to total number of non-viable and viable cells.

To ensure maintenance of cell viability throughout tumor cell induced platelet aggregation (TCIPA) experiments, tumor cells suspended in three separate media were incubated in transparent cuvettes and incubated at 1200rpm and 37°C in the Chrono-log Dual Channel Lumi-Aggregator. Following 30-minute incubation, the cells were collected and stained with 0.4% trypan blue to measure % total cellular viability. The viability prior to incubation and 30-minutes post-incubation was compared to examine the effect of experimental conditions on cellular viability.

3.7.2. Alamar blue

To measure LNCaP cell viability following treatment with bicalutamide, Alamar blue cell viability assay was performed. In this assay, viable cells they maintain a reducing environment within the cytosol. Within viable cells, resazurin which is a non-toxic cell permeable reagent enters the cells and become reduced to fluorescent resorufin. In brief, 5,000 cells treated with either bicalutamide or vehicle controls were incubated in 100µl of complete media in a 96-well plate at 5% CO_2 at 37°C. This experiment was done in triplicates. Following 24-hour incubation, 10µl of resazurin was added to the wells for 4-hours and fluorescence intensity was measured. Bicalutamide treated cell viability was measured relative to viability of untreated vehicle control cells.

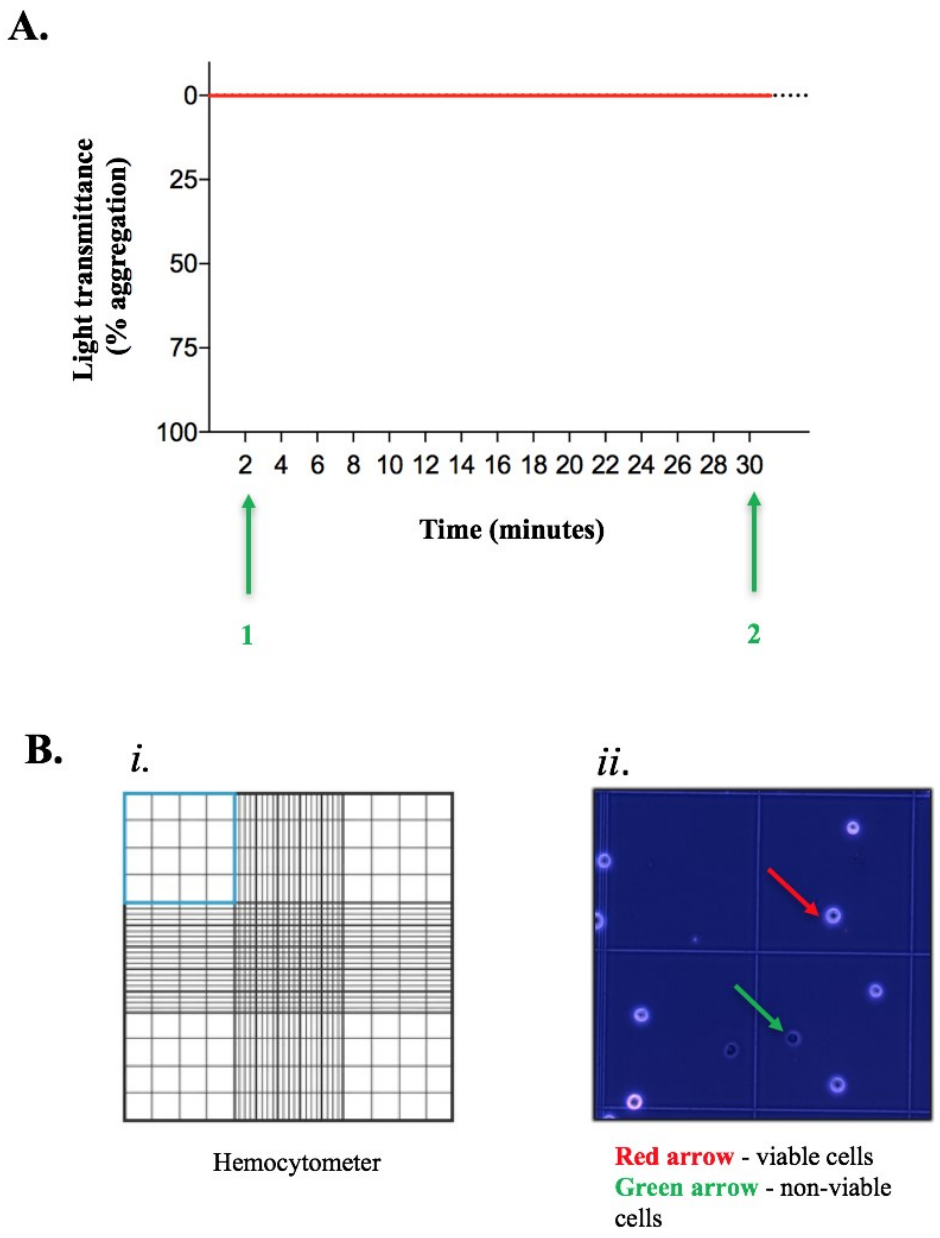


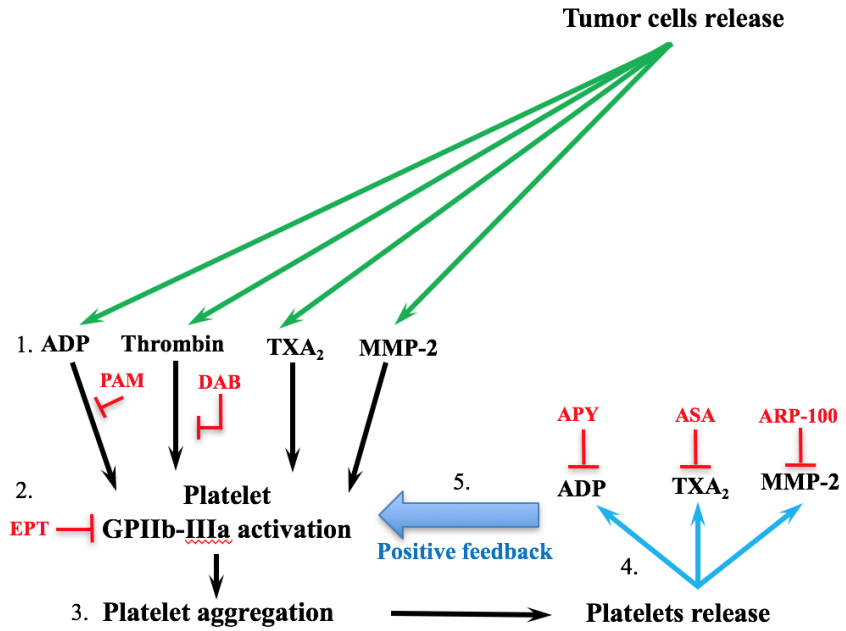
Figure 7: Evaluation of platelet aggregometry experimental conditions on prostate cancer cell viability. To ensure maintenance of cell viability throughout tumor cell induced platelet aggregation (TCIPA) experiments, tumor cells suspended were incubated in transparent cuvettes and incubated at 1200rpm and 37°C in the Chrono-log Dual Channel Lumi-Aggregator for 30 minutes **(A)**. Following incubation, the cells were collected and stained with 0.4% trypan blue to measure % total cellular viability **(B).** The viability prior to incubation **(1)** and 30 minutes post-incubation **(2)** was compared to examine the effect of experimental conditions on cellular viability.

3.8. Mechanism of prostate cancer cell line induced platelet aggregation (TCIPA)

To determine the relative contribution of platelet and tumor cell aggregating pathway to TCIPA induced by prostate cancer cells, five inhibitors of platelet and tumor cell aggregating pathways were utilized. Figure 10A demonstrates a representative schematic of major modulators of TCIPA and the site of action for the inhibitors used, whereas, Figure 10B references the publications from which the *in vitro* dosages of inhibitors were derived. To inhibit adenosine diphoshapate, matrix metalloproteinase-2 (MMP-2), thromboxane (TXA_2), glycoprotein IIb/IIIa, and thrombin-mediated pathways of aggregation, apyrase (11μM), prasurgrel active metabolite (PAM) (30μM), ASA (200μM), ARP-100 (5μM), eptifibatide (50μM), and dabigatran (0.8μM) were used respectively. In brief, 1mL of $2.5x10^8$ platelets/mL platelets were suspended in Tyrodes buffer and incubated with the inhibitors for 2 minutes in the Chrono-log Dual Channel Lumi-Aggregator at 37°C and 1200 rpm. Following 2 minutes, 10μg/mL type I collagen or 250,000 prostate cancer cells were added to initiate the TCIPA and time to initiation of platelet aggregation and % total platelet aggregation in the presence of inhibitors was measured after 30 minutes using the Aggro-Link software.

Since nitric oxide is a potent inhibitor of platelet aggregation and previously shown to be generated by LNCaP cells, we tested the effect of N-Nitro-L-arginine methyl ester hydrochloride (L-NAME) a competitive NOS inhibitor on LNCaP TCIPA (Tong X & Li H 2004). 100μM-500μM of L-NAME was added to LNCaP cells suspended in 1mL of sterile PBS for 20 minutes on ice. Subsequently, 2 million cells were added onto platelets to initiate TCIPA to measure platelet activation and % aggregation.

A.



B.

Inhibitor (dose)	Publication
ASA (200μM)	Sawicki G *et al., 1997* Jurasz P *et al.*, 2001
APY (11μM)	Sawicki G *et al., 1997* Jurasz P *et al.*, 2001
PAM (30μM)	Frelinger III *et al.*, 2011 Frelinger III *et al.*, 2014
ARP-100 (5μM)	Webb *et al.*, 2017
EPT (50μM)	Nobuyuki K *et al.*, 2004
DAB (0.8μM)	Taune V *et al.*, 2018
L-NAME (100-500μM)	Radziwon-Balicka A *et al.*, 2017

Figure 8: Representative schematic of tumor cell induced platelet aggregation and its major modulators and site of action of pharmacological inhibitors. (A) Tumor cells release pro-aggregatory ADP, thrombin, thromboxane, and MMP-2 (1) which leads to activation of platelet glycoprotein IIb/IIIa receptor (2) and subsequent platelet aggregation (3). Platelet activation and aggregation results in the release of platelet derived ADP, thromobaxne, and MMP-2 (4) which exerts positive feedback on platelets to further activate platelet glycoprotein IIb/IIIa (5). APY - apyrase, PAM - presurgrel active metabolite, ASA – acetylsalicylic acid, EPT – eptifibatide, DAB – dabigatran, L-NAME - N-Nitro-L-arginine methyl ester hydrochloride. **(B)** Summary table of publications from which the experimental doses of inhibitors were derived.

3.9. Gelatin zymography on platelet and tumor cell releasates following TCIPA

To generate pro-MMP-2/MMP-2 and 9 standards for gelatin zymography, HT-1080 cells lines were grown in monolayer under serum starved conditions in DMEM with 1% antibiotic (conditioned medium) for 24 hours. The conditioned medium from these cells was isolated, aliquoted in 1mL, and stored at -20°C until gelatin zymography.

All sample preparation was performed on ice. 18µl of collagen or tumor cell activated platelet releasates were added to 6µl of 4X zymography loading buffer (glycerol, SDS, 4x Tris Cl/SDS pH 6.8, double distilled (dd) H_2O, and bromophenol blue). The entire 25 µl sample was then loaded on 20 mg/mL gelatin incorporated 8% SDS-polyacrylmide gel. As molecular weight reference, 15µl of Precision Plus Protein Ladder (Biorad, Hercules, CA, USA) was loaded adjacent to the samples. 9µl of conditioned medium from HT-1080 was diluted in ddH_2O and 4X zymography loading buffer to final volume of 25 µl and used as a pro-MMP-2/MMP-2 and pro-MMP-9/MMP-9 standard. Polyacrylmide gel electrophoresis was performed using Bio-Rad PowerPac HC (Hercules, CA, USA) 150V at 4°C on ice. The gel was then washed three times for 20 minutes with 2% Triton-X-100 (Thermofisher Scientific, Rockford, IL, USA) followed by two 20 minute washes in zymography buffers (2M Tris HCL, NaCl, $CaCl_2 \cdot H_20$, NaN_3). The gel was incubated in fresh zymography buffer at 37°C in the water bath overnight to allow for enzyme activity to degrade gelatin substrate. To visualize bands of gelatinolytic activity, the gel was stained with Coomasie blue for 2 hours then de-stained with 4% methanol and 8% acetic acid destaining solution.

The gels were them imaged using VersaDoc Imaging System (Bio-rad, Hercules, CA, USA) and QuantityOne software was used to analyze the densitometry of bands of gelatinolytic activity.

3.10. Western blot analysis on cellular lysates

3.10.1. Protein assay

The standard curve was prepared using the bovine serum albumin (BSA) standard. The stock solution of BSA (1.5 mg/mL) was serially diluted in ddH_2O to create a standard curve. In brief, 3mL of working reagents A' was prepared by adding 60µl of reagent S into 3mL of reagent A. The assay was set up in 96 well plate in duplicates and 5ul of test sample or 5ul of standard curve sample, 25ul of reagent A', and 200ul of reagent B. The 96 well plate was left in the dark for 15 minutes and then absorbance was read. The protein concentration in each sample was calculated based on BSA standard curve values.

3.10.2. Androgen receptor

Cell pellets were thawed and resuspended in homogenizing buffer (50mM Tris HCL/base, 3.1 mM sucrose, 10% protease inhibitors, and 0.1% SDS). Homogenized lysates were sonicated three times for five seconds and centrifuged 10,000g for 2 minutes. The resultant supernatant was then assayed for protein content as described above and 100ng of protein was aliquoted.

The samples were denatured by 5µl of loading buffer (glycerol, SDS, 4x Tris Cl/SDS pH 6.8, double distilled (dd) H_2O, bromophenol blue, and β-mercaptoethanol) to a final volume of 25µl and by heating up to 100°C for 5 minutes. The sample was loaded into a lane on 8% SDS-polyacrylmide gel. As a molecular weight reference, 15µl of Precision Plus Protein Ladder was loaded adjacent to the samples. Gel electrophoresis was performed using Bio-Rad Powerpac HC system set at 90V for 30 minutes and 120V for 90 minutes.

After completion of the run, the protein was transferred onto 0.45µm PVDF membrane using wet transfer method using Mini-Trans Blot (Biorad, Hercules, CA, USA) and wet transfer buffer (25mM Tris base, 192mM glycine, 0.1%SDS, 20% (v/v) methanol, ddH_2O) at 80V at 4°C

for 2 hours. The membrane was then blocked with Rockland solution diluted in PBS (1:1) for 1 hour. The PVDF membrane was cut at 60kDa molecular weight mark.

The PVDF membrane >60kDa then incubated with androgen monoclonal antibody at 1:500 dilution, whereas, the membrane <60kDa was incubated with polyclonal β-tubulin at 1:10,000 dilution. After incubation with primary antibodies overnight, each membrane was washed three times with PBS contain 0.1% of Tween 20 (0.1% PBST). The membrane was the incubated with AlexaFluor secondary antibody for 1 hour at room temperature protected from light and washed three times with 0.1% PBST. The membrane was scanned for androgen receptor or β-tubulin separately using LI-COR Odyssey Imaging System. To quantify relative protein expression, the densitometry value for androgen receptor was normalized to the level of β-tubulin in each sample. The protein band was quantified using densitometry function in Odyssey Version 3.0.29 (LI-COR).

3.10.3. Prothrombin and thrombin

The benign prostate and prostate cancer cell line pellets were thawed and resuspended in homogenizing buffer (50mM Tris HCL/base, 3.1 mM sucrose, 10% protease inhibitors, and 0.1% SDS). Homogenized lysates were sonicated for five seconds and centrifuged 10,000g for 2 minutes. The resultant supernatant was then assayed for protein content as described above. 1µl platelet poor plasma diluted in ddH_2O was used as positive control and 100ng of protein was aliquoted.

Samples was further denatured by 5µl of loading buffer (glycerol, SDS, 4x Tris Cl/SDS pH 6.8, double distilled (dd) H_2O, bromophenol blue, and β-mercaptoethanol) to a final volume of 25µl and by heating up to 100°C for 5 minutes. The sample was then loaded into a lane on 8% SDS-polyacrylmide gel. As a molecular weight reference, 15µl of Precision Plus Protein Ladder

was loaded adjacent to the samples. Gel electrophoresis was performed using Bio-Rad Powerpac HC system set at 90V for 30 minutes and 120V for 90 minutes. After completion of the run, the protein was transferred onto 0.45μm PVDF using semi-dry method (Trans-Blot, SD) at 25V for 30 minutes. After the transfer, the PVDF membrane was blocked with 5% BSA in Tween tris buffered saline (TTBS) for one hour at room temperature followed by overnight incubation at 4°C with thrombin polyclonal antibody at 1:1000 dilution. Following incubation with antibodies the membrane was washed four times with TTBS for 5 minutes, the probed for one hour at room temperature with anti-mouse IgG conjugated to horse radish peroxidase at 1:2000 dilution. The membrane was developed using ECL kit. Chemiluminescence was detected using enhanced chemiluminescence (ECL) prime wester blotting detection reagent (GE Healthcare, Chicago, IL, USA), and imaged using VersaDoc Imaging System. Band densitometry for prothrombin (72KDa) and active thrombin (36KDa) was analyzed using Quantity One software.

The PVDF membrane was then incubated in stripping buffer (100nM β-mercaptoethanol, 2% SDS, 62.5mM Tris HCL pH 6.7) for 20 minutes. The membrane was then washed with TTBS three times for 10 minutes. Then the membrane was blocked with 5% BSA in TTBS for one hour followed by one hour incubation with monoclonal β-actin antibody conjugated to horse radish peroxidase (1:40,000 dilution). The membrane was developed using ECL kit. Chemiluminescence was detected using enhanced chemiluminescence (ECL) prime wester blotting detection reagent (GE Healthcare, Chicago, IL, USA), and imaged using VersaDoc Imaging System. Band densitometry was analyzed using Quantity One software. To quantify relative expression of prothrombin and thrombin, the densitometry value for protein of interest was normalized to the level of β-actin in each sample.

3.11. Gelatin zymography on prostate cancer cell lysates following exposure to platelets

Prostate cancer cell lines were grown in a monolayer in 25cm^2 flask. The experimental sample was incubated with serum starved conditioned medium containing 3mL of platelets and 1% antibiotic, whereas, the control samples were incubated with equal volume of Tyrodes buffer in the conditioned media and 1% antibiotic. Following, 24-hour incubation with or without washed platelets, the cells were lifted with Gibco™ TripLE Express, and 2 million prostate cancer cells were seeded in a 6 well plate with 1mL of serum starved medium for 24 hrs. The cells were then lifted again with Gibco™ TripLE, collected, and resuspended in homogenizing buffer (50mM Tris HCL/base, 3.1 mM sucrose, 10% protease inhibitors, and 0.1% SDS). Homogenized lysates were sonicated for five seconds and centrifuged 10,000g for 2 minutes. The resultant supernatant was then suspended in 500µl of PBS and filtered with 50K Amicon-Ultra centrifugal units (Thermofisher Scientific, Rockford, IL, USA) to concentrate the samples. In brief, 500µl of cell lysates suspended in PBS were added to the Amicon Ultra filter device and centrifuged at 14,000 RCF for 10 minutes. To recover the concentrated solute at >50KDa, the Amicon Ultra filter device was placed upside down in a clean micro-centrifuge tube and spun at 1,000 RCF for 2 minutes. The resultant 20µl concentrate was collected for gelatin zymography.

All sample preparation was performed on ice. 18µl of concentrated tumor cell lysates was added to 6µl of 4X zymography loading buffer (glycerol, SDS, 4x Tris Cl/SDS pH 6.8, ddH_2O, and bromophenol blue). The 25 µl sample was then loaded on 20mg/ml gelatin incorporated 8% SDS-polyacrylmide gel. As molecular weight reference, 15µl of Precision Plus Protein Ladder was loaded adjacent to the samples. 9µl of conditioned medium HT-1080 was diluted ddH_2O and 4X zymography loading buffer to final volume of 25 µl and used as a pro-MMP-2/MMP-2 and pro-

MMP-9/MMP-9 standard. The polyacrylamide gel electrophoresis was performed using Bio-Rad PowerPac HC 150V at 4°C on ice. The rest of the gelatin zymography was performed as described in section 2.9.

According to previously established protocol by Govindasamy *et al*., 2016, after completion of electrophoresis, the gel was cut at 50kDa mark using the molecular weight marker as a reference. The gel section <50kDa was transferred onto 0.45µm thick PVDF membrane using semi-dry transfer method (Trans-Blot SD) at 25V for 30 minutes. After the transfer, the PVDF membrane was blocked overnight with 5% BSA in TTBS and probed with monoclonal β-actin antibody conjugated with horseradish peroxidase (at 1:40,000 dilution) for 30 minutes followed by four wash cycles in TTBS. Chemiluminescence was detected using enhanced chemiluminescence (ECL) prime wester blotting detection reagent (GE Healthcare, Chicago, IL, USA), and imaged using VersaDoc Imaging System. Band densitometry was analyzed using Quantity One software. The intensity of the pro-MMP-2/MMP-2 and pro-MMP-9/MMP9 bands was normalized to the β-actin during densitometry interpretation.

3.12. Gelatin coated Boyden chamber assay

3.12.1. Preparation of cell culture inserts

Transparent 8µm porous 24-well inserts (Falcon Corning, Tewksbury, MA, USA) were coated with 100µl of 1mg/mL gelatin and incubated at 37°C for 2 hours to allow solidification of gelatin. Excess gelatin was aspirated and inserts were left overnight in tissue culture hood exposed to ultraviolet light for 12 hours and rinsed once with 100µl of sterile PBS.

3.12.2. Prostate cancer cell invasion assay following exposure to platelets

Prostate cancer cells were incubated with platelets suspended in serum starved medium for 24 hours. Following this incubation, cells were rinsed with 3mL of sterile PBS three times to ensure adherent platelets were completely washed away. The cells were then lifted with Gibco™ TripLE, centrifuged at 130 RCF for 7 minutes. The resultant pellet was suspended in 1mL of serum starved medium and counted using a hemocytometer. 20,000 cells were seeded into the upper chamber in serum starved conditioned medium with 1% antibiotic. The bottom chamber was incubated with 500µl of complete medium containing 10%FBS to serve as a chemo attractant (Figure 11). The system was incubated at 37°C 5% CO_2 for 24 hours. After incubation, the top layer was scraped gently with Q-tip to remove the non-migrated cells. Migrated cells at the bottom of the insert were fixed with 4% formaldehyde in PBS for 10 minutes followed by staining with Diffquick stain (Siemens Healthcare Diagnostics Inc. Newark, DE, USA). Stained cells from five fields of view of 24-well inserts were imaged at 10x magnification using Olympic CKX41 microscope (Olympus America Inc. Melville, NY) equipped with Infinity 1 digital camera. Cells were quantified using image J software, and expressed as total migrated cells from five-fields of view and normalized to control cells.

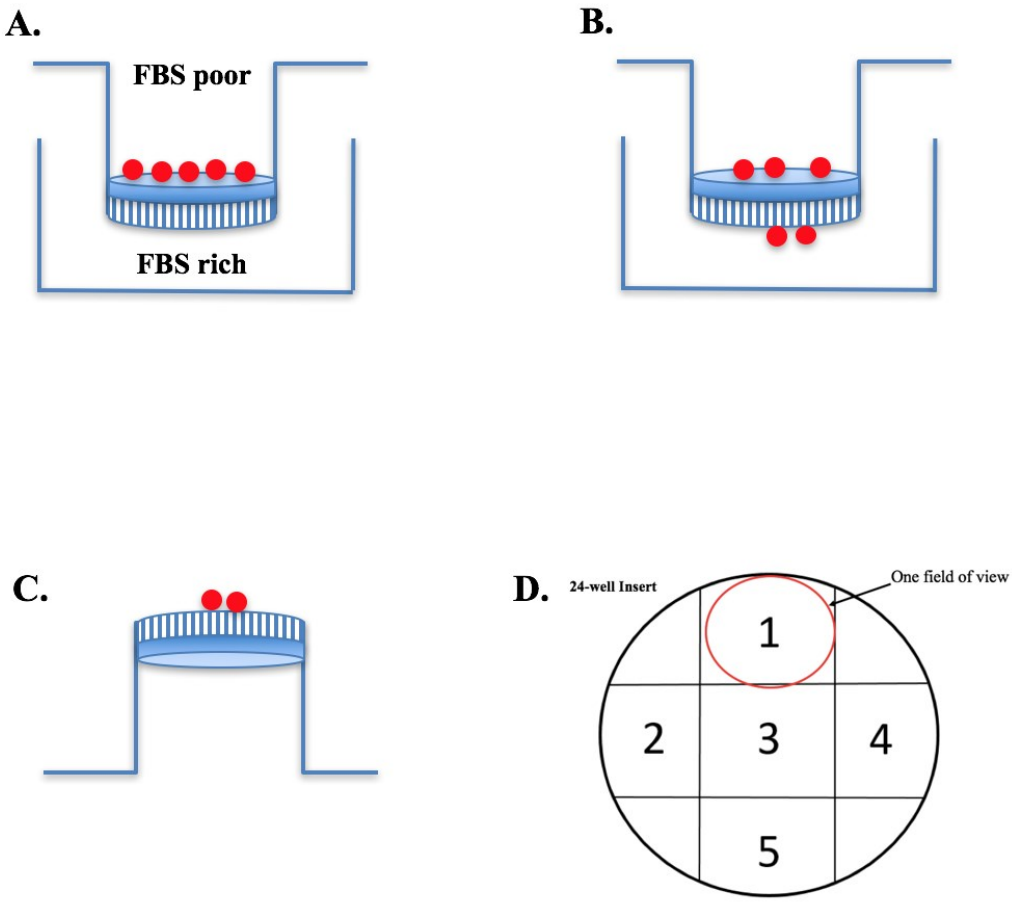


Figure 9: Gelatin coated Boyden chamber. Prostate cancer cells were incubated in FBS poor medium in the upper chamber. The bottom chamber was incubated with 500ul of complete medium containing FBS to serve as a chemo attractant. The system was incubated at 37°C 5% CO_2 for 24 hours. After incubation, the top layer was scraped gently with Q-tip to remove the non-migrated cells. Migrated cells at the bottom of the insert were fixed with 4% formaldehyde in PBS for 10 minutes followed by staining with Diffquick stain (Siemens Healthcare Diagnostics Inc. Newark, DE, USA). Stained cells from five fields of view of 24-well inserts were imaged at 10x magnification using Olympic CKX41 microscope (Olympus America Inc. Melville, NY) equipped with Infinity 1 digital camera. Cells were quantified using image J software, and expressed as total migrated cells from five-fields of view normalized to control. FBS – fetal bovine serum.

3.13. Statistics

Statistical analysis was performed using Graphpad Prism 7.0. Results were expressed as mean $\pm$ standard error of the mean. Comparison between two groups were performed using two-tailed t-test. Comparison of variance between multiple groups was performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or Dunnett's multiple comparison test. Comparison between more than one variable was performed using two-way ANOVA with Sidak's multiple comparison test. P value less than 0.05 was accepted as statistically significant.

4. Results

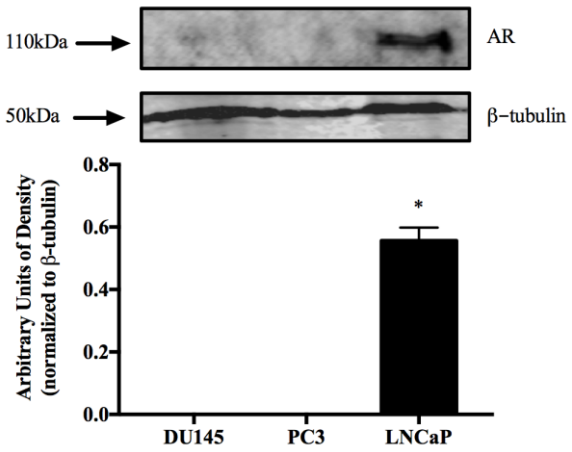
4.1. Characterization of PCa cell lines based on AR expression and invasiveness.

To confirm previous studies suggesting that LNCaP express AR, whereas, DU145 and PC3 lack AR expression, we conducted a western blot analysis on PCa cellular lysates. Western blot analysis of PCa cellular lysates demonstrates high expression of AR in LNCaP cells (arbitrary units of density (AUD) 0.55±0.04) whereas AR was undetectable by immunoblot in DU145 (Figure 10A). Therefore, LNCaP cells express AR, whereas, DU145 and PC3 cells do not.

Pharmacological inhibition of AR with bicalutamide demonstrates no significant effect on LNCaP cellular viability. LNCaP cell lines incubated with 20μM bicalutamide for 24-hours demonstrated no significant loss in cellular viability compared LNCaP cells exposed to vehicle control (0.001% DMSO) for 24-hours (95.67±2.96% vs 100%, respectively) (Figure 10B).

Gelatin coated Boyden chamber assay demonstrate that DU145 are significantly more invasive compared to LNCaP, LNCaP + 20μM bic, and PC3 cells. LNCaP; 26.75±6.81 cells/5 fields of view, LNCaP + 20μM Bic; 28.0±5.05 cells/5 fields of view, DU145; 391.0±36.60 cells/5 fields of view; PC3 86.13±21.11 cells/5 fields of view. DU145 cells are the most invasive prostate cancer cell line *in vitro* (Figure 11).

A.



B.

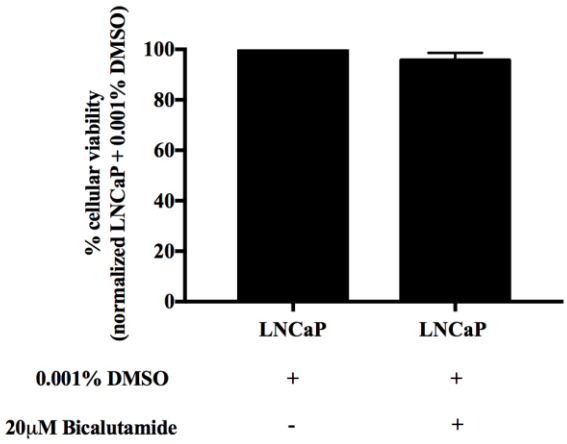


Figure 10: Baseline expression of androgen receptor (AR) in prostate cancer cell line cellular lysates and the effect of pharmacological AR blockade on LNCaP cellular viability. (A) Cellular lysates of prostate cancer cell lines demonstrate significantly higher expression of AR in LNCaP compared to DU145 and PC3. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=3, *$p<0.05$. **(B)** Alamar blue cellular viability assay demonstrates no significant effect on LNCaP viability following inhibition of AR with 20μM of bicalutamide *in vitro* for 24-hours compared to control LNCaP cells exposed to 0.001% DMSO (solvent of bicalutamide) for 24-hours. Statistics: Two-tailed, paired t-test, N=3, $p>0.05$.

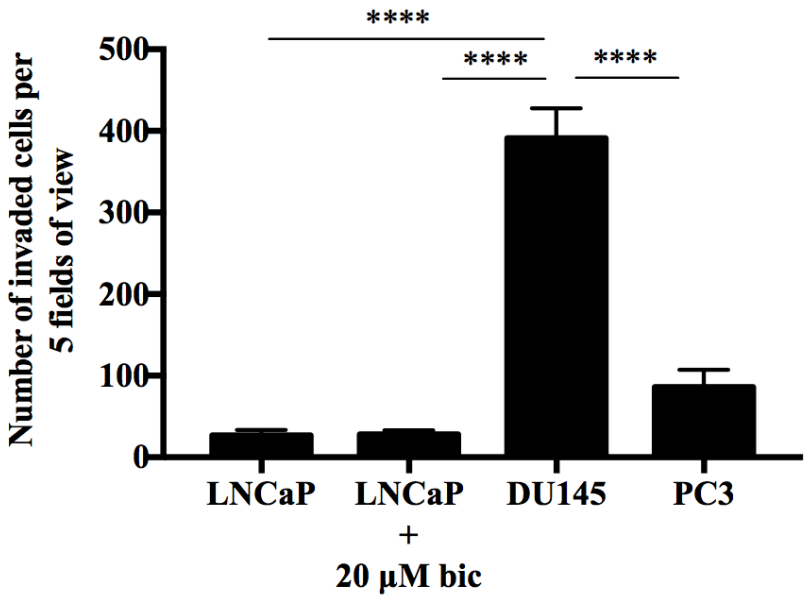
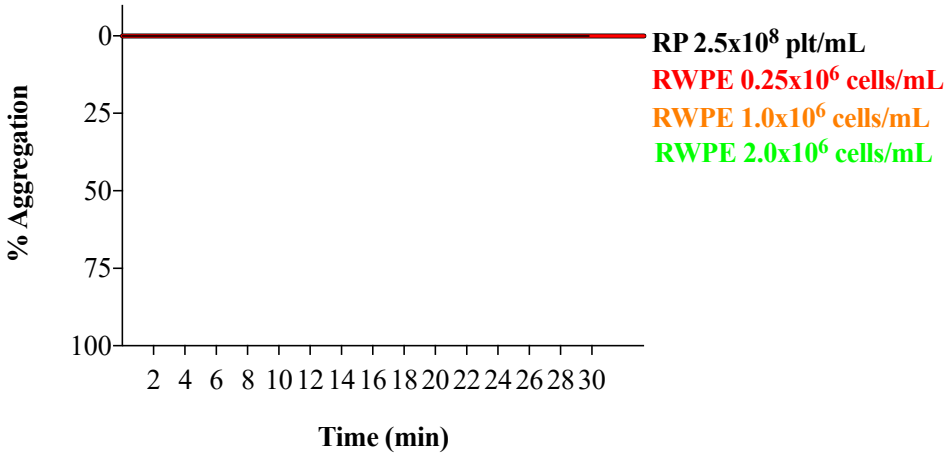


Figure 11: Comparison of prostate cancer cell line invasiveness in vitro. *In vitro* gelatin coated Boyden chamber assay demonstrate that DU145 cell are significantly more invasive than LNCaP, LNCaP + 20µM bic, and PC3 cells. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=4, ****$p<0.0001$.

4.2. Characterization of AR positive PCa cell line TCIPA

Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to increasing concentration of RWPE and LNCaP cells ($0.25\text{-}2.0x10^6$ cells/mL) demonstrate no significant effect on initiation of platelet activation and % aggregation.

A.



B.

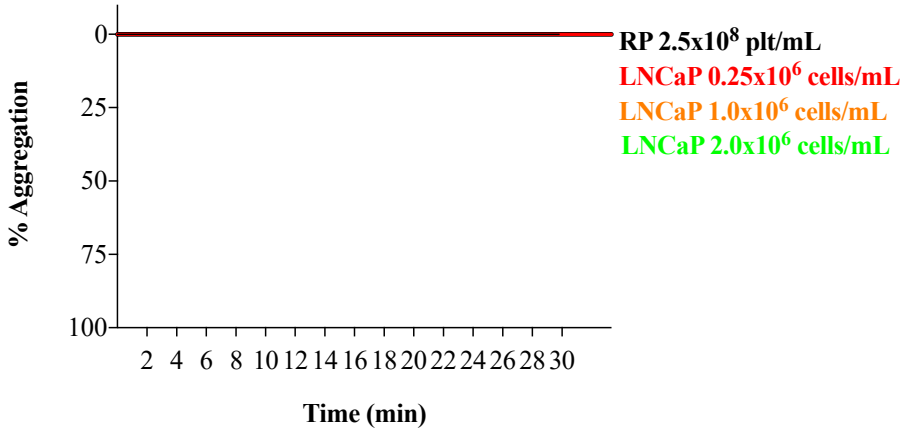


Figure 12: RWPE and LNCaP TCIPA profile. Representative platelet aggregometry traces of isolated human platelets stimulated by **(A)** RWPE or **(B)** LNCaP cells (0.25-2.0x10^6 cells/mL). Addition of increasing concentration of RWPE or LNCaP cell lines to human platelets (2.5x10^8 platelets/mL) demonstrates no effect on platelet activation and % aggregation. N=6.

4.3. Characterization of AR negative and AR inhibited PCa cell line TCIPA

DU145 potently stimulate platelet aggregation in a concentration-dependent manner. Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to increasing concentration of DU145 cells ($0.005x10^6$ – $2.0x10^6$ cells/mL) demonstrate significant increase in % platelet aggregation compared to $0.001x10^6$ cells/mL; $0.001x10^6$ cells/mL (54.04 $\pm$ 1.55%), $0.005x10^6$ cells/mL (62.23 $\pm$ 2.03%), $0.010x10^6$ cells/mL (64.14 $\pm$ 1.04%), 0.10 $x10^6$ cells/mL (61.81 $\pm$ 1.46%), 0.25 $x10^6$ cells/mL (64.06 $\pm$ 0.88%), 0.50 $x10^6$ cells/mL (63.73 $\pm$ 0.76%), 1.0 $x10^6$ cells/mL (63.17 $\pm$ 0.86%), 1.5 $x10^6$ cells/mL (62.60 $\pm$ 1.04%), and $2.0x10^6$ cells/mL (61.05 $\pm$ 0.99%) (Figure 13B).

Exposure of isolated human platelets ($2.5x10^8$ platelet/mL) to increasing concentration of DU145 cells ($0.010x10^6$ – $2.0x10^6$ cells/mL) demonstrates significant reduction in time to initiation of platelet aggregation compared to $0.001x10^6$ cells/mL; $0.001x10^6$ cells/mL (1519 $\pm$ 90 seconds), $0.005x10^6$ cells/mL (802 $\pm$ 165.1 seconds), $0.010x10^6$ cells/mL (678.8$\pm$ 71.16%), 0.10 $x10^6$ cells/mL (629.8 $\pm$ 67.77 seconds), 0.25 $x10^6$ cells/mL (423.8 $\pm$ 73.75 seconds), 0.50 $x10^6$ cells/mL (417.3 $\pm$ 67.15 second), 1.0 $x10^6$ cells/mL (422.7 $\pm$ 69.79 seconds), 1.5 $x10^6$ cells/mL (389.9 $\pm$ 55.87 seconds), and $2.0x10^6$ cells/mL (393.8 $\pm$ 55.01 seconds) (Figure 13C).

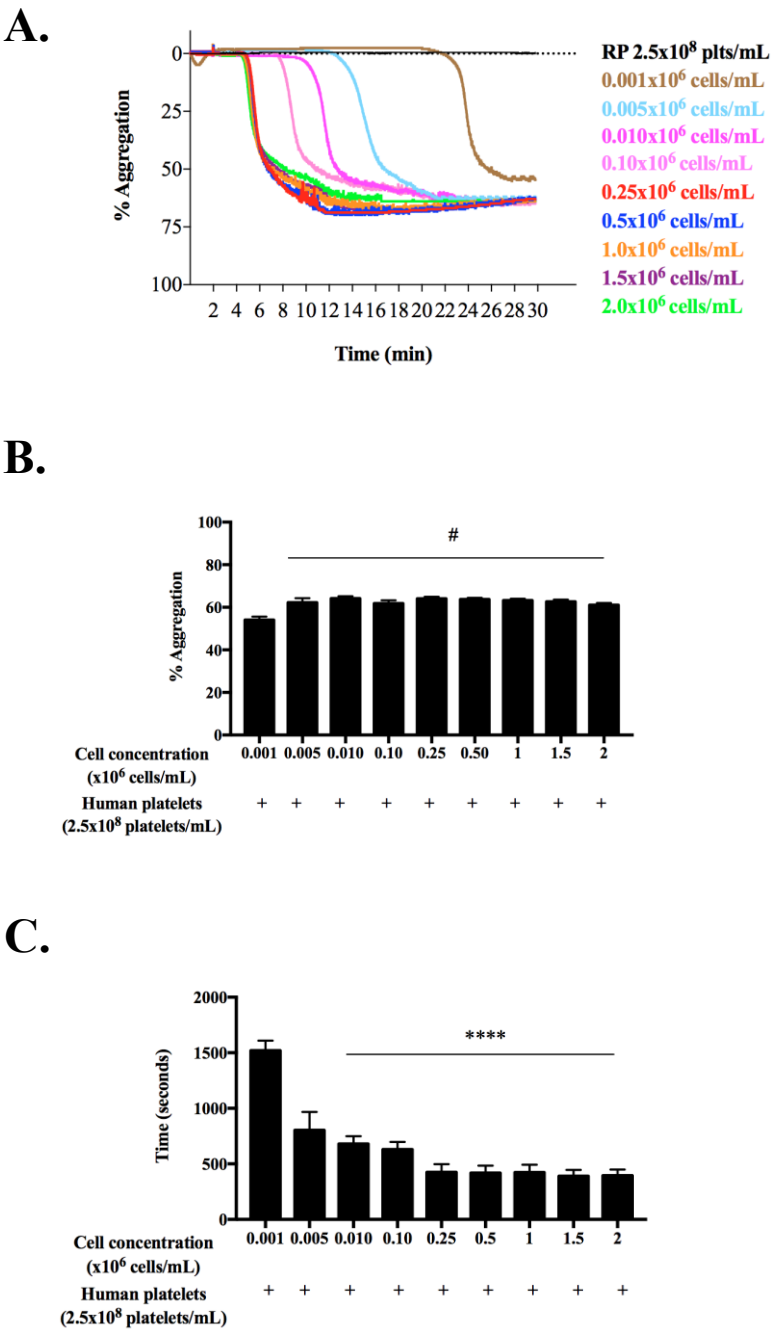


Figure 13: DU145 TCIPA profile. (A) Representative platelet aggregation traces of isolated human platelets exposed to increasing concentration of DU145 cells (0.001-2.0x10^6 cells/mL). **(B)** Addition of 0.005-2.0x10^6 DU145 cells/mL results in significant increase in % platelet aggregation compared to 0.001x10^6 cells/mL. **(C)** Addition of 0.010-2.0x10^6 DU145 cells/mL to isolated human platelets results in significant reduction in time to initiation of platelet aggregation compared to 0.001x10^6 cells/mL. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=6, #p<0.001, ****p<0.0001.

PC3 also stimulate platelet aggregation in a concentration-dependent manner. Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to increasing concentration of PC3 cells ($0.25x10^6$ – $2.0x10^6$ cells/mL) demonstrates significant increase in % platelet aggregation compared to $0.001x10^6$ – $0.10x10^6$ cells/mL; $0.001x10^6$ cells/mL (0 $\pm$ 0%), $0.005x10^6$ cells/mL (0 $\pm$ 0%), $0.010x10^6$ cells/mL (0 $\pm$ 0%), 0.10 $x10^6$ cells/mL (0 $\pm$ 0%), 0.25 $x10^6$ cells/mL (26.34 $\pm$ 11.78%), 0.50 $x10^6$ cells/mL (36.83 $\pm$ 11.9%), 1.0 $x10^6$ cells/mL (43.16 $\pm$ 8.88%), 1.5 $x10^6$ cells/mL (47.47 $\pm$ 3.17%), and $2.0x10^6$ cells/mL (39.02 $\pm$ 5.10%) (Figure 14B).

Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to increasing concentration of PC3 cells (0.25 – $2.0x10^6$ cells/mL) demonstrates significant reduction in time to initiation of platelet aggregation compared to $0.001x10^6$ – $0.10x10^6$ cells/mL; $0.001x10^6$ cells/mL (0 $\pm$ 0 seconds), $0.005x10^6$ cells/mL (0 $\pm$ 0 seconds), $0.010x10^6$ cells/mL (0 $\pm$ 0 seconds), 0.10 $x10^6$ cells/mL (0 $\pm$ 0 seconds), 0.25 $x10^6$ cells/mL (1397 $\pm$ 185.6 seconds), 0.50 $x10^6$ cells/mL (1194 $\pm$ 223.6 second), 1.0 $x10^6$ cells/mL (1007 $\pm$ 57.75 seconds), 1.5 $x10^6$ cells/mL (937 $\pm$ 63.84 seconds), and $2.0x10^6$ cells/mL (909.9 $\pm$ 69.17 seconds) (Figure 14C).

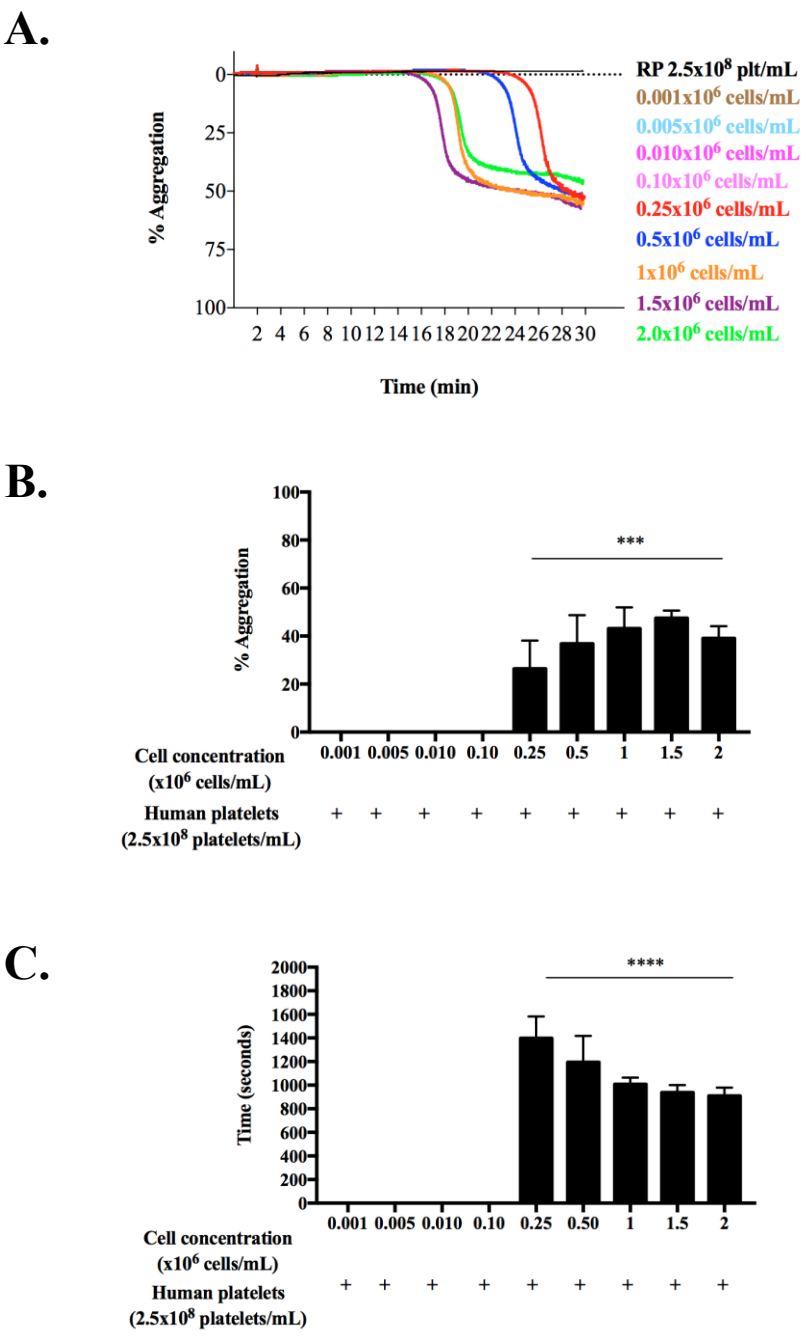


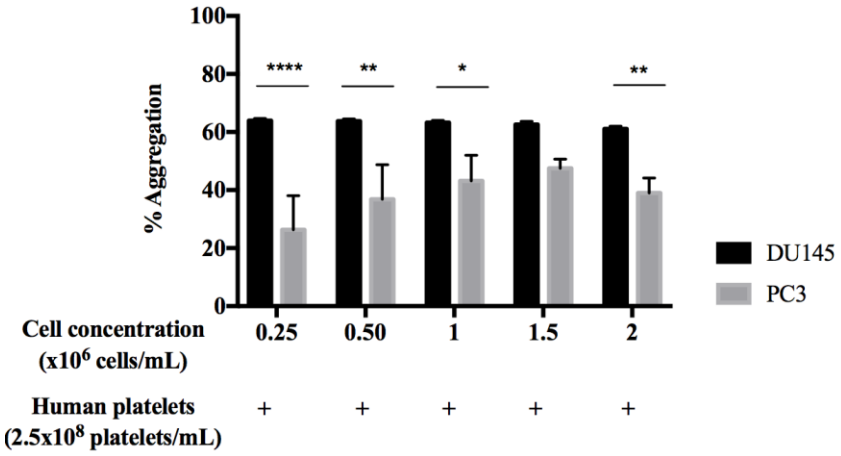
Figure 14: PC3 TCIPA profile. (A) Representative platelet aggregation traces of isolated human platelets exposed to PC3 cells (0.001-2.0x10^6 cells/mL). **(B)** Addition of increasing concentration of PC3 cells to isolated human platelets results in significant increase in % total platelet aggregation at concentration 0.25-2.0x10^6 cells/mL. **(C)** Addition of increasing concentration of PC3 cells to human platelets at concentrations 0.25-2.0x10^6 cells/mL demonstrates significant reduction in time to initiation of platelet activation. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=6, ***p<0.001.

Comparison of AR negative TCIPA profile demonstrate that DU145 have higher aggregation potency compared to PC3.

Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to increasing concentration of DU145 demonstrate significantly higher % platelet aggregation compared to PC3 cells at cellular concentrations 0.25 $x10^6$ – 1.0$x10^6$ cells/mL and 2.0$x10^6$ cells/mL; 0.25$x10^6$ cells/mL (DU145 64.06 $\pm$ 0.88% vs PC3 26.34 $\pm$ 11.78%), 0.50 $x10^6$ cells/mL (DU145 63.73 $\pm$ 0.76% vs PC3 36.83 $\pm$ 11.9%), 1.0 $x10^6$ cells/mL (DU145 63.17 $\pm$ 0.86% vs PC3 43.16 $\pm$ 8.88%), and 2.0$x10^6$ cells/mL (DU145 61.05 $\pm$ 0.99% vs PC3 39.02 $\pm$ 5.10%). However, there is no significant difference in % platelet aggregation between 1.5$x10^6$ cells/mL (DU145 62.60 $\pm$ 1.04% vs PC3 47.47 $\pm$ 3.17%) (Figure 15A).

Exposure of isolated human platelets to increasing concentration of DU145 demonstrate significantly shorter time to initiation of platelet aggregation compared to PC3 cells at cellular concentrations 0.25$x10^6$ cells/mL – 2.0$x10^6$ cells/mL; 0.25 $x10^6$ cells/mL (Du145 423.80 $\pm$ 73.75 seconds vs PC3 1397.00 $\pm$ 185.60 seconds), 0.50 $x10^6$ cells/mL (DU145 417.30 $\pm$ 67.15 second vs 1194.00 $\pm$ 223.60 seconds), 1.0 $x10^6$ cells/mL (DU145 422.70 $\pm$ 69.79 seconds vs PC3 1007.00 $\pm$ 57.75 seconds), 1.5 $x10^6$ cells/mL (DU 145 389.90 $\pm$ 55.87 seconds vs PC3 937.00 $\pm$ 63.84 seconds), and 2.0$x10^6$ cells/mL (DU145 393.80 $\pm$ 55.01 seconds vs PC3 909.90 $\pm$ 69.17 seconds) (Figure 15B).

A.



B.

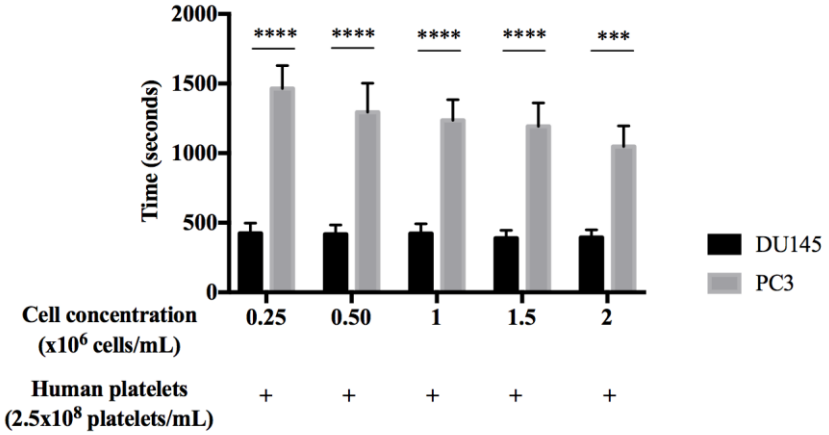


Figure 15: DU145 and PC3 TCIPA profile comparison. (A) DU145 cells demonstrate significantly higher % platelet aggregation compared to PC3 cells at concentrations 0.25-1.0x10^6 tumor cells/mL and 2.0x10^6 tumor cells/mL. **(B)** Upon addition of 0.25-2.0x10^6 tumor cells/mL to isolated human platelets, DU145 cells demonstrate significantly shorter time to initiation of platelet aggregation. Statistics: Two-way ANOVA, Sidak's multiple comparison test, N=6, *p<0.05, **p<0.01,***p<0.001,****p<0.0001.

To explain whether differences in PCa AR signaling affects the ability to induce platelet aggregation, LNCaP cells were treated with 20μM of bicalutamide and added to isolated human platelets.

Isolated human platelets ($2.5x10^8$ platelets/mL) exposed to $0.25x10^6$ cells/mL LNCaPs treated with 20μM bicalutamide for 24-hours *in vitro* demonstrate significant increase in platelet aggregation and time to initiation of platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) exposed to LNCaP cell lines exposed to 0.001% DMSO for 24-hours (43.73$\pm$8.62% vs 0$\pm$0%, 911.30$\pm$198.70 seconds vs 0$\pm$0 seconds, respectively) (Figure 16B-C).

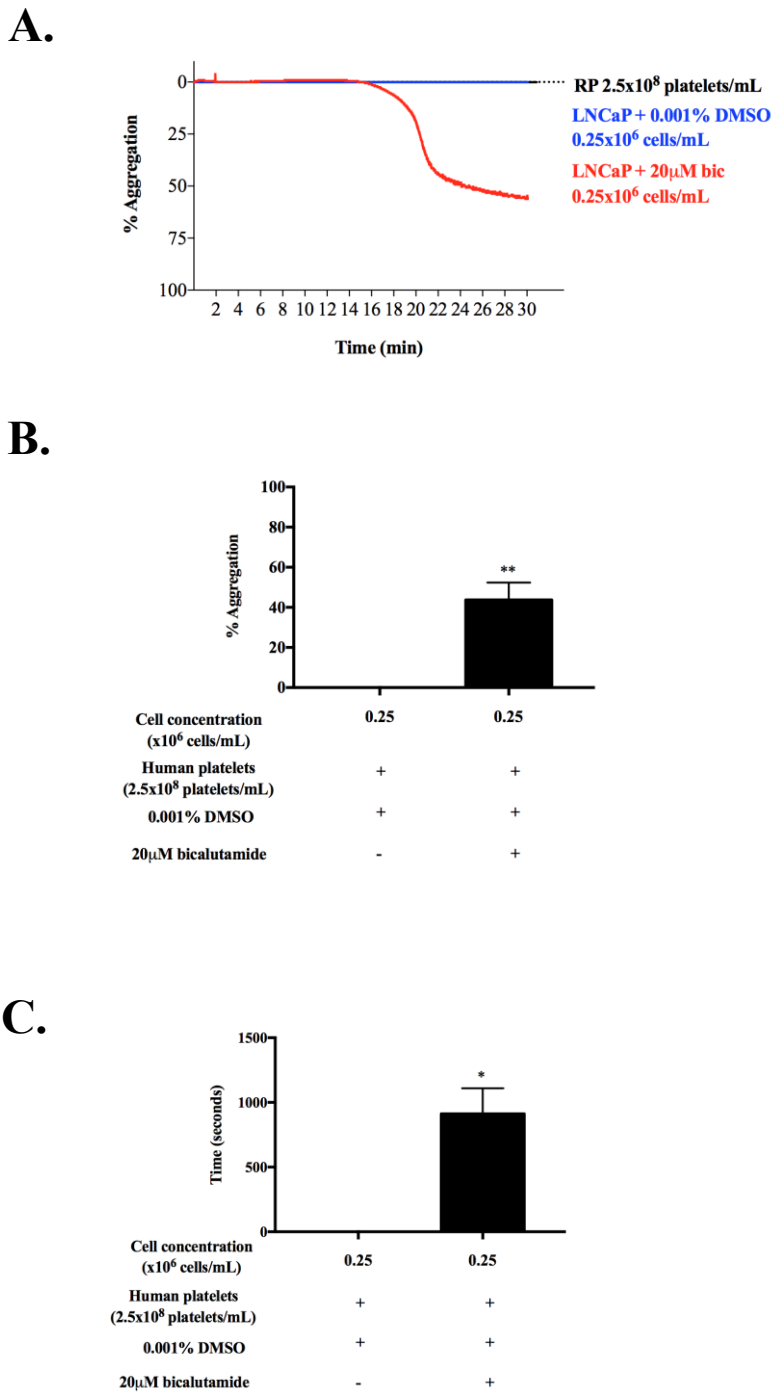


Figure 16: TCIPA profile of LNCaP cells treated with 20 µM bicalutamide for 24-hours in vitro. (A) Representative platelet aggregation traces of isolated human platelets stimulated by LNCaP treated with 20 µM bicalutamide and isolated human platelets exposed to 0.001% DMSO. Addition of $0.25x10^6$ cells/mL of LNCaP treated with 20µM bicalutamide demonstrates significant increase in % platelet aggregation**. (B)** and significant increase in time to initiation of platelet aggregation **(C)** compared to LNCaP cells treated with 0.001% DMSO. Statistics: Two-tailed, paired t-test, N=6, *$p<0.05$ and **$p<0.01$. Bic – bicalutamide.

Comparison of TCIPA profile demonstrate that addition of $0.25x10^6$ cells/mL of LNCaP + 20μM bicalutamide, DU145, and PC3 to isolated human platelets ($2.5x10^8$ platelets/mL) results in significant increase in % platelet aggregation (43.73±8.62%, 64.06±0.88%, 26.34±11.78%, respectively) compared to RWPE and LNCaP cells (0±0%, 0±0%, respectively). Furthermore, addition $0.25x10^6$ cells/mL of DU145 to isolated human platelets ($2.5x10^8$ platelets/mL) demonstrate significant increase in % platelet aggregation compared to $0.25x10^6$ cells/mL of PC3 (64.06±0.88%, 26.34±11.78%, respectively) (Figure 17B).

Comparison of TCIPA profile demonstrate that addition of $0.25x10^6$ cells/mL of LNCaP + 20μM bicalutamide, DU145, PC3 to isolated human platelets ($2.5x10^8$ platelets/mL) results in significant increase in time to initiation of platelet aggregation (842.8±176.1 seconds, 423.8±73.75 seconds, 1403±151.6 seconds, respectively) compared to RWPE and LNCaP cells (0±0 seconds, 0±0 seconds, respectively). Furthermore, addition $0.25x10^6$ cells/mL of DU145 and LNCaP + 20μM bicalutamide to isolated human platelets ($2.5x10^8$ platelets/mL) demonstrate significant reduction in time to initiation of platelet aggregation compared to $0.25x10^6$ cells/mL of PC3 (DU145 423.8±73.75 seconds vs PC3 1403±151.6 seconds, LNCaP + 20μM bicalutamide 1403±151.6 seconds vs PC3 1403±151.6 seconds) (Figure 17C).

Overall, comparison of PCa TCIPA profile demonstrate that DU145 cells have the greatest effect on isolated platelet activation.

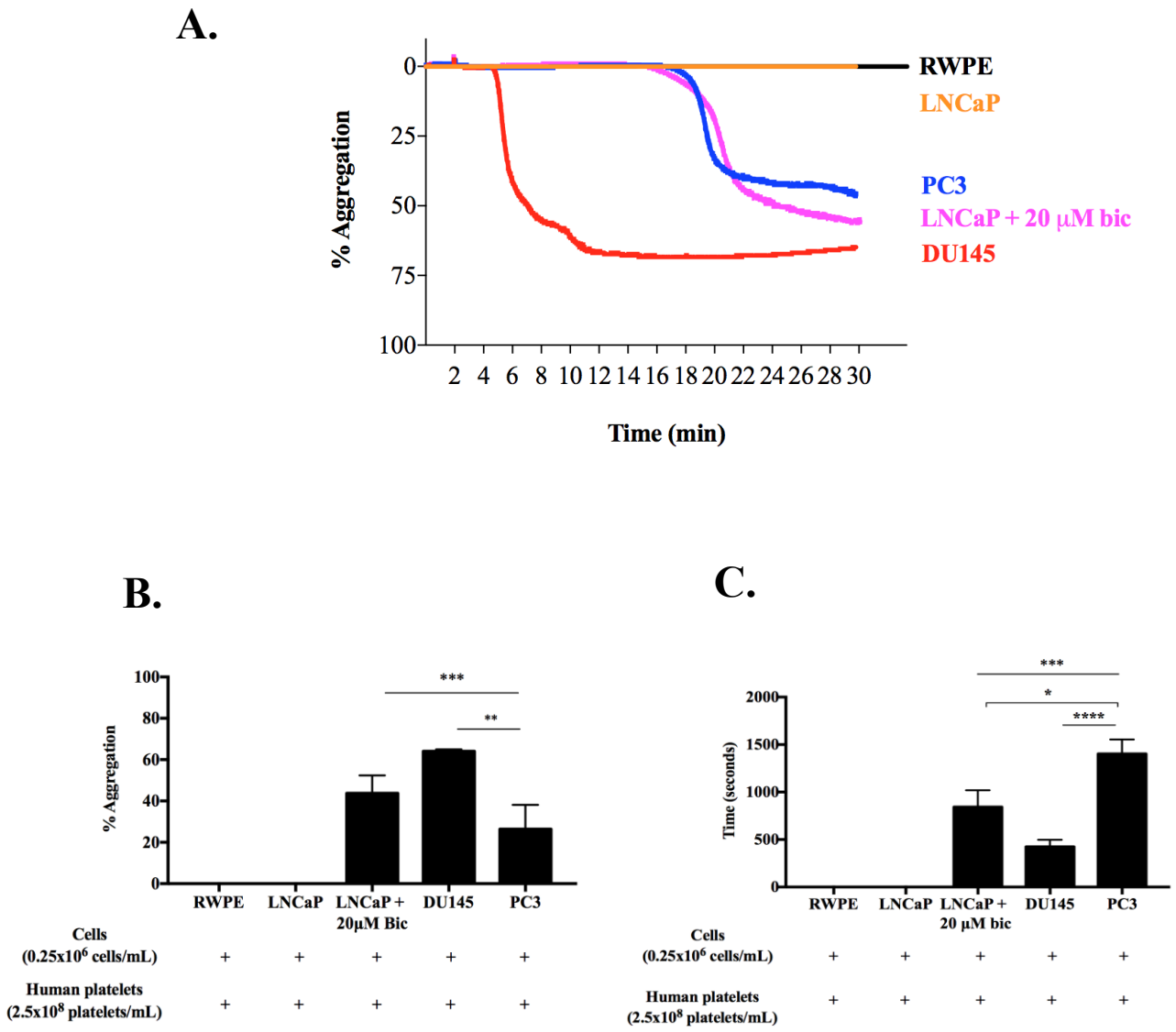


Figure 17: Benign prostate and prostate cancer cell line TCIPA profile comparison. (A) Representative platelet aggregation traces of isolated human platelets stimulated by RWPE, LNCaP + 20μM bic, LNCaP, DU145, and PC3. **(B)** Addition of $0.25x10^6$ cells/mL of LNCaP + 20 μM bic, DU145, and PC3 cells to isolated human platelets results in significant increase in % platelet aggregation compared to RWPE and LNCaP. Addition of $0.25x10^6$ tumor cells/mL demonstrate that DU145 incite significantly higher % aggregation compared to PC3. **(C)** Addition of $0.25x10^6$ tumor cells/mL of LNCaP + bic, DU145, and PC3 cells to isolated human platelets demonstrate that PC3 cells have significantly prolonged time to initiation of platelet activation compared to LNCaP + 20 μM bic and DU145. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=6, *p<0.05, **p<0.01,***p<0.001.

4.4. Investigating the role of MMP-2, 9 and NO in PCa induced TCIPA

Having established that PCa cell lines induce TCIPA, we proceeded to study the some of the common molecular mechanisms responsible for this interaction such as MMP-2 and 9. Isolated human platelets ($2.5x10^8$ platelets/mL) exposed to 0.25-$2.0x10^6$ cells/mL release significantly higher level of pro-MMP-2 compared to resting isolated platelets over 30 minutes in the platelet aggregometer. Resting platelets; 100$\pm$0%, $0.25x10^6$ cells/mL; 218.30$\pm$37.68%, $1.0x10^6$ cells/mL; 235.70$\pm$20.92%, $2.0x10^6$ cells/mL; 217.7$\pm$35.57% (Figure 18B).

Isolated human platelets ($2.5x10^8$ platelets/mL) exposed to 0.25-$2.0x10^6$ cells/mL do not release significantly higher amount of pro-MMP-9 compared to resting isolated platelets over 30 minutes in the platelet aggregometer. Resting platelets; 100$\pm$0%, $0.25x10^6$ cells/mL; 133.70$\pm$35.62%, $1.0x10^6$ cells/mL; 112.70$\pm$24.54%, $2.0x10^6$ cells/mL; 120.5$\pm$35.63% (Figure 18C).

Platelets incubated with increasing concentration of LNCaP release a significantly higher amount of pro-MMP-2, but not pro-MMP-9, compared to resting platelets.

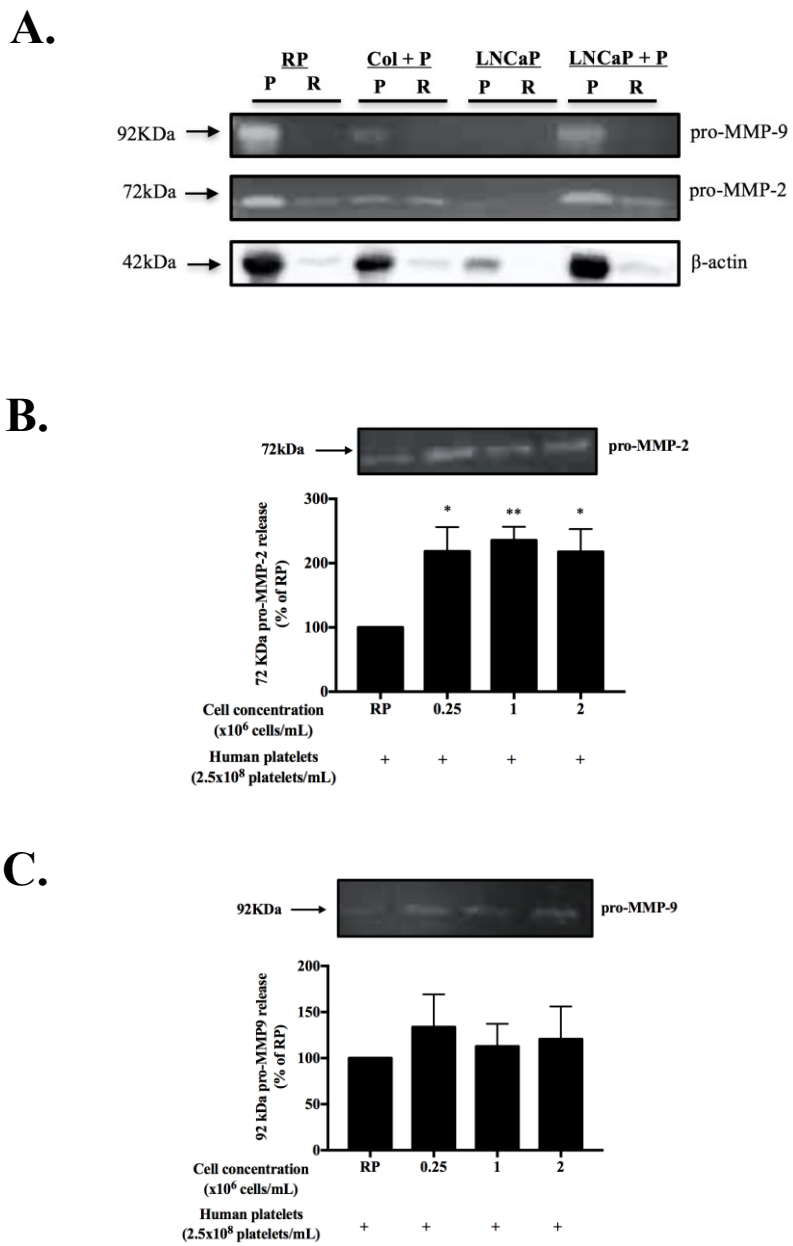


Figure 18: Platelet derived pro-MMP-2 and 9 release during incubation of LNCaP with isolated human platelets. (A) Representative gelatin zymogram of isolated human platelet ($2.5x10^8$ platelets/mL) secreted pro-MMP-2 and 9 following type I collagen (10µg/mL) or LNCaP cell ($1.0x10^6$ cells/mL) stimulation. (P) denotes pellet, (R) denotes releasate, RP+C denotes resting platelets stimulated with type I collagen, and (+) P denotes addition of physiologic concentration of human platelets ($2.5x10^8$plts/mL). **(B)** Addition of $\geq 0.25x10^6$ tumor cells/mL to isolated human platelets results in significant increase in platelet pro-MMP-2 release compared to resting platelets. (C) Addition of LNCaP cells to isolated human platelets does not results in significant increase in platelet pro-MMP-9 release. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6, *$p<0.05$, **$p<0.01$.

Isolated human platelets ($2.5x10^8$ platelets/mL) exposed to 0.25-$2.0x10^6$ cells/mL release significantly higher level of pro-MMP-2 compared to resting isolated platelets over 30 minutes in the platelet aggregometer. RP; 100±0%, $0.25x10^6$ cells/mL; 149.50±13.68%, $0.50x10^6$ cells/mL; 164.70±22.44%, $1.0x10^6$ cells/mL; 179.30±30.34%, $1.5x10^6$ cells/mL 184.0±22.43%, $2.0x10^6$ cells/mL; 192.0±20.60% (Figure 19B).

Isolated human platelets exposed to 0.25-$2.0x10^6$ cells/mL demonstrate no significant increase in pro-MMP-9 release compared to resting isolated platelets ($2.5x10^8$ platelets/mL) over 30 minutes in the platelet aggregometer. RP; 100±0%, $0.25x10^6$ cells/mL; 95.28±13.39%, $0.50x10^6$ cells/mL; 89.40±19.31%, $1.0x10^6$ cells/mL; 95.0±24.62%, $1.5x10^6$ cells/mL; 98.40±21.75%, $2.0x10^6$ cells/mL; 106.20±19.52% (Figure 19C).

Platelets incubated with increasing concentration of DU145 release a significantly higher amount of pro-MMP-2, but not pro-MMP-9, compared to resting platelets.

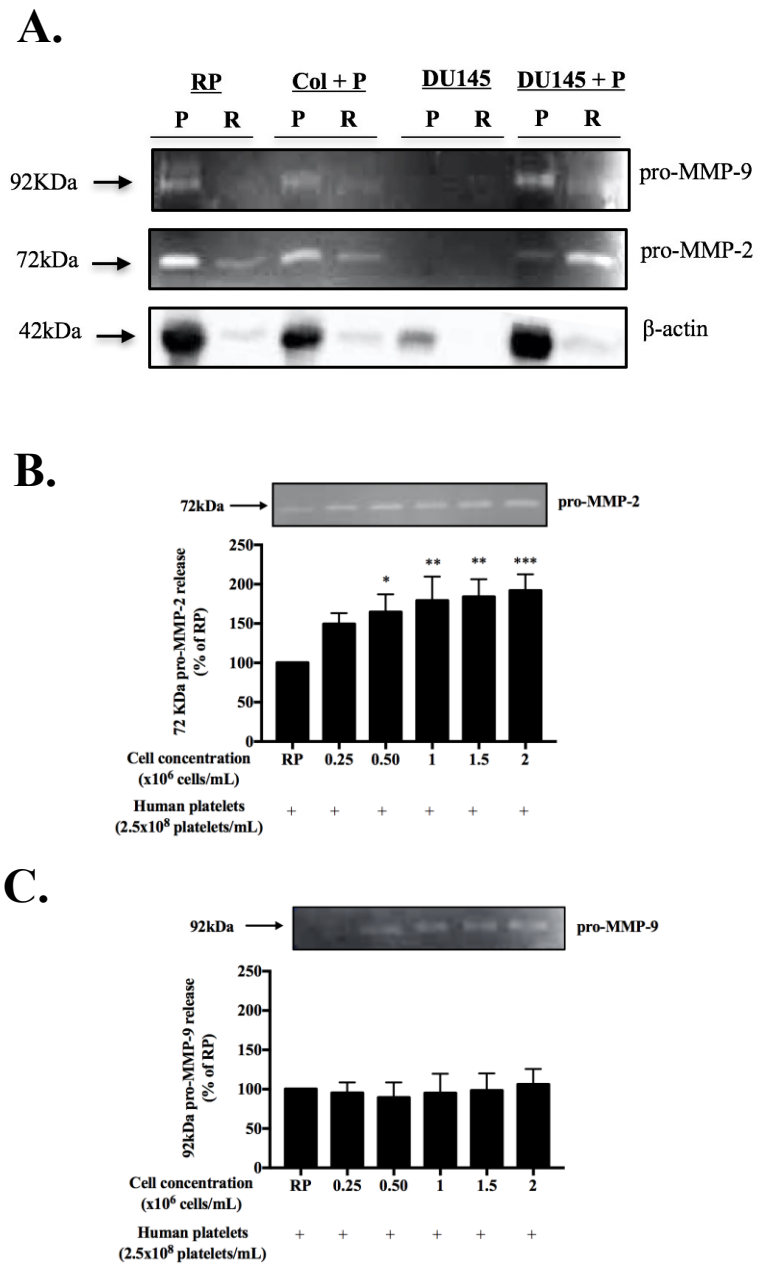


Figure 19: Platelet derived pro-MMP-2 and 9 release during DU145 TCIPA. (A) Representative gelatin zymogram of isolated human platelet ($2.5x10^8$ platelets/mL) secreted pro-MMP-2 and 9 following type I collagen (10µg/mL) or DU145 cell ($1.0x10^6$ cells/mL) stimulation. (P) denotes pellet, (R) denotes releasate, RP+C denotes resting platelets stimulated with type I collagen, and (+) P denotes addition of physiologic concentration of human platelets ($2.5x10^8$plts/mL). **(B)** Addition of $\geq 0.50x10^6$ tumor cells/mL to isolated human platelets results in significant increase in platelet pro-MMP-2 release compared to resting platelets. **(C)** Addition of DU145cells to isolated human platelets does not results in significant increase in platelet pro-MMP-9 release. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Isolated human platelets exposed to 0.25-2.0x10^6 cells/mL release significantly higher level of pro-MMP-2 compared to resting isolated platelets (2.5x10^8 platelets/mL) over 30 minutes in the platelet aggregometer. RP; 100$\pm$0%, 0.25x10^6 cells/mL; 173.80$\pm$14.49%, 0.50x10^6 cells/mL; 156.0$\pm$20.30%, 1.0x10^6 cells/mL; 172.20$\pm$23.45%, 1.5x10^6 cells/mL 165.80$\pm$19.55%, 2.0x10^6 cells/mL; 152.20$\pm$12.71% (Figure 34B).

However, isolated human platelets incubated with increasing concentration of PC3 in the platelet aggregometer release significant amount of pro-MMP-9 at 0.25x10^6 cells/mL; RP; 100$\pm$0% and 0.25x10^6 cells/mL; 301.0$\pm$83.72%. At cellular concentration $\geq$0.25x10^6 cells/mL there is no significant release of pro-MMP-9 from isolated human platelets compared to resting platelets; RP; 100$\pm$0%, 0.50x10^6 cells/mL; 208.30$\pm$38.32%, 1.0x10^6 cells/mL; 213.30$\pm$49.76%, 1.5x10^6 cells/mL; 180.30$\pm$64.85%, 2.0x10^6 cells/mL; 121.70$\pm$43.43% (Figure 34C).

Platelets incubated with increasing concentration of PC3 release a significantly higher amount of pro-MMP-2, but not pro-MMP-9, compared to resting platelets.

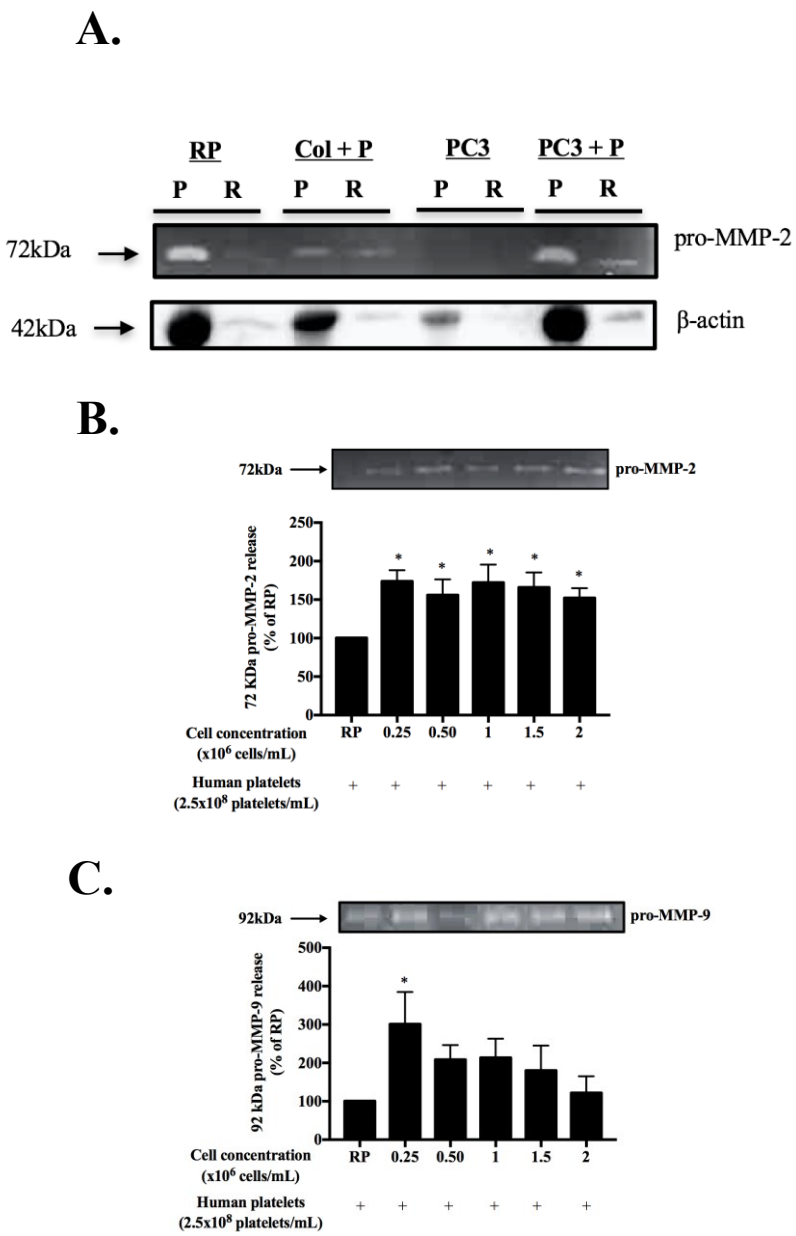


Figure 20: Platelet derived pro-MMP-2 and 9 release during PC3 TCIPA. (A) Representative gelatin zymogram of isolated human platelet ($2.5x10^8$ platelets/mL) secreted pro-MMP-2 and 9 following type I collagen (10µg/mL) or PC3 cell ($1.0x10^6$ cells/mL) stimulation. (P) denotes pellet, (R) denotes releasate, RP+C denotes resting platelets stimulated with 10µg/mL of type I collagen, and (+)P denotes addition of physiologic concentration of human platelets ($2.5x10^8$plts/mL). **(B)** Addition of $\geq 0.25x10^6$ tumor cells/mL to isolated human platelets results in significant increase in platelet pro-MMP-2 release compared to resting platelets. (C) Addition of $0.25x10^6$ tumor cells to human platelets results in significant increase in platelet pro-MMP-9 release. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6, *$p<0.05$.

To explain the lack of aggregation by LNCaPs we explored the role of cancer cell derived NO pathway in inhibition of platelet aggregation. Incubation of LNCaP cells with 100 μM or 500 μM of L-NAME demonstrates no significant effect on LNCaP TICPA profile (Figure 21).

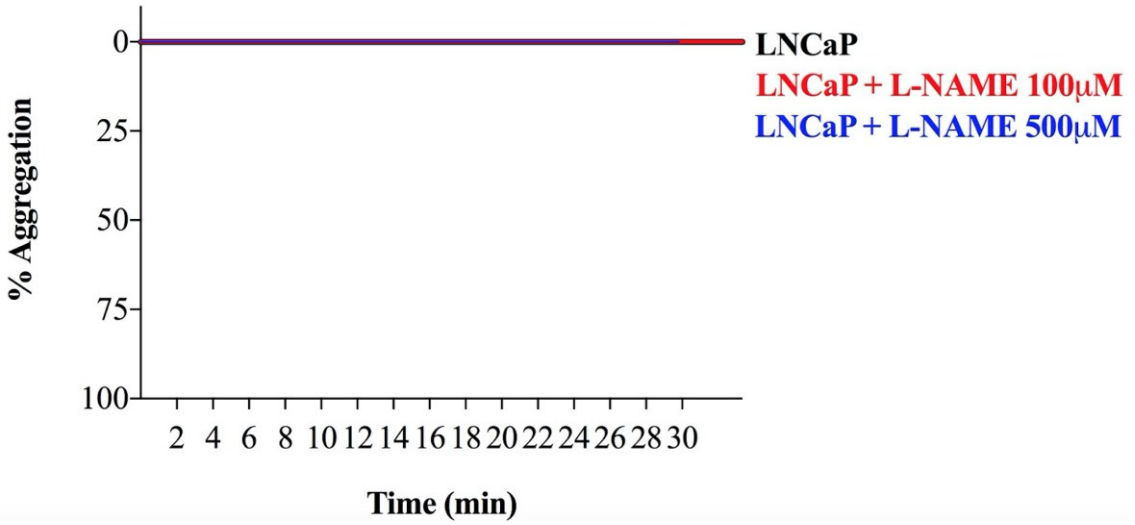


Figure 21: Expression of eNOS and the role of eNOS in LNCaP TCIPA mechanism. (A) Cellular lysates of LNCaP and LNCaP treated with 20μM bic *in vitro* for 24-hours demonstrate no significant difference in expression of eNOS. **(B)** Incubation of LNCaP cells with L-Nomega-Nitro-L-arginine methyl ester (L-NAME) to inhibit eNOS demonstrates no observable effect on % isolated human platelet aggregation. Statistics: Two-tailed, paired t-test, N=6, $p>0.05$. HUVEC – human umbilical vein endothelial cells.

4.5. Pharmacological characterization of PCa cell line induced TCIPA

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 200µM acetylsalicylic acid, or 5µM ARP-100, or 11µM apyrase, or 30µM Prasugrel active metabolite, or combination of 200µM acetylsalicylic acid, 5µM ARP-100, 11µM apyrase then stimulated with LNCaP + 20µM bic demonstrate no significant effect on % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with LNCaP + 20µM bic alone; LNCaP + 20µM bic; 43.73±8.62%, 200µM ASA 51.67±1.66%; 5µM ARP-100 51.67±1.66%; 11µM APY 48.67±4.315%; 30µM PAM 49.67±4.31%; 200µM ASA and 5µM ARP-100 and 11µM APY 40.83±3.51%. On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) treated with 50µM eptifibatide or 0.8µM dabigatran then stimulated with LNCaP + 20µM bic demonstrate significant reduction in % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with LNCaP + 20µM bic alone; 50µM EPT 23.28±0.82%, 0.8µM DABI 0±0% (Figure 22A).

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 200µM acetylsalicylic acid, or 5µM ARP-100, or 11µM apyrase, or 30µM prasugrel active metabolite, or combination of 200µM acetylsalicylic acid, 5µM ARP-100, 11µM apyrase then stimulated with LNCaP + 20µM bic demonstrate no significant effect on time to initiation of platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with LNCaP + 20µM bic alone; LNCaP + 20µM bic 842.80±176.10 seconds, 200µM ASA 909.20±137.80 seconds, 5µM ARP-100 908.80±137.80 seconds, 11µM APY 828.30±106.60 seconds, 30µM PAM 843.70±92.04 seconds, 200µM ASA and 5µM ARP-100 and 11µM APY 1069.0±151.30 seconds. On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) exposed to 50µM eptifibatide then stimulated with LNCaP + 20µM bic demonstrate significant prolongation in time to initiation of platelet

aggregation compared to isolated human platelets stimulated with LNCaP + 20μM bic alone; 50μM EPT 1530.0±34.79 seconds (Figure 22B).

Pharmacological inhibition of platelet surface GPIIb/IIIa and thrombin inhibits LNCaP + 20μM bic induced TCIPA.

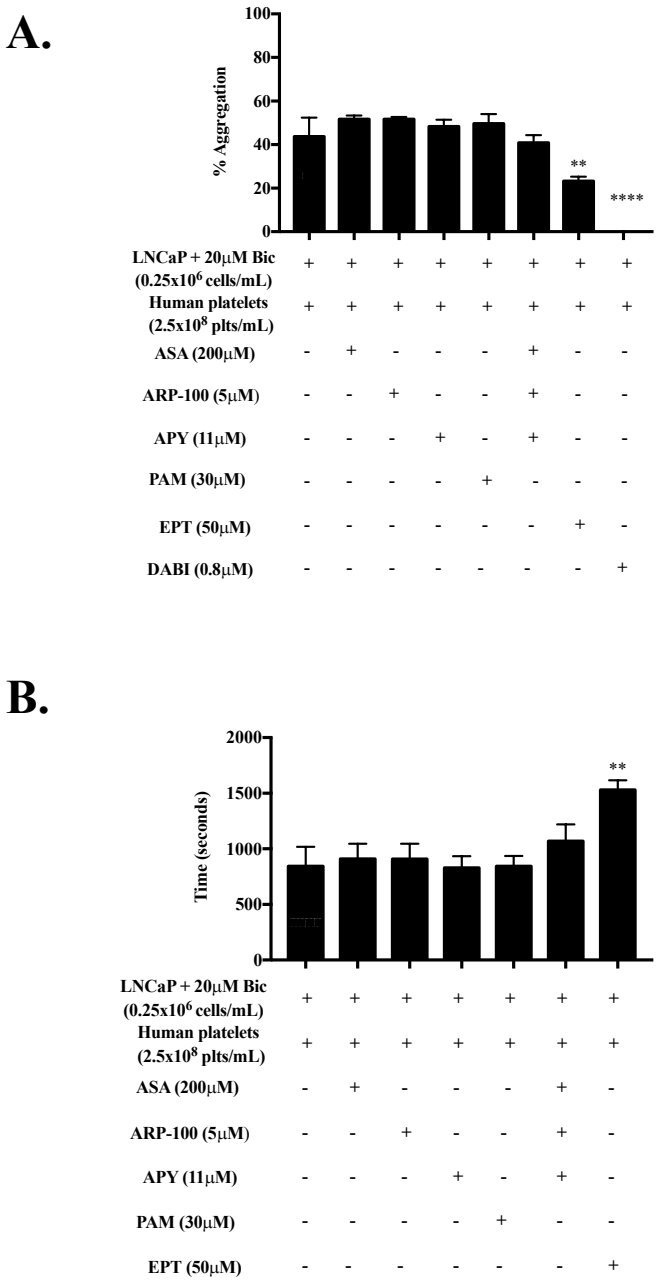


Figure 22: Contribution of platelet pathways to LNCaP + 20μM bic TCIPA mechanism. (A) Treatment of isolated human platelets with EPT and DABI then stimulation with LNCaP + 20μM bic demonstrates significant reduction in % platelet aggregation compared to isolated human platelets stimulated with LNCaP + 20μM bic alone. **(B)** Treatment of isolated human platelets with EPT results in significant prolongation in time to initiation of isolated human platelet aggregation compared to isolated human platelets stimulated with LNCaP + 20μM bic alone. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6,**p<0.01,****p<0.0001. (acetylsalicylic acid (ASA), ARP-100, apyrase (APY), Prasurgrel active metabolite (PAM), eptifibatide (EBT), and dabigatran (DABI).

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 200μM acetylsalicylic acid, or 5μM ARP-100, or 11μM apyrase, or 30μM prasugrel active metabolite, then stimulated with DU145 demonstrate no significant effect on % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with DU145 alone; DU145 64.06±0.88%, 200μM ASA 57.23±6.77%, 5μM ARP-100 53.16±3.47%, 11μM APY 57.56±3.68%, 30μM PAM 59.88±2.20%. On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) treated with combination of 200μM acetylsalicylic acid and 5μM ARP-100 and 11μM apyrase, or 50μM eptifibatide or 0.8μM dabigatran then stimulated with DU145 demonstrate significant reduction in % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with DU145 alone; DU145 64.06±0.88%, 200μM ASA and 5μM ARP-100 and 11μM APY 44.14±6.67%, 50μM EPT 31.21%±8.49, 0.8μM DABI 0±0% (Figure 23A).

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 200μM acetylsalicylic acid, or 11μM apyrase, or 30μM prasugrel active metabolite then stimulated with DU145 demonstrate no significant effect on time to initiation of platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with DU145 alone; DU145 423.80±73.75 seconds, 200μM ASA 519.80±49.01 seconds, 11μM APY 616.30±83.43 seconds, 30μM PAM 679.0±92.81 seconds.

On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) treated with 5μM ARP-100, or combination of 200μM acetylsalicylic acid and 5μM ARP-100 and 11μM apyrase, or 50μM eptifibatide then stimulated with DU145 demonstrate significant prolongation in time to initiation of platelet aggregation compared to isolated human platelets stimulated with DU145 alone; DU145 423.80±73.75 seconds, 5μM ARP-100 871.80±164.50 seconds, 200μM ASA and

5μM ARP-100 and 11μM APY 791.70±102.0 seconds, 50μM EPT 810.20±58.18 seconds (Figure 23C).

Combined pharmacological inhibition of platelet derived MMP-2, ADP, and TXA_2, and GP IIb/IIIa, as well as, cancer cell derived thrombin inhibit DU145 TCIPA.

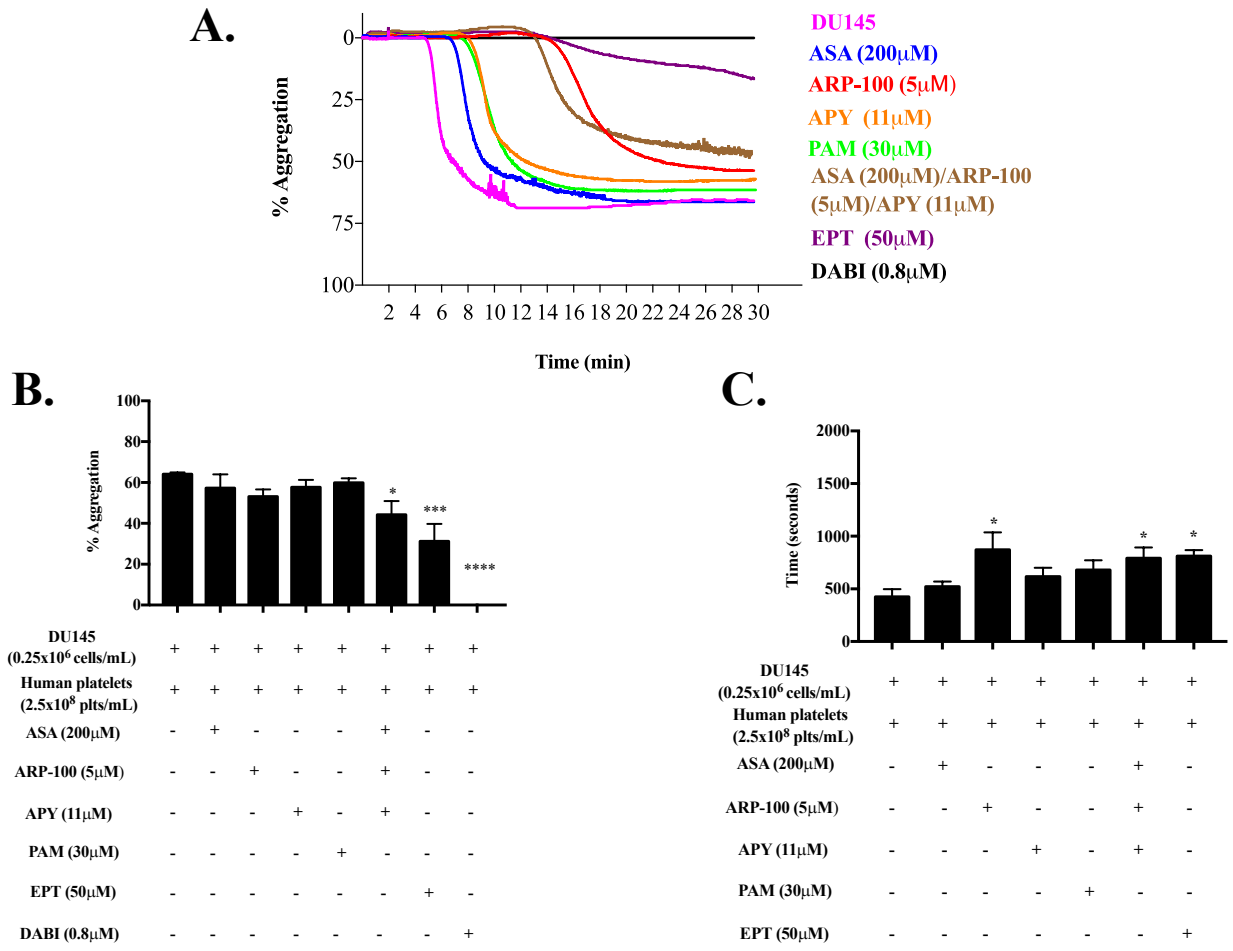


Figure 23: Contribution of platelet pathways to DU145 TCIPA mechanism. (A) Representative platelet aggregation traces of isolated human platelets stimulated with DU145 cells at $0.25x10^6$ cells/mL in the presence of acetylsalicylic acid (ASA), ARP-100, apyrase (APY), prasurgrel active metabolite (PAM), eptifibatide (EBT), and dabigatran (DABI). **(B)** Combination treatment of isolated human platelets with ASA/ARP-100/APY, EPT, or DABI then stimulation with DU145 cells demonstrate significant reduction in % aggregation compared to isolated human platelets stimulated with DU145 alone. **(C)** Isolated human platelets treated with ARP-100, ASA/ARP-100/APY, EPT demonstrate significant prolongation in time to initiation of isolated platelet aggregation. Treatment of isolated human platelets with DABI results in lack of platelet aggregation. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6, *p<0.05, ***p<0.001, ****p<0.0001.

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 200μM acetylsalicylic acid, or 5μM ARP-100, or 11μM apyrase, or 30μM Prasugrel active metabolite, then stimulated with PC3 demonstrate no significant effect on % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with PC3 alone; PC3 50.63±5.71%, 200μM ASA 48.93±4.74%, 5μM ARP-100 47.0±2.69%, 11μM apyrase 49.51±5.52%, 30μM PAM 50.23±4.11%. On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) treated with combination of 200μM acetylsalicylic acid and 5μM ARP-100 and 11μM apyrase, or 50μM eptifibatide or 0.8μM dabigatran then stimulated with PC3 demonstrate significant reduction in % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with PC3 alone; PC3 50.63±5.71%, 200μM ASA and 5μM ARP-100 and 11μM APY; 32.70±1.11%, 50μM EPT; 27.21±5.07%, 0.8μM DABI; 0±0% (Figure 30A).

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 200μM acetylsalicylic acid, or 5μM ARP-100, or 11μM apyrase, or 30μM prasugrel active metabolite, or 50μM eptifibatide then stimulated with PC3 demonstrate no significantly effect on time to initiation of platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with PC3 alone; PC3 1390.0±151.70 seconds, 200μM ASA 1392.0±133.20 seconds, 5μM ARP-100 1411±89.81 seconds; 11μM APY 1439.0±55.33 seconds, 30μM PAM 1381.0±69.13 seconds, 50μM EPT 1321.0±94.58 seconds. On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) treated with combination of 200μM acetylsalicyclic acid and 5μM ARP-100 and 11μM apyrase then stimulated with PC3 demonstrate significant prolongation in time to initiation of platelet aggregation compared to isolated human platelets stimulated with PC3 alone; PC3 1390.0±151.70 seconds, 200μM ASA and 5μM ARP-100 and 11μM apyrase; 1770±55.02 seconds (Figure 30B).

Combined pharmacological inhibition of platelet derived MMP-2, ADP, and TXA_2, and GP IIb/IIIa, as well as, cancer cell derived thrombin inhibit PC3 TCIPA.

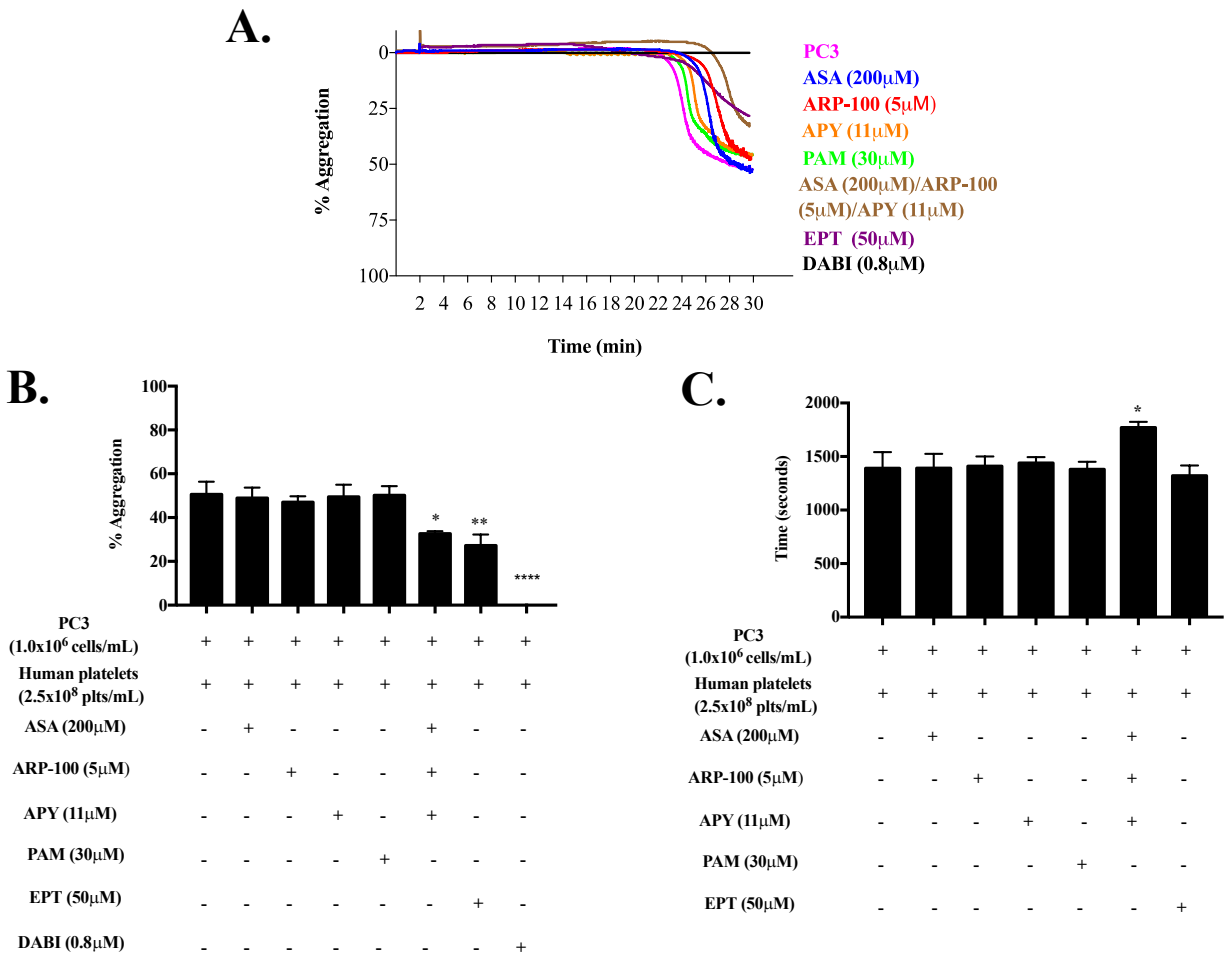


Figure 24: Contribution of platelet and PC3 released factors on TCIPA mechanism. (A) Representative platelet aggregation traces of human platelets stimulated with PC3 cells at $1.0x10^6$ cells/mL in the presence of of acetylsalicylic acid (ASA), ARP-100, apyrase (APY), prasurgrel active metabolite (PAM), eptifibatide (EBT), and dabigatran (DABI). Combination treatment of isolated human platelets with ASA/ARP-100/APY, EPT, or DABI then stimulation with PC3 cells demonstrate significant reduction in % aggregation compared to isolated human platelets stimulated with PC3 alone. **(C)** Isolated human platelets treated with ASA/ARP-100/APY or EPT demonstrate significant prolongation in time to initiation of isolated platelet aggregation. Treatment of isolated human platelets with DABI results in lack of platelet aggregation. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6, *p<0.05, **p<0.01, ****p<0.0001.

4.6. Determination of prothrombin and active thrombin expression in PCa cell lines

Since DU145, PC3, and LNCaP + 20μM bicalutamide induced TCIPA was completely abolished with dabigatran, we wanted to determine the expression of prothrombin and thrombin in PCa cell lines.

Western blot analysis of prostate cancer cellular lysates demonstrates significantly higher expression of active thrombin in DU145, PC3, and LNCaP + 20μM bicalutamide compared to RWPE. RWPE 100±0%; DU145 374.10±95.18%; PC3 279.30±15.60%; LNCaP + 20μM bicalutamide 264±33.51%. Furthermore, incubation of LNCaP cells with 20μM *in vitro* for 24-hours demonstrates significantly higher level of active thrombin expression compared to LNCaP cells alone (LNCaP 161±15.89% vs LNCaP + 20μM bicalutamide 264±33.51%) (Figure 25). DU145, PC3, and LNCaP + 20μM of bicalutamide express significantly higher levels of pro- and active thrombin compared to RWPE.

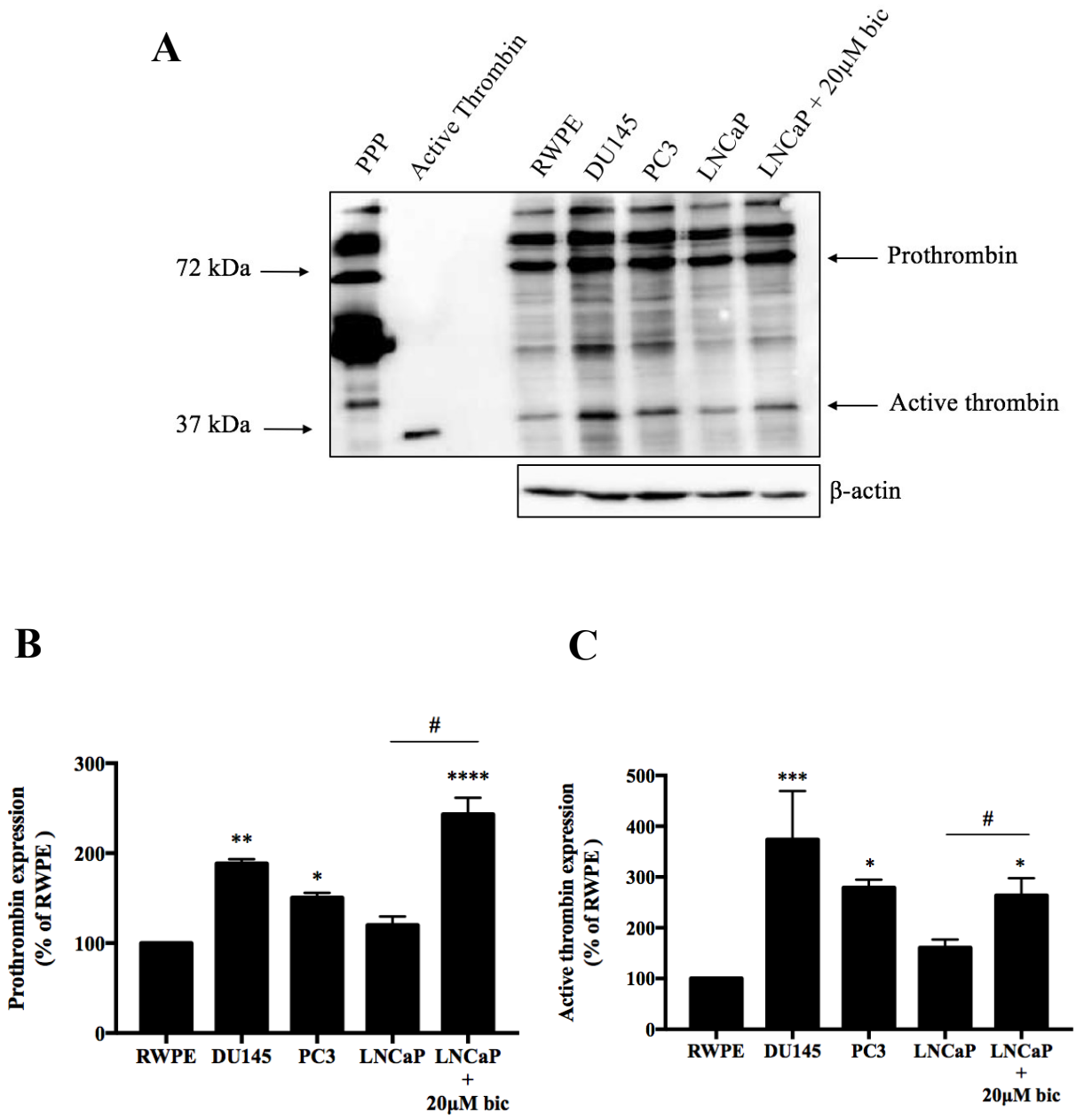


Figure 25: Expression of prothrombin and active thrombin in benign prostate and prostate cancer cell line cell lysates. Cellular lysates of prostate cancer cell lines demonstrate that DU145, PC3, and LNCaP + 20μM bic express significantly higher level of active thrombin compared to RWPE cells. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=7, **$p<0.05$. Cellular lysates of LNCaP + 20μM Bic demonstrate significantly higher level of active thrombin expression compared to LNCaP cells. Statistics: Two-tailed, paired t-test, #$p<0.05$. PRP – platelet rich plasma.

4.7. The effect of platelets on PCa MMP expression and invasion

To determine the reciprocal effect of platelets on PCa cell lines, cells were incubated with platelets then assayed for MMP expression and invasive capacity using Boyden Chamber assay Co-incubation of DU145 with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours results in significant upregulation of pro-MMP-2 in DU145 cellular lysates: DU145 AUD 368.9±43.92, DU145 + isolated human platelets ($2.5x10^6$ platelets/mL) AUD 664.4±125.6 (Figure 26A). Co-incubation of DU145 with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours results in significant upregulation of pro-MMP-9 in DU145 cellular lysates; DU145 AUD 239.1±31.72, DU145 + isolated human platelets ($2.5x10^6$ platelets/mL); AUD 664.8±143.5 (Figure 26B). DU145 exposed to isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours demonstrates significant increase in % invasion: DU145 100±0%, DU145 + isolated human platelets ($2.5x10^6$ platelets/mL) 176±20.88% (Figure 26C).

Overall, exposure of DU145 to isolated human platelets results in significant increase in pro-MMP-2 and pro-MMP-9 cell expression, and invasion *in vitro*.

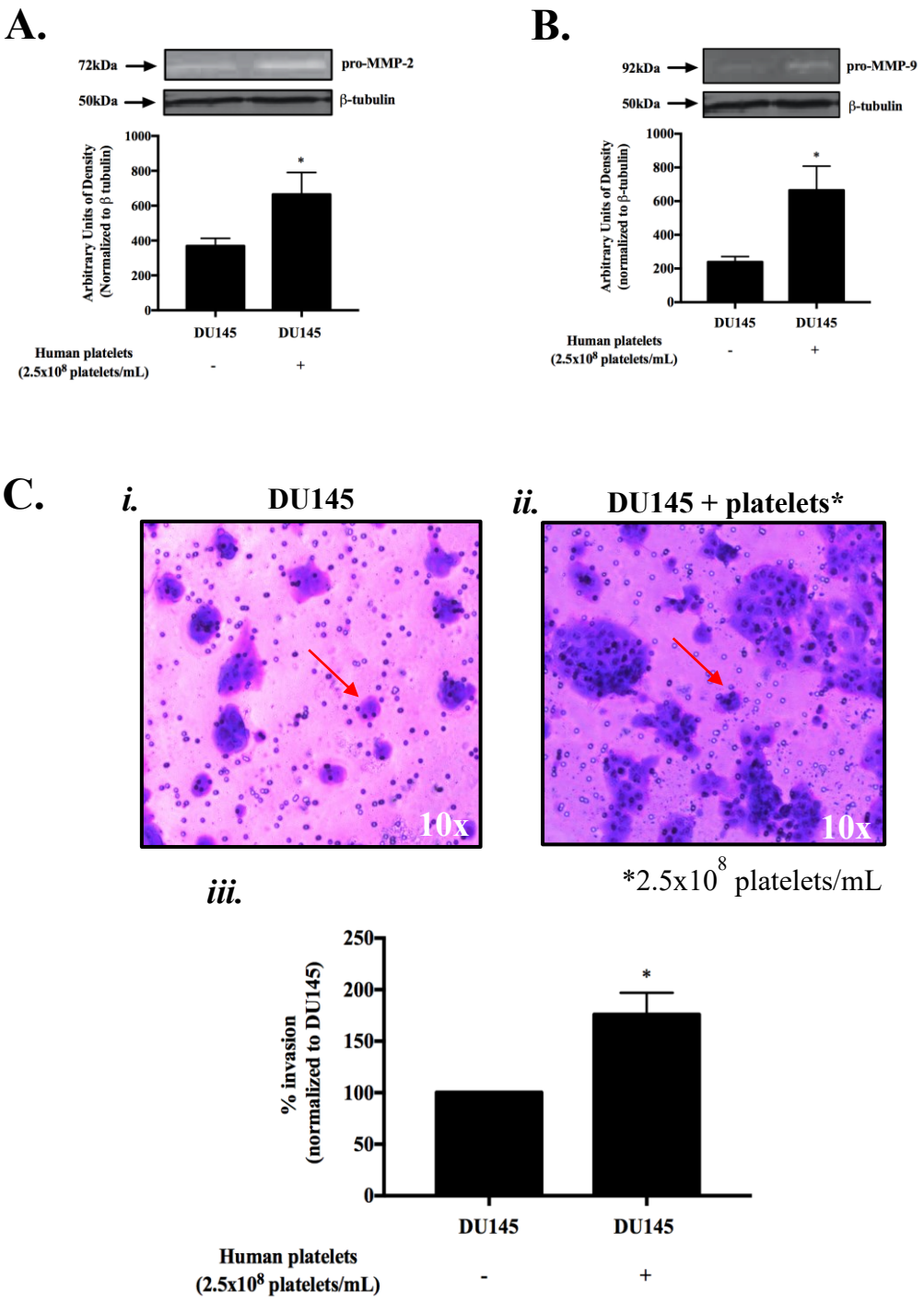


Figure 26: The effect of platelets on DU145 pro-MMP-2/9 expression and invasion. Co-incubation of DU145 cells with human platelets results in significant upregulation of **(A)** pro-MMP-2 and **(B)** pro-MMP-9 expression in cell lysates. **(C)** DU145 cells exposed to human platelets for 24 hours exhibit significant increase in % invasion compared to unexposed DU145 cells. Statistics: Two-tailed, paired t-test, N=6, *p<0.05.

Co-incubation of PC3 with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours results in significant upregulation of pro-MMP-2 in PC3 cellular lysates: PC3 AUD 564.7±186.7, PC3 + isolated human platelets ($2.5x10^6$ platelets/mL) AUD 1886±388.9 (Figure 27A).

Co-incubation of PC3 with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours results in significant upregulation of pro-MMP-9 in PC3 cellular lysates: PC3 AUD 80.83±14.93, PC3 + isolated human platelets ($2.5x10^6$ platelets/mL) AUD 502.2±105.4 (Figure 27B).

PC3 exposed to isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours demonstrates significant increase in % invasion: PC3 100±0%, PC3 + isolated human platelets ($2.5x10^6$ platelets/mL) 304.8±60.65% (Figure 27C).

Exposure of PC3 to isolated human platelets results in significant increase in pro-MMP-2 and pro-MMP-9 cell expression, and invasion *in vitro*.

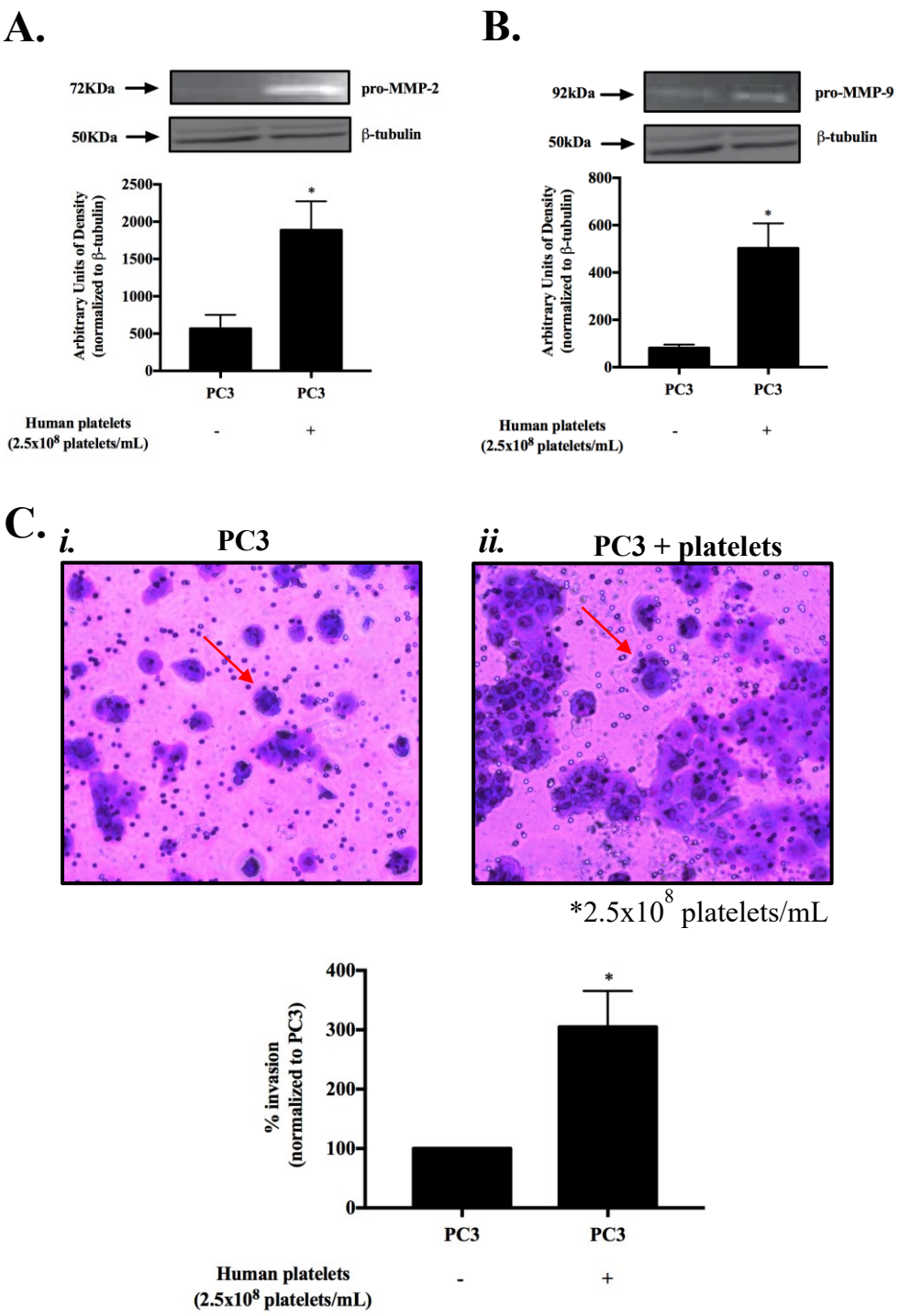


Figure 27: The effect of platelets on PC3 pro-MMP-2/9 expression and invasion. Co-incubation of PC3 cells with isolated human platelets ($2.5x10^8$ platelets/mL) results in significant upregulation of **(A)** pro-MMP-2 and **(B)** pro-MMP-9 expression. **(C)** PC3 cells exposed to isolated human platelets ($2.5x10^8$ platelets/mL) for 24 hours exhibit significant increase in % invasion compared to PC3 cells. Statistics: Two-tailed, paired t-test, N=6, *$p<0.05$.

Co-incubation of LNCaPs with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours does not result in significant upregulation of pro-MMP-2 in LNCaP cellular lysates; LNCaP; AUD 1021.0±342.3, LNCap + isolated human platelets ($2.5x10^6$ platelets/mL); AUD 1190.0±251.10 (Figure 28A).

Co-incubation of LNCaPs with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours *in vitro* does not result in significant upregulation of pro-MMP-9 in LNCaP cellular lysates; LNCaP; AUD 163.20±27.41, LNCap + isolated human platelets ($2.5x10^6$ platelets/mL); AUD 268.90±60.65 (Figure 28B).

LNCaP exposed to isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours do not exhibit significant increase in % invasion: LNCaP; 100±0%, LNCaP + isolated human platelets ($2.5x10^6$ platelets/mL) 117±25.85%.

Exposure of LNCaP to platelets does not result in significant increase in pro-MMP-2 and pro-MMP-9 cell expression, and invasion *in vitro* (Figure 28C)

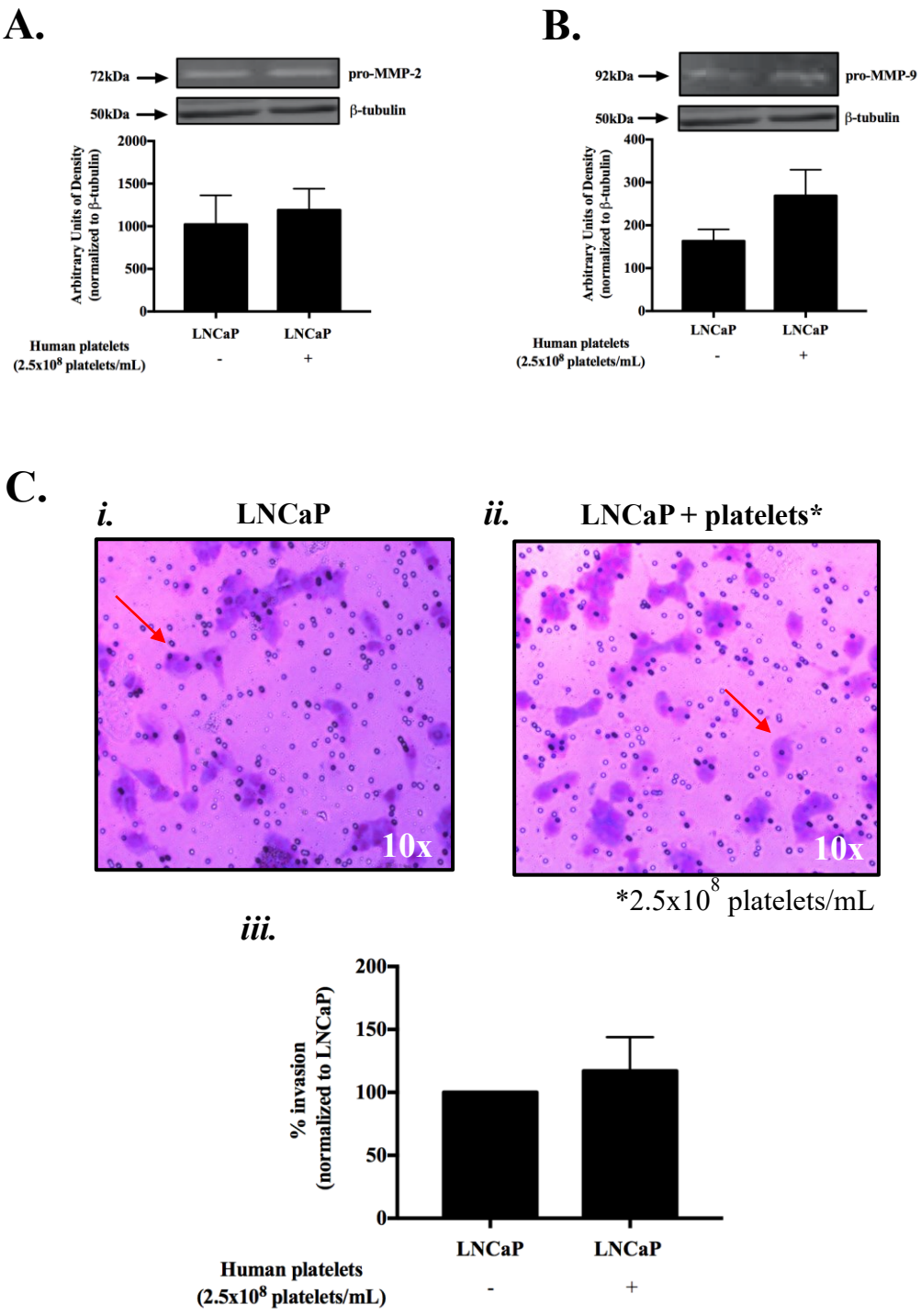


Figure 28: The effect of platelets on LNCaP pro-MMP-2/9 expression and invasion. Co-incubation of LNCaP cells with isolated human platelets ($2.5x10^8$ platelets/mL) does not result in significant upregulation of **(A)** pro-MMP-2 and **(B)** pro-MMP-9 expression in cellular lysates. **(C)** LNCaP cells exposed to human platelets for 24 hours do not exhibit significant increase in % invasion compared to unexposed LNCaP cells. Statistics: Two-tailed, paired t-test, N=6, $p>0.05$.

Co-incubation of LNCaP + 20μM bic with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours results in significant upregulation of pro-MMP-2 in LNCaP + 20μM bic cellular lysate: LNCaP + 20μM bic; AUD 1068±118.7, LNCaP + 20μM Bic + isolated human platelets ($2.5x10^6$ platelets/mL); AUD 1686±159.2 (Figure 29A).

Co-incubation of LNCaP + 20μM bic with isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours results in significant upregulation of pro-MMP-9 in LNCaP + 20μM bic cellular lysates: LNCaP + 20μM bic AUD 144.3±43.8, LNCaP + 20μM bic + isolated human platelets ($2.5x10^6$ platelets/mL) AUD 2098±81.18 (Figure 29A).

LNCaP exposed to isolated human platelets for 24-hours do not exhibit significant increase in % invasion: LNCaP; 100±0%, LNCaP + 20μM Bic + isolated human platelets ($2.5x10^6$ platelets/mL) 185±13.26% (Figure 29C).

Exposure of LNCaP + 20μM bic to platelets results in significant increase in pro-MMP-2 and pro-MMP-9 cell expression, and invasion *in vitro*.

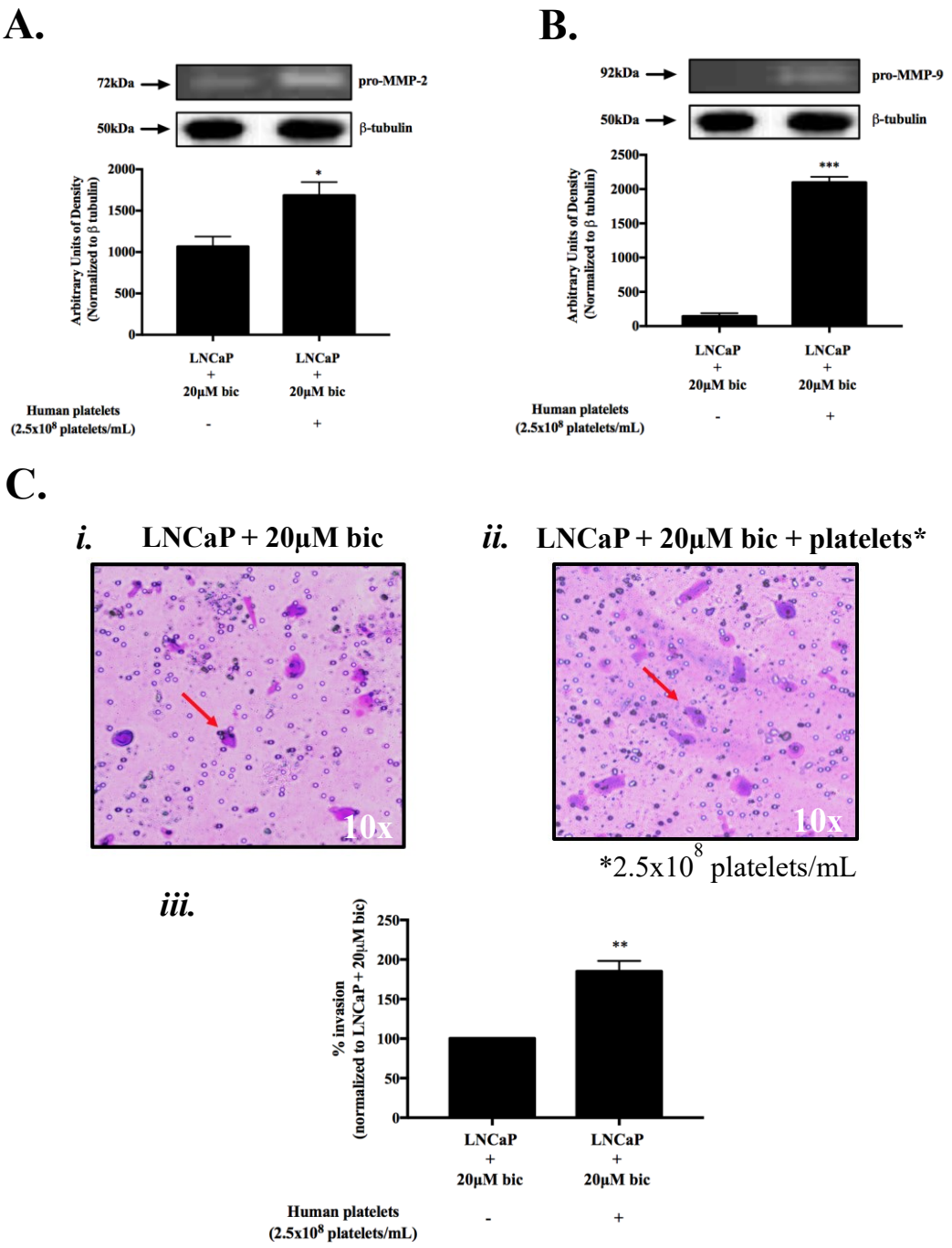


Figure 29: The effect of platelets on LNCaP + 20μM bic pro-MMP-2/9 expression and invasion. Co-incubation of LNCaP + 20μM bic with isolated human platelets ($2.5x10^8$ platelets/mL) results in significant upregulation of **(A)** pro-MMP-2 and **(B)** pro-MMP-9 expression in cellular lysates. **(C)** LNCaP+ 20μM bic cells exposed to isolated human platelets ($2.5x10^8$ platelets/mL) for 24-hours exhibit significant increase in % invasion compared to unexposed LNCaP + 20μM bic cells. Statistics: Two-tailed, paired t-test, N=6, *p<0.05, **p<0.01, ***p<0.001.

5. Discussion

In addition to their role in hemostasis, there is evidence to suggest that platelets contribute to pathophysiology of cancer metastasis. Cancer patients have increased platelet reactivity which can cause disseminated intravascular coagulation, migratory thrombophlebitis, and pulmonary embolism (Rickles FR *et al.,* 2001; Jurasz P *et al.,* 2004; Ay C *et al.,* 2008; Riedl J *et al.,* 2014). The incidence of cancer associated thrombosis is higher in patients with distant metastasis contributing to cancer associated mortality and morbidity (Khorana AA & Fine RL 2004). There are five major steps involved in metastatic dissemination of PCa with emerging body of literature suggesting that circulating human platelets contribute to these steps through TCIPA. Initially, platelets have been shown to aid in transformation of epithelial neoplastic cells into invasive and aggressive malignant phenotype by epithelial mesenchymal transformation (EMT) (Mol AJ *et al.,* 2007). Within the tumor neovasculature, platelets activate and secrete numerous factors that stabilize and prevent intra-tumoral hemorrhage (Kisucka J *et al.,* 2006; Ho-Tin-No *et al.,* 2008). Invasion and extravasation are further promoted by platelet-stimulated transcriptional up-regulation of cancer cell matrix metalloproteinase (Jurasz P *et al.,* 2004; Yan M & Jurasz P 2016). Within the circulation, TCIPA aids in the ability of the tumor cells to evade the immune system, arrest, extravasate, then grow and disseminate in the secondary sites (Nieswandt B *et al.,* 1999; Palumbo JS 2005). Furthermore, platelets store, generate, and secrete numerous pro-angiogenic growth factors, which promote successful tumor metastasis, growth at a secondary site, and even escape from apoptosis (Rickles FR *et al.,* 2001; Kopp HG *et al.,* 2009; Placke T *et al.,* 2012; Schumacher D *et al.,* 2013; Kerr BA *et al.,* 2013). There are diverse mechanisms involved in initiation of TCIPA and the metastatic potential of cancer cells is closely associated with the extent of platelet activation (Gasic GJ 1973).

Advancing PCa invading through to the surrounding tissue has the propensity to recur after local therapy. Upon further advancement, metastatic PCa (mPCa) becomes incurable and the current therapeutic paradigm is centered around controlling the disease burden to prolong overall survival. Given these limitations in prevention and treatment of mPCa, there is a need for identification of novel pathways involved in metastasis to better predict and understand the mechanisms behind the development of mPCa. Sine there is a strong body of literature suggesting participation of platelets in cancer metastasis, delineating the mechanism by which PCa induce TCIPA could be a potential avenue for identification of novel therapeutic targets with significant chance for impact. However, there is a paucity in the literature with regards to the role of platelets in PCa metastasis and even less is known about interaction of platelets with PCa during its androgen dependent (AR+) and more aggressive castrate resistant states (AR-). The main objective of this study is to understand the role of AR signaling in PCa induced TCIPA and platelet-induced PCa cell invasion. Given the more aggressive nature of hormone insensitive tumors, my hypothesis is that PCa cell lines lacking AR expression will exhibit higher platelet aggregation potency compared to cell lines with intact AR signaling.

The main findings in our study are: 1) hormone sensitive (AR+) cell lines such as RWPE and LNCaP do not induce platelet activation and aggregation, whereas, 2) hormone independent (AR-) cell lines such as DU145 and PC3 activate and aggregate platelets 3) pharmacological inhibition of eNOS in LNCaP demonstrate no effect on TCIPA, whereas, inhibition of AR induces platelet activation and aggregation 4) Platelet derived pro-MMP-2 does not play a major role in DU145, PC3, and LNCaP induced TCIPA 5) DU145 have significantly higher aggregation potency compared to PC3 and LNCaP+bic 6) thrombin plays a major role in DU145, PC3 and LNCaP+bic induced TCIPA 7) DU145, PC3, and LNCaP+bic express significantly higher level

of prothrombin and thrombin compared to RWPE 8) Exposure of LNCaP to bicalutamide results in significant upregulation of prothrombin and thrombin compared to LNCaP, and 9) platelets enhance DU145, PC3, and LNCaP+bic invasion, as well as, MMP-2 and 9 expression, but not that of LNCaP. Overall, these findings suggest that AR plays an important role in PCa TCIPA perhaps through its influence on prothrombin and active thrombin expression. Reciprocally, platelets enhance invasion of AR-negative and AR-inhibited PCa cells by increasing their MMP-2 and 9 expression.

In the first set of experiments, we validated our cell line model by demonstrating detectable AR in LNCaP and lack of AR expression in DU145 and PC3. This finding is concordant with previously published reports characterizing the molecular profile of commercially available PCa cell lines (Stone KR *et al.,* 1978; Kaighn ME et al., 1979; Horoszewicz JS *et al.,* 1980; Connolly JM & Rose DP 1990). Androgen insensitivity in advanced PCa can occur in the presence of functional AR or complete loss of AR expression (Kinoshita H *et al.,* 2000; Culig Z & Bartsch G 2006; Bonaccorsi L *et al.,* 2008). In our study, we utilized the AR lacking cell lines such as DU145 and PC3 to represent the AR independent state of PCa. In a subsequent experiment, we showed that androgen insensitive DU145 are the most invasive prostate cancer cell line *in vitro*. In support of this data, there is evidence suggesting that downregulation of AR levels in AR expressing cells enhances PCa cell invasion *in vitro* and *in vivo* (Niu Y *et al.,* 2008). In addition, restoring AR expression in PC3 results in decreased invasion in bone lesion assays and *in vivo* mouse models (Niu Y *et al.,* 2008). This was further corroborated by Huo and colleagues where AR was re-expressed in PC3 which resulted in suppression of motility, migration, and invasion (Huo C *et al.,* 2015). In a study by Moroz and colleagues, the authors demonstrate similar findings by demonstrating higher invasive potential of DU145 compared to PC3 and LNCaP in a matrigel

invasion assay (Moroz A *et al.,* 2013). In our experimental model, LNCaP reflect early stage PCa based on their expression of AR, whereas, DU145 and PC3 are representative of advanced stage PCa based on their metastatic origin, higher invasive potential, and lack of AR expression (Kaighn ME *et al.,* 1979; Horoszewicz JS *et al.,* 1983; Nunlist EH *et al.,* 2004). The use of multiple PCa cell lines with differences in AR expression and metastatic origin, allowed us to investigate the interaction between platelets and PCa cells along the spectrum of PCa progression.

To study the role of AR signaling in PCa induced TCIPA, we utilized a nonsteroidal antiandrogen, bicalutamide, to inhibit AR within LNCaP. Our data suggest that exposure of LNCaP to bicalutamide for 24 hours did not impact cellular viability nor the invasive potential when compared to LNCaP. This finding could be attributed to short duration of exposure to the anti-androgen treatment. Our experimental goal was to delineate the role of AR signaling on TCIPA through pharmacological disruption of AR in LNCaP while ensuring minimal impact of AR inhibition on cellular viability. Therefore, to balance this fine line, we chose to expose LNCaPs to high *in vitro* dose of bicalutamide over a short period of time.

The first major finding in our study was that both benign and cancerous hormone sensitive (AR+) cell lines such as RWPE and LNCaP do not induce platelet activation and aggregation. The lack of platelet aggregation by benign RWPE was expected, however, lack of TCIPA induction by LNCaP at very high cellular concentrations was unanticipated. On the other hand, hormone independent (AR-) DU145 and PC3 readily activate and aggregate platelets. Overall, this data suggests that perhaps PCa in its hormone refractory phase has greater impact on TCIPA compared to when it is in a castrate sensitive state. Furthermore, within the AR independent PCa cells, platelet aggregation potency was significantly higher in DU145 compared to PC3, possibly implying that the location of metastatic deposit and the invasiveness of AR lacking cells may play

a critical role in TCIPA potency. Similarly, to highlight the link between invasive capacity and platelet aggregation, Jurasz P and colleagues demonstrated that highly invasive fibrosarcoma HT1080 have high platelet aggregation potency *in vitro* compared to less invasive small cell lung carcinoma cell line, A549 (Jurasz P *et al.,* 2001). To further delineate whether the observed differences in TCIPA were associated with AR signalling, we demonstrated that exposure of LNCaP to bicalutamide results in induction of TCIPA. This is an important and novel observation highlighting the role of AR signalling in the ability of PCa cells to activate ad aggregate platelets.

Having established that PCa cell lines induce TCIPA, we then proceeded to study the some of the common molecular mechanisms responsible for this interaction. Previous *in vitro* studies with other adenocarcinoma cell lines, MCF-7 and Caco-2, as well as, non-adenocarcinoma cells A549, and HT1080 demonstrate that MMP-2 dependent pathway is critical for TCIPA (Jurasz P *et al.,* 2001; Alonso-Escolano D *et al.,* 2004; Medina C *et al.,* 2006;). In addition, MMP-2 was also suggested to play a role in increased platelet reactivity in patients with metastatic prostate adenocarcinoma (Jurasz P *et al.,* 2003). Similarly, in our current study, we have shown that MMP-2 is released during DU145 and PC3 TCIPA, however, despite lack of platelet aggregation, LNCaP cell line also induces platelet pro-MMP-2 secretion. The proposed lack of platelet aggregating activity despite release of pro-MMP-2 when LNCaP are added could be attributed to lack of its conversion to active MMP-2, high level of TIMP release, or the presence of a more predominant pathway involved in inhibition of platelet activation such as release of NO. In addition, we also found that PCa induced platelet aggregation did not result in significant release of pro-MMP-9, suggesting that pro-MMP-2 is the dominant gelatinase released from platelets. The limitation of this of the study is that we did not explore the role of other pro-aggregatory MMPs including MMP-1, MT1-MMP, nor the expression or activity of TIMP in PCa cell line induced TCIPA.

To explain the lack of aggregation by LNCaPs we explored the role of cancer cell derived NO pathway in inhibition of platelet aggregation. Furthermore, NO has been shown to inhibit HT1080 induced TCIPA. It has been shown that eNOS, which is the enzyme responsible for NO production, is highly expressed in LNCaP but not in DU145 and PC3. However, pre-treatment of LNCaPs with an eNOS inhibitor, L-NAME, did not result in induction of platelet aggregation. Therefore, we suggest that lack of TCIPA by LNCaP is not associated with inhibition of platelet aggregation by tumor cell derived NO.

To further explore the precise mechanism behind LNCaP+bic, DU145, and PC3 induced TCIPA, major platelet activating pathways were pharmacologically inhibited. Combined disruption of platelet derived ADP, MMP-2, and TXA_2 resulted in attenuation of DU145 and PC3 TCIPA. Similar interactions of these major pathways of platelet activation was observed in HT1080 induced TCIPA (Jurasz P *et al.,* 2001). Antagonism of GP IIb-IIIa with eptifabatide also resulted in reduction in the extent of TCIPA and prolonged time to initiation of LNCaP+bic, DU145, and PC3 induced TCIPA. The lack of a complete inhibition of platelet aggregation by eptifabatide could be attributed to the fact that only one concentration was tested and perhaps higher doses would achieve a more profound effect on TCIPA. In addition, eptifabatide blocks the final pathway of platelet aggregation rather than the initial stimulus inciting platelet activation. Therefore, even in the presence of eptifabatide, it is possible that once platelets are activated, exposure of α-granules derived receptors such as P-selectin may also contribute to PCa TCIPA. The most profound effect on LNCaP+bic, DU145, and PC3 induced TCIPA was observed with addition of a direct thrombin inhibitor, dabigatran. Taken together, these findings suggest that thrombin has a predominant role in AR independent and AR inhibited PCa induced TCIPA. Interestingly, in a similar study by Swaim MW and colleagues, PC3 induced TCIPA in platelet

rich plasma was completely inhibited by a natural thrombin inhibitor, hirudin, also implicating thrombin as a main player in PCa induced TCIPA (Swaim, M *et al.,* 1996). The novelty of our study lies in the fact that we suggest a link between AR signaling and thrombin induced PCa TCIPA.

Since addition of dabigatran completely abolished PCa induced TCIPA, we proceeded to explore the level of prothrombin and thrombin expression in benign RWPE and PCa cell lines. Overall, AR independent DU145, PC3 and AR inhibited LNCaP+bic demonstrate significantly higher level of prothrombin and thrombin expression compared to RWPE. Furthermore, disruption of AR signaling with bicalutamide in LNCaP resulted in upregulation of prothrombin and thrombin expression. Taken together, these observations suggest that prothrombin expression is suppressed by AR signaling. Furthermore, perhaps upon PCa progression to AR independent stage with loss of AR, there is marked increase in prothrombin and thrombin generation by PCa cells. Kohli and colleagues suggest that both localized and advanced PCa tissue is a rich reservoir of thrombin based on immunohistochemical analysis and real time polymerase chain reaction (PCR) of PCa patient biopsy samples (Kohli, M *et al.,* 2011). In a study by Al Saleh and colleagues, DU145 overexpressing EGFRvIII are shown to indirectly generate thrombin through release of extracellular vesicles containing TF into the tumor microenvironment (Al Saleh H *et al.,* 2018). However, in our study we show that PCa cell can directly generate and release functional thrombin to initiate TCIPA *in vitro*. Interestingly, thrombin has also been suggested to act on PCa expressed PAR-1 to increase tumor invasion, cellular migration, angiogenesis, and metastasis (Shi X *et al.,* 2004; Tantivejkul K *et al.,* 2005). Therefore, perhaps it is not a surprise to observe higher level of thrombin expression among AR independent cell lines which have higher invasive potential. In addition to TF and thrombin expression by PCa, anti-thrombin (AT) has been shown to be

expressed by both benign and malignant prostatic tissue (Cao Y *et al.,* 2002). The role of AT has been implicated in inhibition tumor angiogenesis and growth. Cao Y and colleagues suggest that AT expression is gradually lost in tumors with high Gleason grade (Cao Y *et al.,* 2002). This finding could suggest that PCa progression is linked to gradual loss of AT which acts as an inhibitory stimulus on PCa generated thrombin resulting in higher level of active thrombin available for initiation of TCIPA. Overall, the novelty of our finding is that the level of prothrombin and thrombin expression within PCa cell lines could be regulated by AR signaling. However, these findings need to be further validated by either knockdown of AR in LNCaP with short interfering RNA or overexpression of AR in DU145 or PC3s.

Lastly, we investigated the reciprocal role of platelets on PCa cell MMP expression and invasion. Interestingly, platelets enhanced DU145 and PC3, and AR-inhibited LNCaP invasion and MMP-2 and 9 expression, however, not that of LNCaP. Similar results were observed by Alonso-Escolano D and colleagues, who demonstrated that platelets upregulate MCF-7 MMP-9 through protein kinase C δ activation which subsequently resulted in increased cancer cell invasion *in vitro* (Alonso-Escolano D *et al.,* 2006). In another experiment by Dashevsky O and colleagues, pre-incubation of prostate cancer cell line with platelet derived micro particles resulted in increased expression of MMP-2 within the cancer cells which then translated to increased invasiveness *in vitro* (Dashevsky O *et al.,* 2009). The main limitation of this set of experiments is lack of quantitative PCR data to confirm upregulation of MMP-2 and 9 mRNA within cancer cells to validate gelatin zymography findings. Nevertheless, our results suggest a novel finding that platelet induced increase in invasive capacity of PCa cell lines is dependent on the status of AR signaling. These results perhaps imply that platelets have a greater effect on increasing invasive potential of PCa in a castrate-resistant phase rather than castrate sensitive phase.

The major strength of the study is that we investigated the interaction between platelets and PCa cells along the spectrum of PCa progression by incorporating both hormone sensitive and insensitive PCa cell lines. This has allowed us to gain a more comprehensive understanding of how PCa cells activate and aggregate platelets depending on their AR expression and signalling status.

The limitation of the study is that we did not incorporate castrate resistant PCa cell lines expressing AR such as LNCaP-AI. These cell lines are generated through androgen deprivation of cell culture medium in LNCaP and require long time for expansion. Instead we focused on extreme model of castrate resistance by utilizing cells with lack AR expression. Furthermore, our TCIPA findings were not further validated under laminar flow conditions which more closely resemble blood flow through small capillaries *in vivo.* Lastly, we did not silence the expression of AR in LNCaP to validate finding observed with pharmacological inhibition of AR.

6. Concluding remarks

Overall, inhibition or loss of AR signaling within PCa results in increased thrombogenicity due to upregulation of prothrombin and thrombin expression. Reciprocally, platelets enhance invasion of AR-negative or AR-inhibited PCa cells by increasing their MMP expression. Therefore, further research into investigating the impact of silencing thrombin generation by PCa *in vivo* could pave the way for development of novel anti-metastatic therapies in PCa.

The summary of main findings are:

1) PCa cell induced TCIPA is dependent on AR expression or AR signaling.

2) Platelet derived pro-MMP-2 and 9 does not play a major role in DU145, PC3, and LNCaP induced TCIPA. Similarly, LNCaP derived NO is not a major contributor to observed lack of platelet aggregation.

3) Inhibition of AR in LNCaP results in platelet activation and aggregation.

4) DU145 has significantly higher aggregation potency compared to PC3 and AR inhibited LNCaP.

5) Thrombin appears to play a key role in DU145, PC3 and LNCaP+bic induced TCIPA.

6) DU145, PC3, and LNCaP+bic express significantly higher level of prothrombin and thrombin compared to benign prostate cells. Furthermore, AR antagonism within LNCaPs results in prothrombin and thrombin upregulation.

7) Platelets enhance DU145, PC3, and LNCaP+bic MMP expression and invasion, but not that of LNCaP.

7. Future Directions

Prostate cancer is a heterogeneous disease with multiple genetically distinct foci that may be initiated by cancer cells with stem cell-like properties. First identified in hematological malignancies, cancer stem cells (CSCs) are capable of tumor initiation, self-renewal, and differentiation into cancer cells that form the bulk of a tumor (Lapidot T *et al.,* 1994; Bonnet D 1997; Kreso A *et al.,* 2014; Pattabiraman DR & Weinberg RA 2014). CSCs have also been identified in PCa (Richardson GD *et al.,* 2004). Although contentious, numerous PCa cell lines such as DU145, PC3, and LNCaPs have also been reported to contain cells with stem-*like* properties (Wu C *et al.,* 2008). The presence of CSCs within PCa tumor bulk may provide us with insight into the understanding of tumor recurrence, the resistance to conventional therapies such as ADT, to the progression to hormone refractory disease, and metastasis. (Lapidot T *et al.,* 1994; Bonnet D 1997; Richardson GD *et al.,* 2004; Wu C *et al.,* 2008; Kreso A *et al.,* 2014; Pattabiraman DR & Weinberg RA 2014). Therefore, targeting CSCs in PCa may have significant implications in prevention of PCa dissemination.

In addition to secreting factors that promote metastasis, platelets secrete factors (mainly SDF-1α) known to mobilize bone marrow-derived hematopoietic stem/progenitor cells that increase MMP-2 and 9 expression (Heissig B *et al.,* 2002; Jin DK *et al.,* 2006; Stellos K *et al.,* 2007). There is emerging evidence which suggests that SDF-1α is one of the key regulators in PCa dissemination and maintenance of PCSCs (Dubrovska A *et al.,* 2012). Recently, Dubrovska A and collegues, demonstrated that PC3 and DU145 FACSs sorted stem-cell like CD133+ and CD44+ population expressed significantly higher level of CXCR-4 compared to CD44 and CD133 negative counterparts (Dubrovska A *et al.,* 2012). Together with our data, this suggests that a stem-cell like PCa cell subpopulation may be preferentially recruited by activated platelets which release

high levels of SDF-1α.

Therefore, I propose that selective inhibition of platelet interactions with a specific subset of prostate cancer cells (ie. CSCs) may be an effective strategy in suppressing PCa metastasis. I hypothesize that platelet-secreted SDF-1α preferentially promotes metastasis of PCSCs and inhibition of platelet-PCSC interactions by concomitant inhibition of platelet secretion and of SDF-1α:CXCR4-signalling is a novel anti-metastatic target. To test this hypothesis, I plan to pursue the following objectives:

1) To characterize DU145, PC3, LNCaP, and LNCaP+bic subpopulation based on CD133+ and CD44+ surface expression.

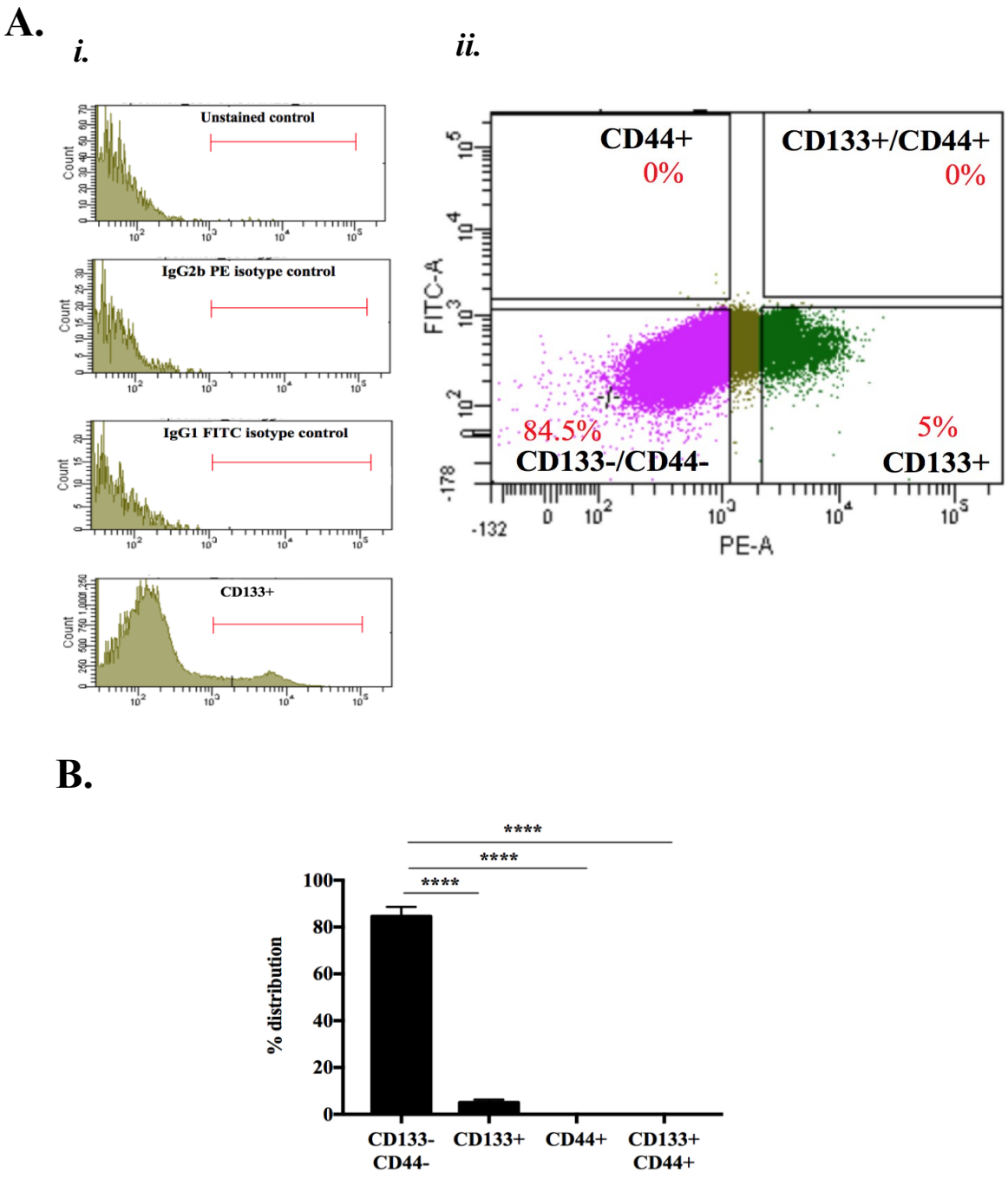
2) To characterize PCa subpopulation TCIPA to determine if CD133+/CD44+ stem-cell like subpopulation is more thrombogenic compared to CD133-/CD44-, CD133+, and CD44+ subpopulations.

3) To determine contributing factors, such as thrombin, to differences observed in subpopulation TCIPA.

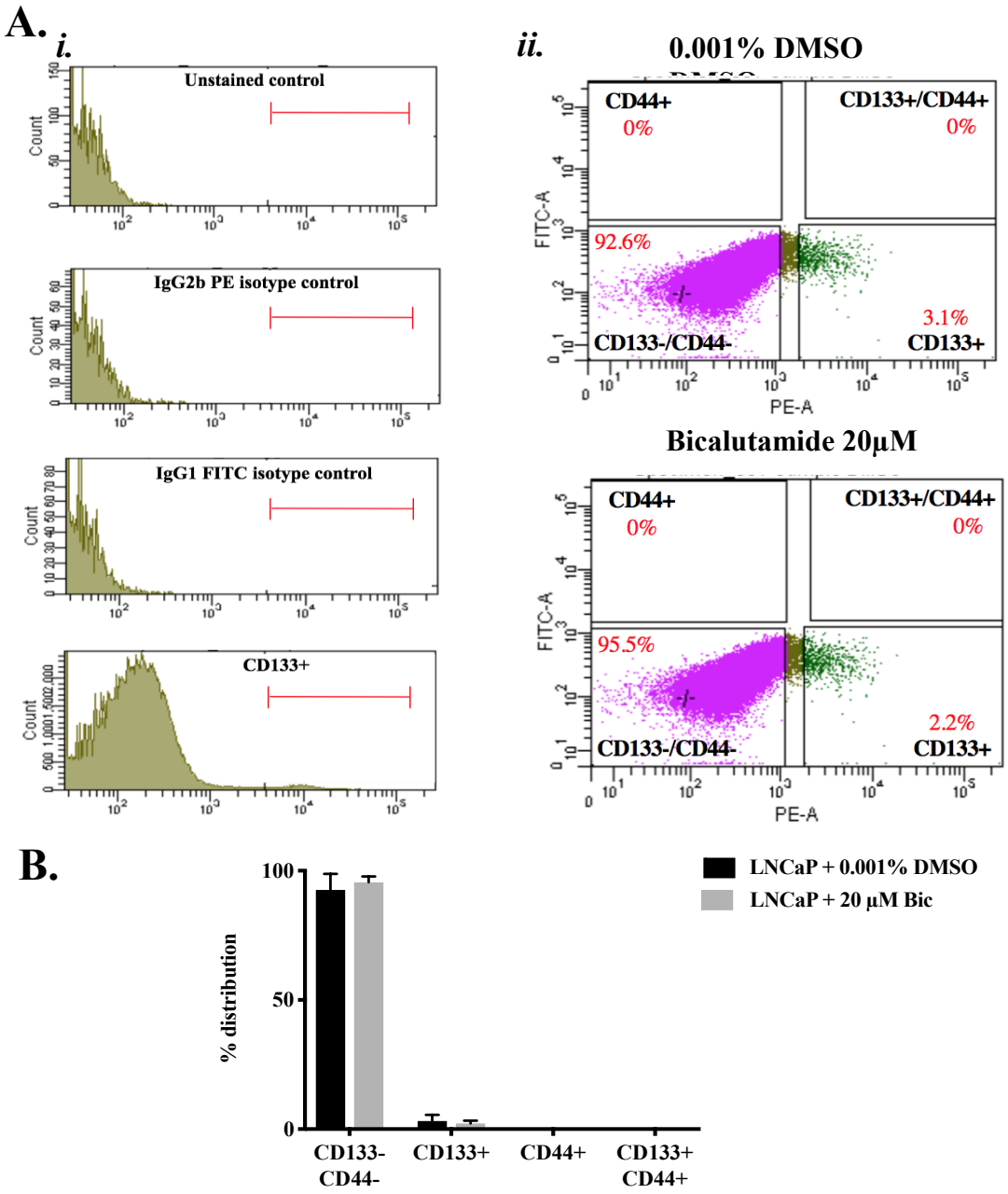
4) To determine if platelets preferentially promote PCSC invasion *in vitro*.

5) To characterize CXCR-4 surface expression in DU145, PC3, LNCaP, and LNCaP+bic.

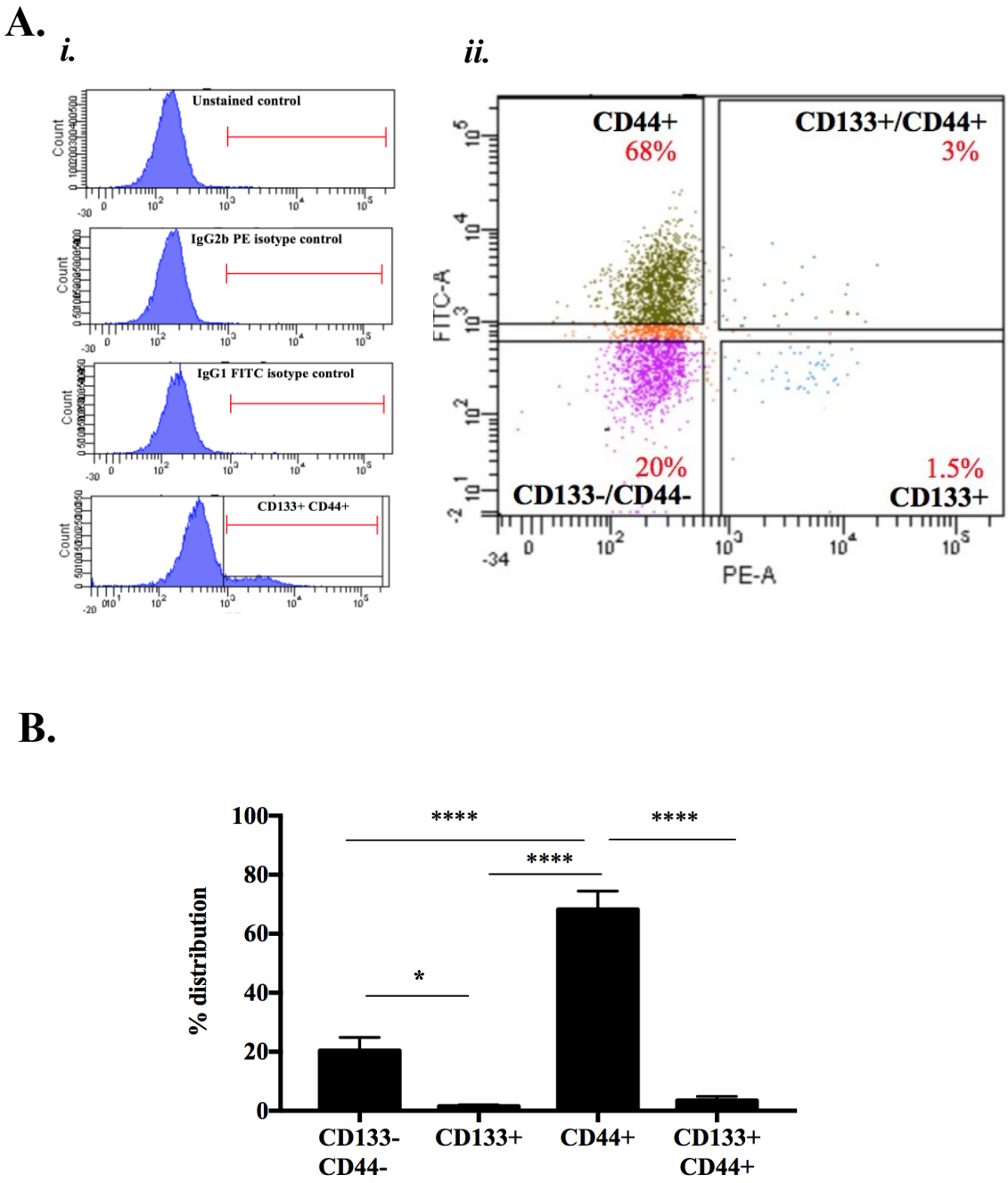
6) To investigate if inhibition of platelet-PCSC interaction and SDF-1α:CXCR4-signalling suppresses experimental invasion *in vitro.*



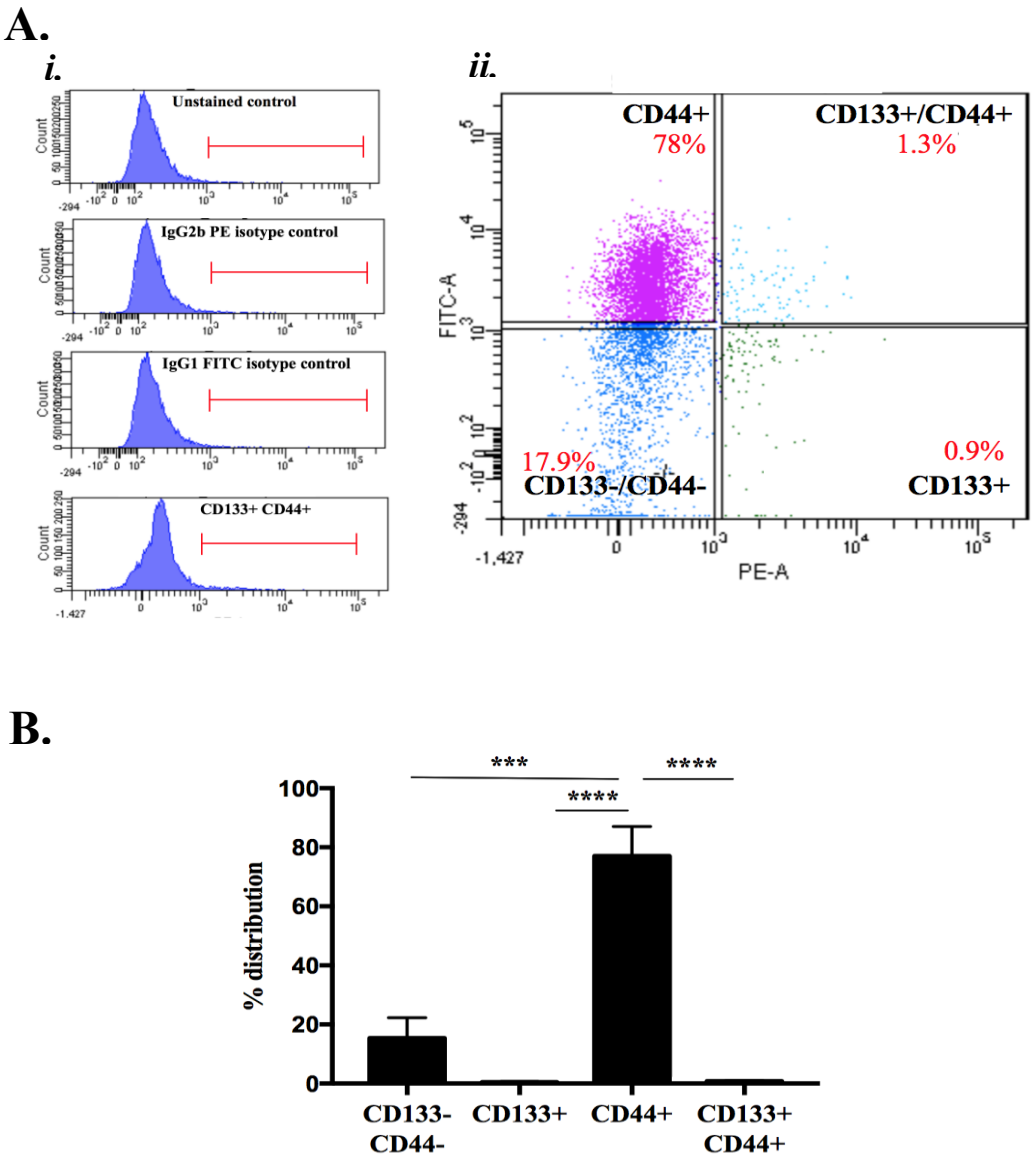
Supplementary figure 1: Characterization of LNCaP subpopulations based on CD133 and CD44 surface expression. (A-*i*) Specificity of antibodies for characterization of LNCaP subpopulation based on CD133 and CD44 surface expression. **(A-*ii*)** Representative flow cytometry histogram of LNCaP subpopulations based on CD133 and CD44 surface expression. **(B)** Significantly greater proportion of LNCaP cells are CD133-/CD44- compared to CD133+, CD44+, and CD133+/CD44+. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=4, ****p<0.0001.



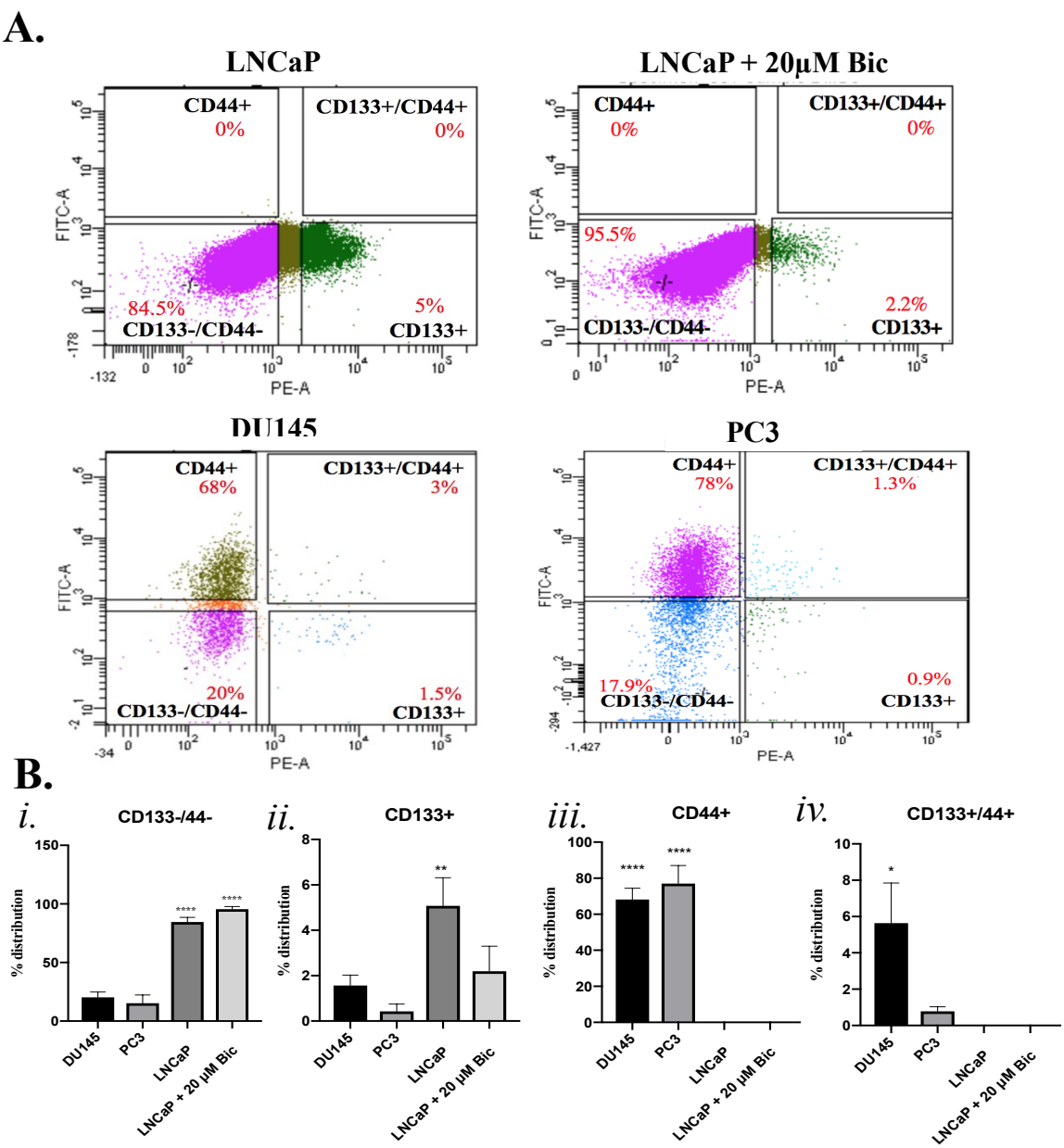
Supplementary figure 2: Characterization of LNCaP + 20µM Bic subpopulations based on CD133 and CD44 surface expression. (A-*i*) Specificity of antibodies for characterization LNCaP + 0.001% DMSO/20µM Bic subpopulation based on CD133 and CD44 surface expression. **(A-*ii*)** Representative flow cytometry histogram of LNCaP+0.001% DMSO/20µM Bic subpopulations based on CD133 and CD44 surface expression. **(B)** There are no significant differences in % distribution of cells with CD133 and CD44 surface expression between LNCaP treated with 0.001% DMSO and 20µM Bic. Statistics: Two tailed, paired, t-test N=3, $p>0.05$.



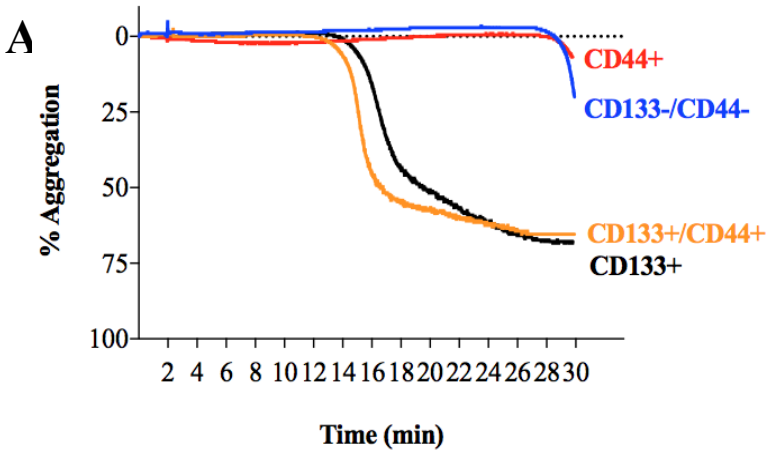
Supplementary figure 3: Characterization of DU145 subpopulations based on CD133 and CD44 surface expression. **(A-*i*)** Specificity of antibodies for characterization DU145 subpopulation based on CD133 and CD44 surface expression. **(A-*ii*)** Representative flow cytometry histogram of DU145 subpopulations based on CD133 and CD44 surface expression. **(B)** Significantly greater proportion of DU145 cells express CD44+ compared to CD133-/CD44-, CD133+, and CD133+/CD44+. In addition, there is significantly higher proportion of CD133-/CD44- compared to CD133+. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=7, *p<0.05, ****p<0.0001.

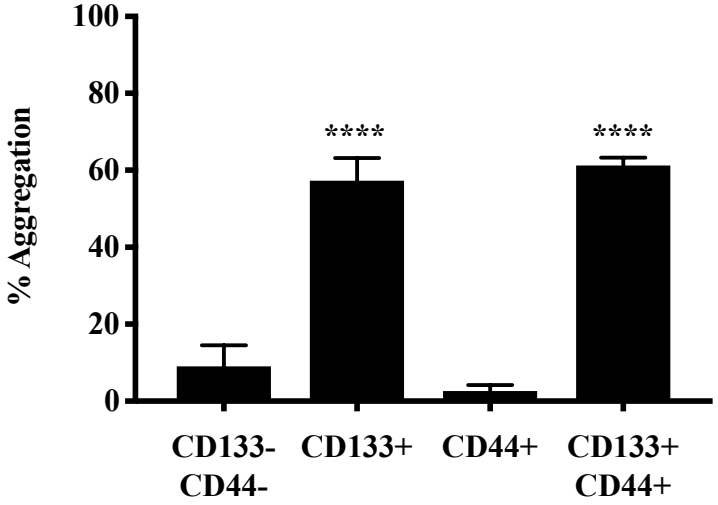


Supplementary figure 4: Characterization of PC3 subpopulation based on CD133 and CD44 surface expression. (A-*i*) Specificity of antibodies for characterization DU145 subpopulation based on CD133 and CD44 surface expression. **(A-*ii*)** Representative flow cytometry histogram of PC3 subpopulations based on CD133 and CD44 surface expression. **(B)** Significantly greater proportion of PC3 cells expressed CD44+ compared to CD133-/CD44-, CD133+, and CD133+/CD44+. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=4, ***p<0.001, **** p<0.0001.

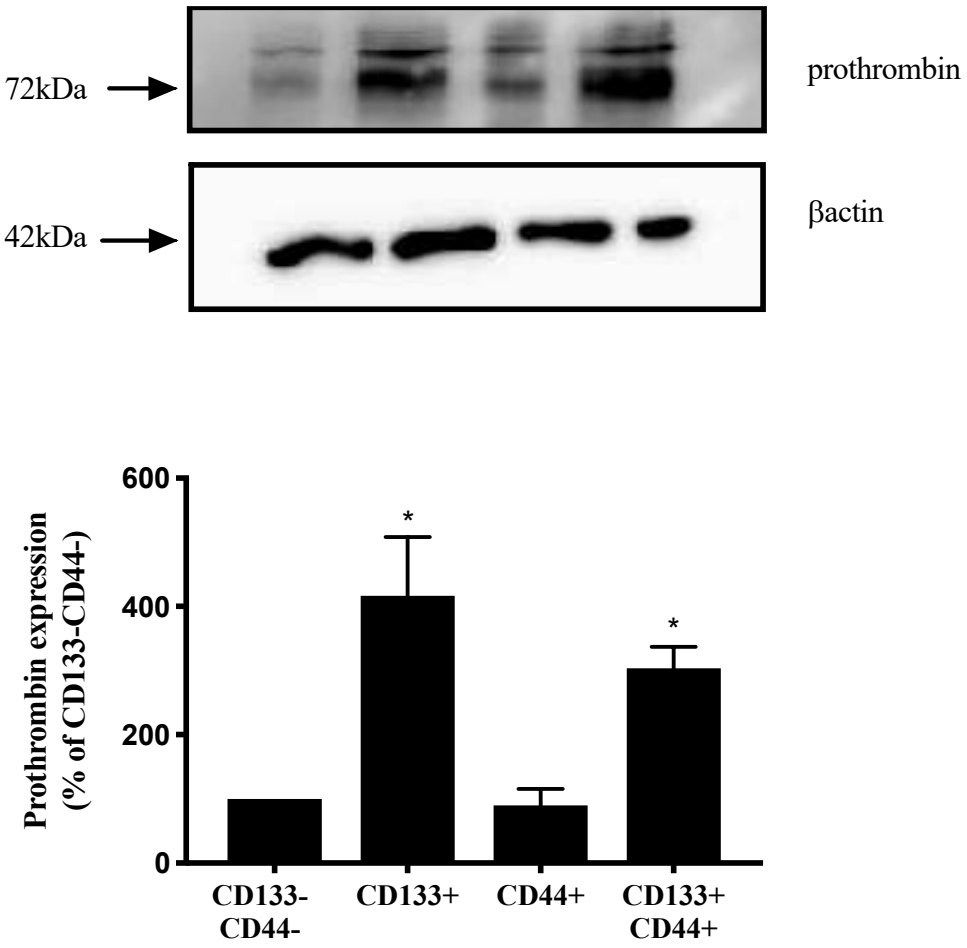


Supplementary figure 5: Comparison of prostate cancer cell line subpopulations based on CD133 and CD44 surface expression. (A) Representative flow cytometry histogram of prostate cancer cell line subpopulations based on CD133 and CD44 surface expression. **(B*i*)** Significantly greater proportion of LNCaP and LNCaP + 20µM Bic lack CD133 and CD44 surface expression compared to DU145 and PC3. **(Bii)** Significantly greater proportion of LNCaP express CD133+ alone compared to DU145, PC3 and LNCaP + 20µM Bic. **(B*iii*)** However, significantly greater proportion of DU145 and PC3 express CD44+ compared to LNCaP and LNCaP + 20µM. **(B*iv*)** Significantly greater proportion of DU145 express CD133+ and CD44+ compared to PC3, LNCaP and LNCaP + 20µM Bic. Statistics: One-way ANOVA, Tukey's multiple comparison test, *p<0.05, **p<0.01, **** p<0.0001.

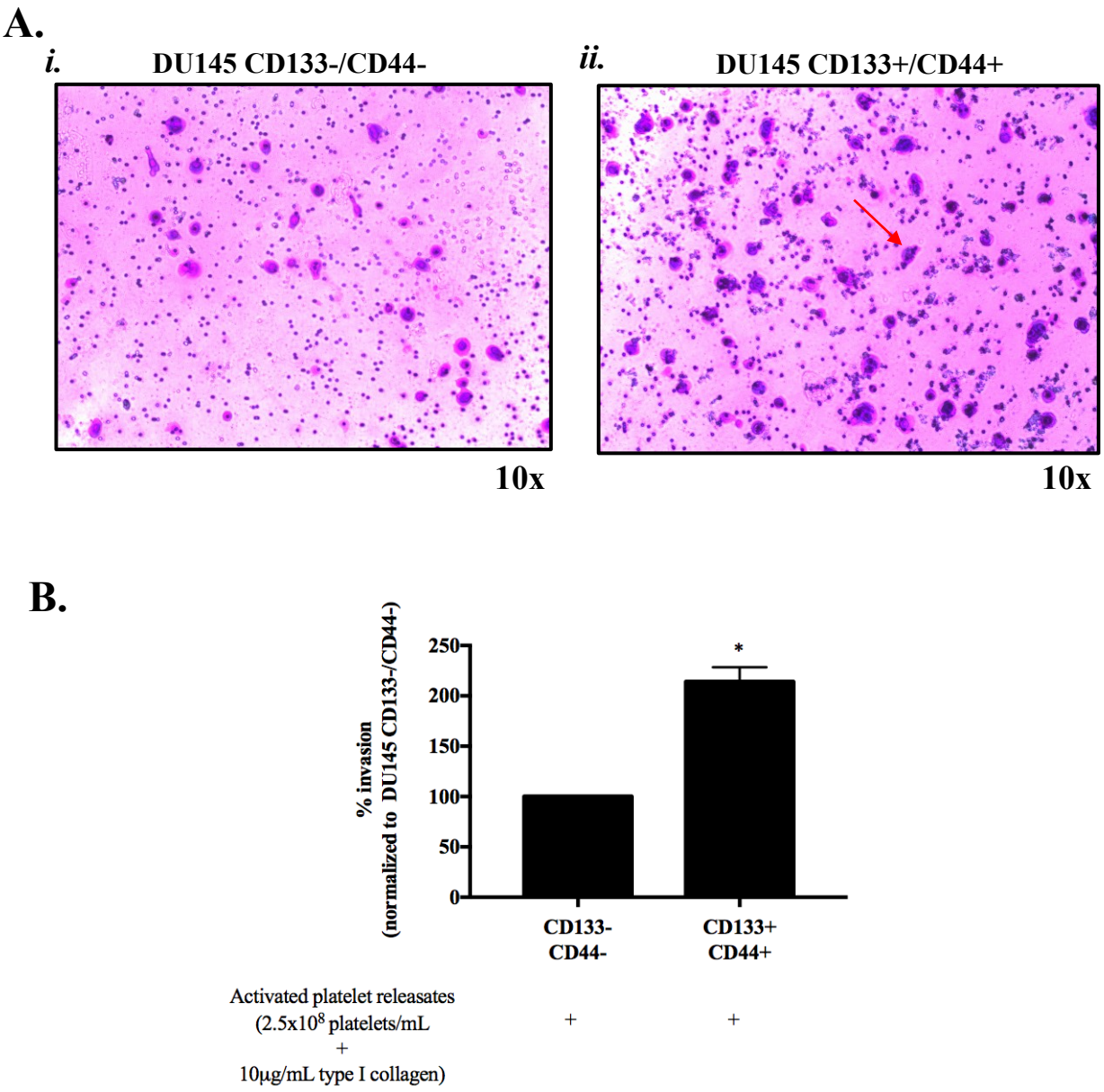




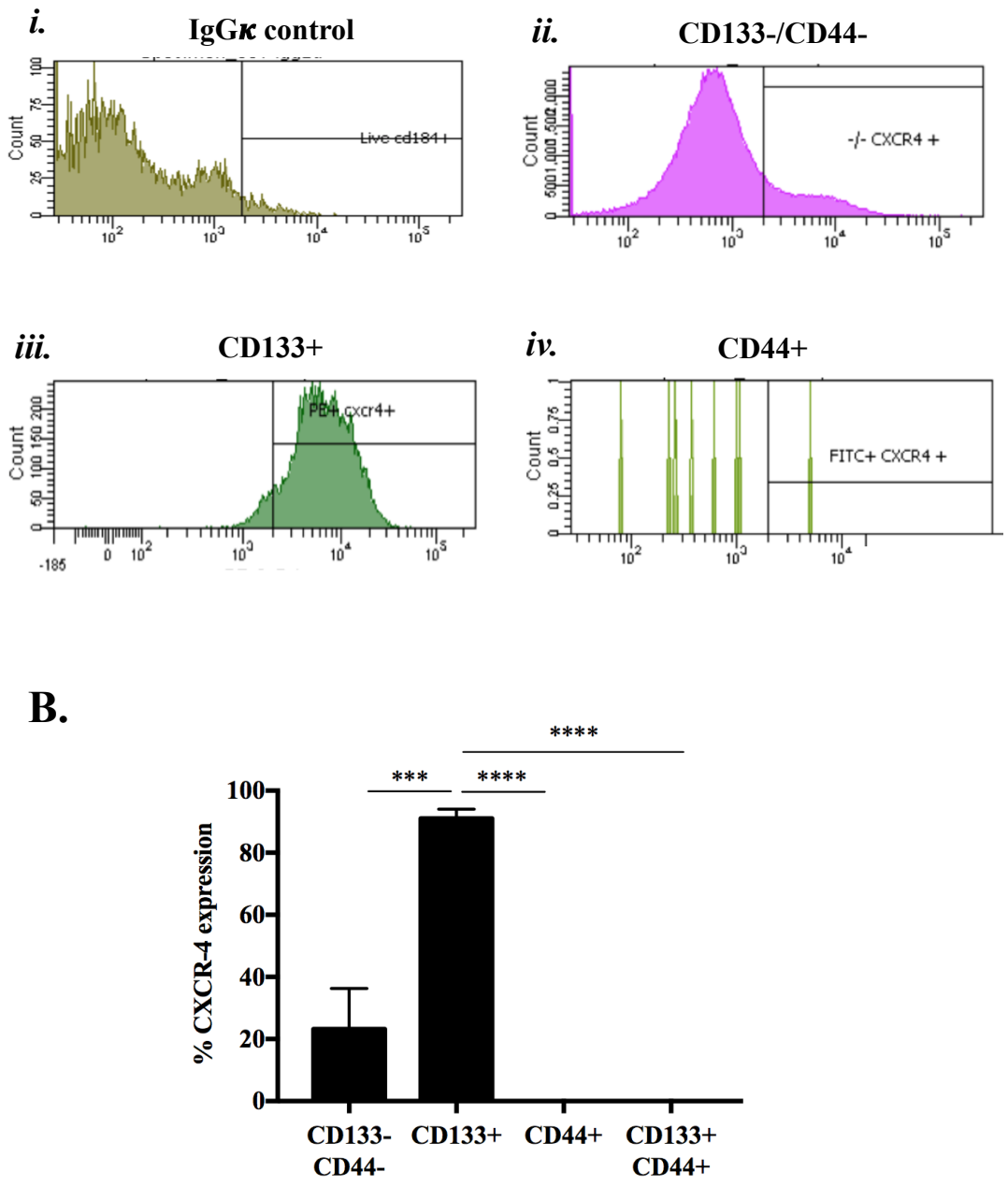
Supplementary figure 6: Characterization of DU145 subpopulation TCIPA. (A) Representative platelet aggregometry trace of DU145 subpopulations TCIPA profile. **(B)** DU145 CD133+ and CD133+/CD44+ demonstrate significantly higher % total aggregation compared to CD44+ and CD133-/CD44- at 0.001×10^6 cells/mL. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=5, ****$p<0.0001$.



Supplementary figure 7: Expression of prothrombin by DU145 subpopulations. DU145 CD133+ and CD133+/44+ subpopulations exhibited significantly higher levels of prothrombin expression compared to CD133-/44- and CD44+ subpopulations. Statistics: One-way ANOVA, Tukey's post-test, N=5, *$p<0.05$.



Supplementary figure 8: DU145 subpopulation invasion towards activated isolated platelet releasates. (A) Representative light microscopy images (10X magnification) of DU145 CD133-/CD44- and CD133+/CD44+ subpopulation migrating towards isolated activated platelet releasates. **(B)** Significantly greater proportion of CD133+/CD44+ invade and migrate through 0.1% gelatin coated Boyden Chamber towards activated isolated human platelet releasates compared to CD133-/CD44-. Statistics: Two tailed, paired, t-test N=3, *p<0.05.



Supplementary figure 9: Characterization of LNCaP subpopulation CXCR-4 surface expression. (A) Specificity of antibodies for characterization of LNCaP subpopulation CXCR-4 expression. **(B)** LNCaP CD133+ demonstrate significantly higher % expression of CXCR-4 compared to CD133-/CD44-, CD44+, and CD133+/CD44+ subpopulations. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=4, ****p<0.0001.

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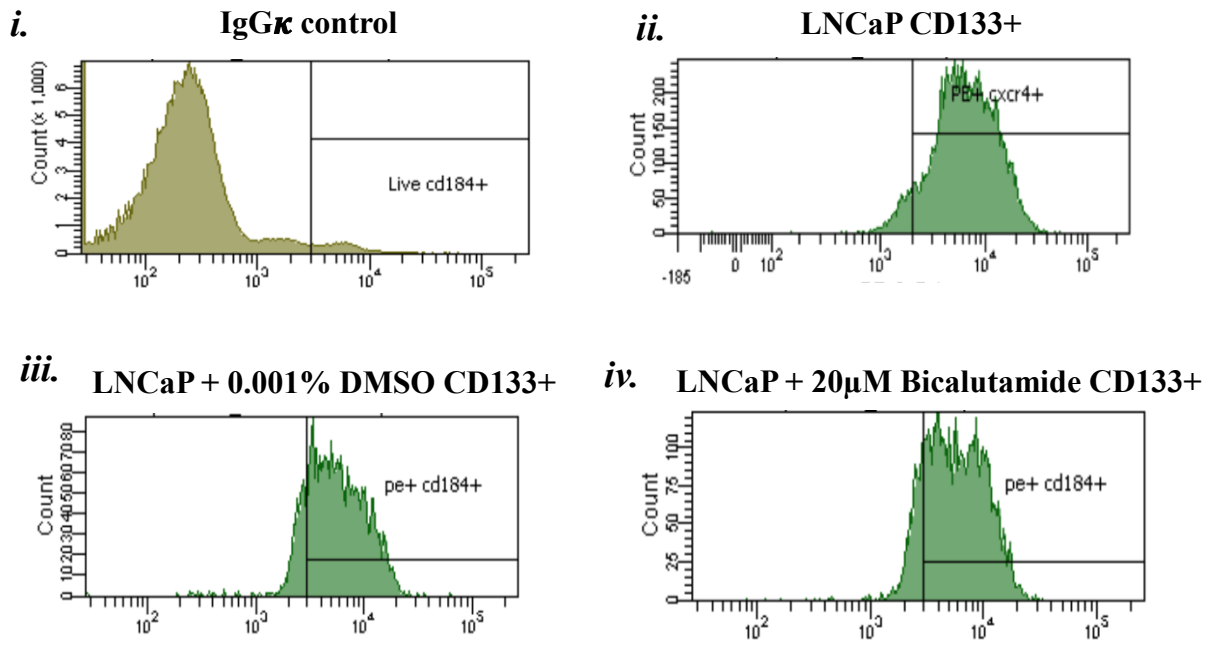

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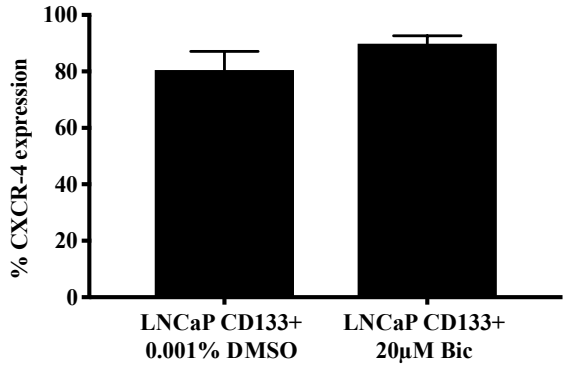

Supplementary figure 10: Characterization of LNCaP CD133+ subpopulation CXCR-4 surface expression following 0.001% DMSO or 20µM Bic exposure. (A) Specificity of antibodies for LNCaP, LNCaP + 0.001% DMSO, LNCaP + 20µM Bicalutamide CD133+ subpopulation % CXCR-4 expression. **(B)** Exposure of LNCaP to 0.001% DMSO or 20µM Bic for 24-hours *in vitro* does not have a significant effect on CD133+ subpopulation CXCR-4 expression. Statistics: Two-tailed, paired, t-test, N=3, $p>0.05$.

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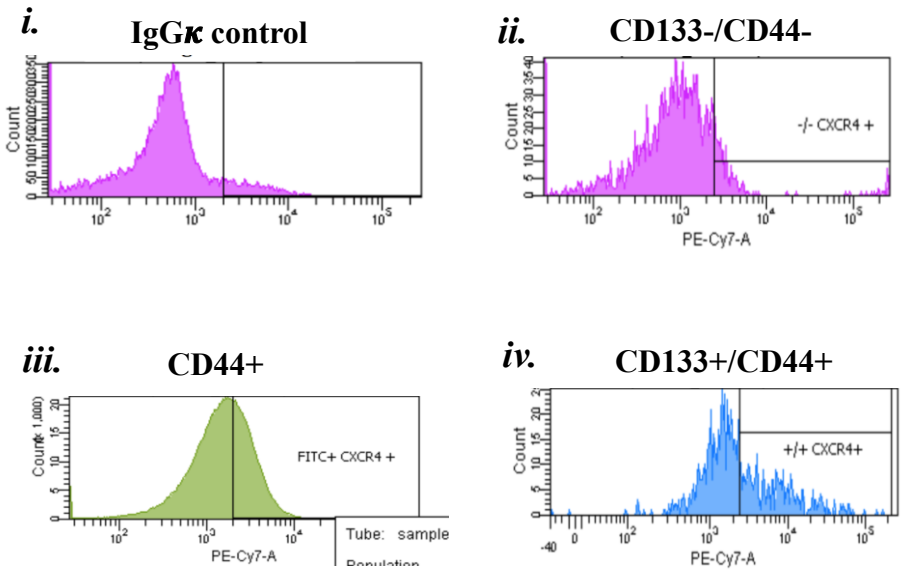

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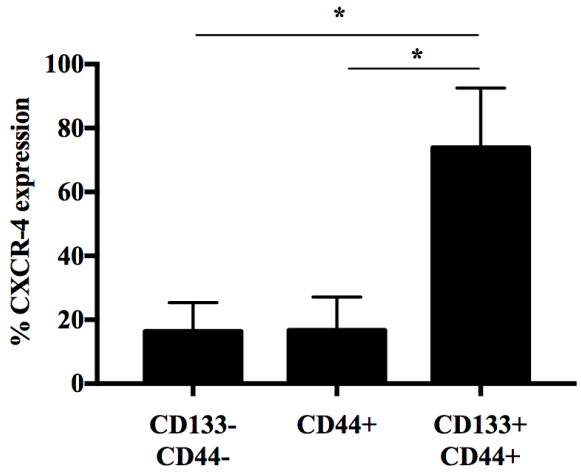
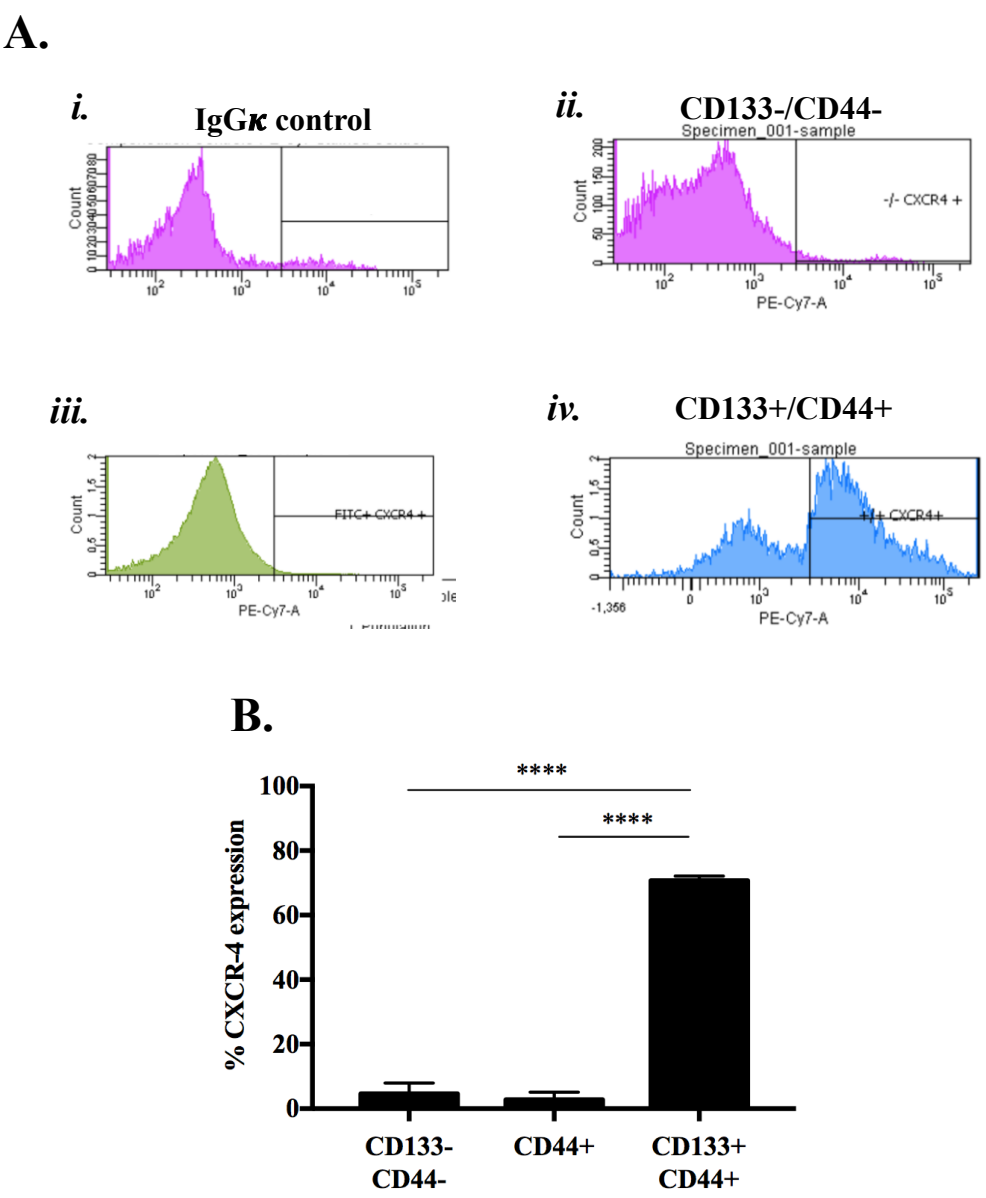
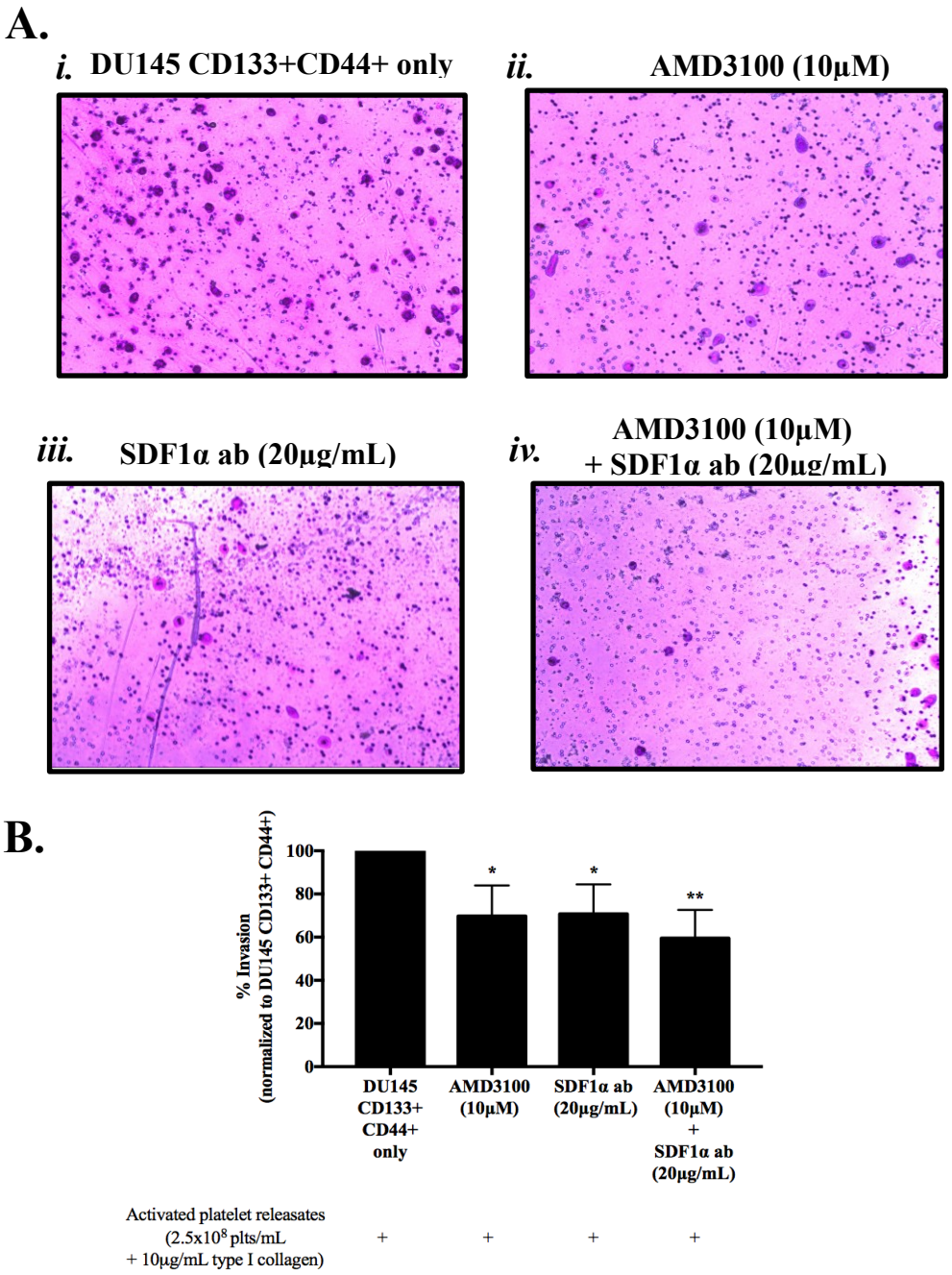


Supplementary figure 11: Characterization of DU145 subpopulation CXCR-4 surface expression. (A) Specificity of antibodies for DU145 subpopulation CXCR-4 expression. **(B)** DU145 CD133+/CD44+ cells demonstrate significantly higher % expression of CXCR-4 compared to CD44+ and CD133-/CD44- subpopulations. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=3, *p<0.05.



Supplementary figure 12: Characterization of PC3 subpopulation CXCR-4 surface expression. (A) Specificity of antibodies for PC3 subpopulation CXCR-4 expression. **(B)** PC3 CD133+/CD44+ cells demonstrate significantly higher % expression of CXCR-4 compared to CD44+ and CD133/CD44- subpopulations. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=3, ****$p<0.05$.



Supplementary figure 13: DU145 CD133+/CD44+ subpopulation invasion in response to Type I collagen activated isolated platelet releasates. (A) Representative light microscopy images (10X magnification) of DU145 CD133+/CD44+ subpopulation invading towards 10µg/mL type I collagen activated isolated platelet releasates in 1% gelatin coated Boyden chambers. AMD3100 – CXCR-4 antagonist, SDF1α ab – SDF1α neutralizing antibody. **(B)** DU145 CD133+/CD44+ cells treated with AMD3100, SDF1α ab, and AMD3100 with SDF1α ab demonstrate significantly reduced % invasion towards Type I collagen activated isolated platelet releasates compared to DU145 CD133+/CD44+. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=4, ****$p<0.05$.

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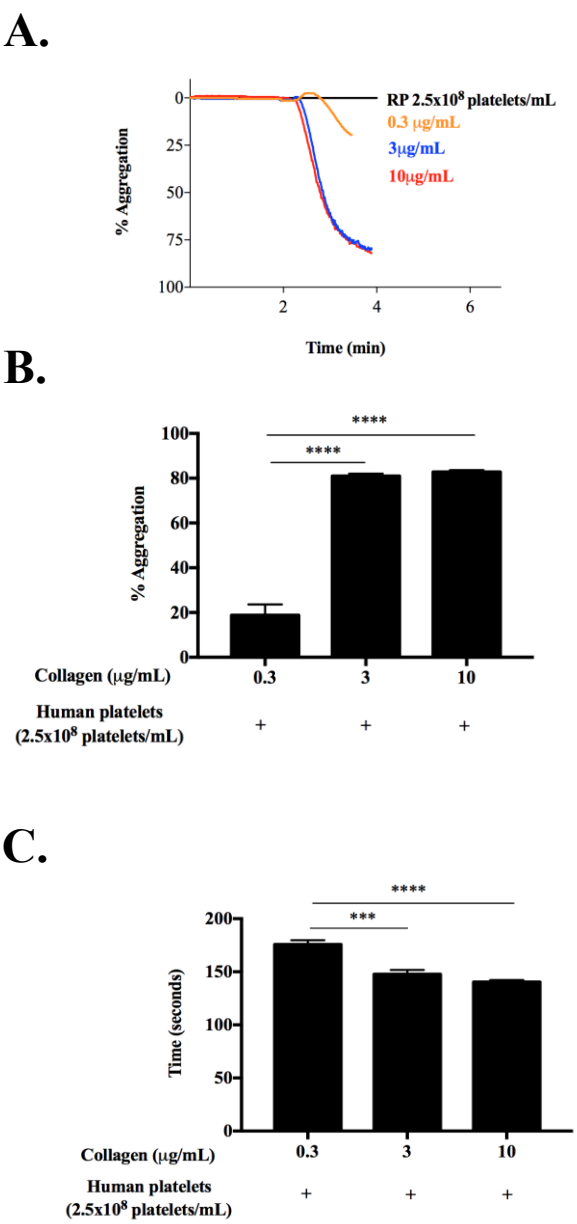
9. Appendix

Appendix 1. Type I collagen induces a concentration-dependent increase in platelet aggregation *in vitro*.

Isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with 10 µg/mL and 3 µg/mL of type I collagen demonstrate significant increase in % platelet aggregation (82.75%±0.86%, 80.97%±1.09%, respectively) compared to isolated human platelets stimulated with 0.3 µg/mL of type I collagen (18.76%±4.81%) (Figure appendix 1A).

Isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with 10 µg/mL and 3 µg/mL of type I collagen demonstrate significant shortening in time to initiation of platelet aggregation (140.40±1.80 seconds, 147.80±4.00 seconds, respectively) compared to 0.3 µg/mL (175.70±4.00 seconds) (Figure appendix 1B).

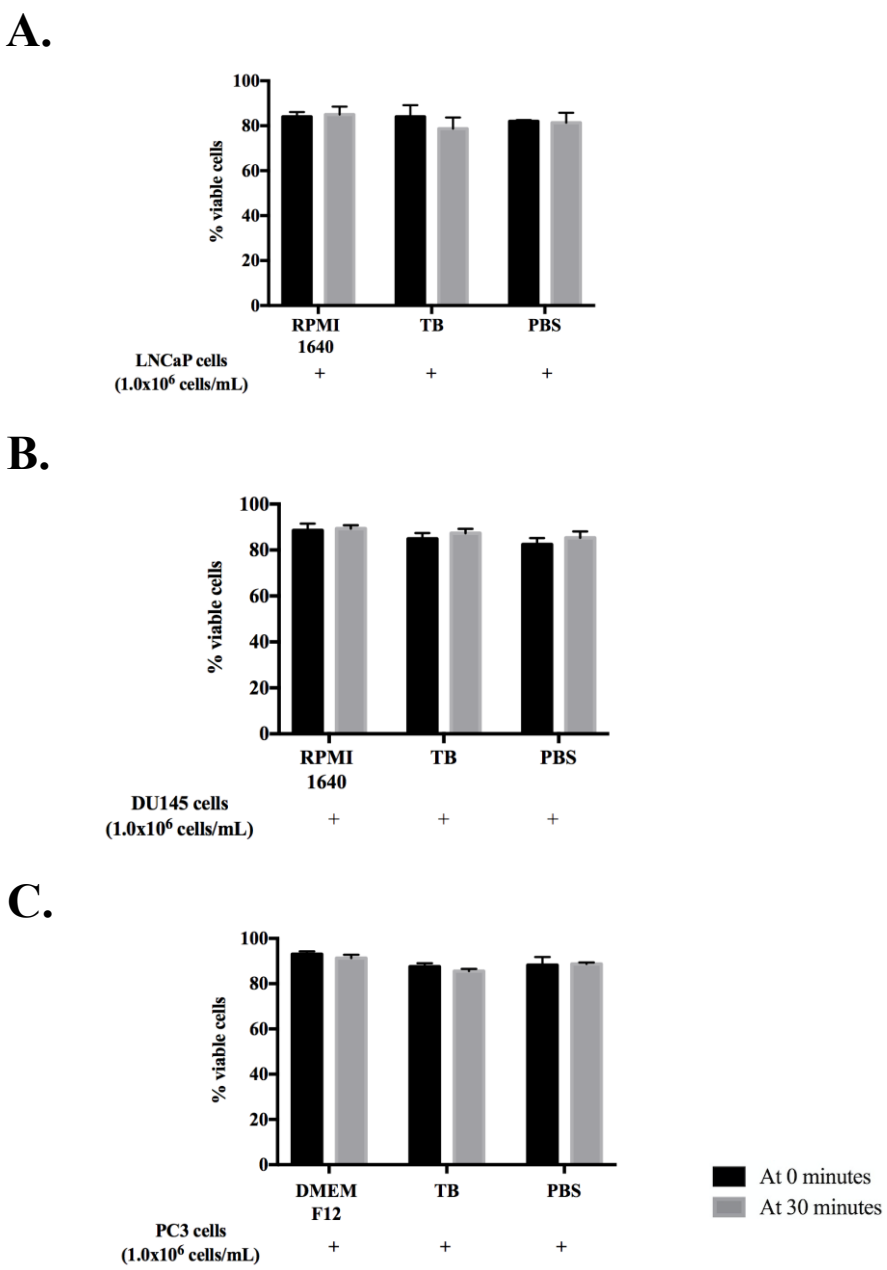


Appendix figure 1: Stimulation of isolated human platelets with type I collagen (A) Representative platelet aggregation traces of isolated human platelets stimulated with increasing concentration of type I collagen (0.3µg/mL – 10µg/mL). (B) Isolated human platelets stimulated with 3µg/mL and 10µg/mL of type I collagen demonstrated significantly higher % aggregation compared to 0.3µg/mL. (C) Isolated human platelets stimulated with 0.3µg/mL of type I collagen demonstrated significantly prolonged time to initiation of platelet aggregation compared to 10µg/mL and 3µg/mL. Time to initiation of platelet aggregation - measured as time after completion of platelet shape change. Statistics: One-way ANOVA, Tukey's multiple comparison test, N=6, ***$p<0.001$ and ****$p<0.0001$.

Appendix 2. Experimental conditions within the platelet aggregometer do not affect prostate cancer cell viability.

Trypan blue cellular viability demonstrate that duration of exposure of prostate cancer cells to experimental conditions in the platelet aggregometer (1200rpm and 37°C) and type of buffer used to suspend cells has no significant effect on prostate cancer cell viability; LNCaP RPMI 1640 (0 min 84.0±2.08% vs 30min 85.0±3.51%), Tyrode's buffer (0 min 84.0±5.13 vs 30 min 78.67±4.97%), phosphate buffered saline (0 min 82.0±0.57% vs 30 min 81.3±4.41%) (Figure 2A). DU145 RPMI 1640 (0 min 88.50±3.01% vs 30 min 89.30±1.45%), Tyrode's buffer (0 min 84.80±2.58% vs 30 min 87.30±1.85%), phosphate buffered saline (0 min 82.43±2.81% vs 30 min 85.30±2.72%) (Figure 2B). PC3 DMEM F12 (0 min 93.07±1.22% vs 30 min 91.27±1.50%), Tyrode's buffer (0 min 87.57±1.44% vs 30 min 85.57±0.97%), phosphate buffered saline (0 min 88.17±3.65 vs 30 min 88.67±0.66%) (Appendix figure 2).

Overall, these results demonstrate maintenance of prostate cancer cellular viability during the TCIPA assay.

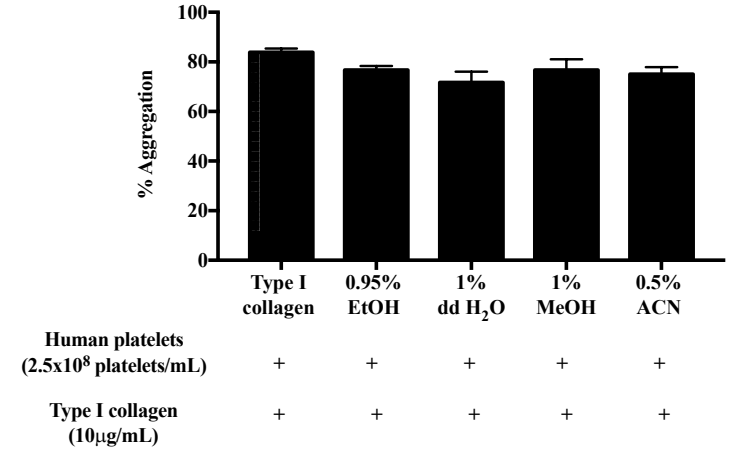


Appendix figure 2: Prostate cancer cell viability in platelet aggregometer. To ensure cellular viability during TCIPA experiments, prostate cancer cells were suspended in RPMI 1640, DMEM F12, Tyrode's buffer, or phosphate buffered saline and exposed to 1200 rpm at 37˚C. The % viable cells were compared at the start (0 min) and at the end (30 min) of the assay following exposure to experimental conditions and exposure different buffers using Trypan blue cellular viability assay. Duration of exposure to experimental conditions and type of buffer used to suspend the cells demonstrate no significant impact on **(A)** LNCaP **(B)** DU145 **(C)** PC3 cellular viability in the platelet aggregometer. Statistics: Two-way ANOVA, Sidak's multiple comparison test, N=6, $p>0.05$. TB – Tyrode's buffer, PBS – phosphate buffered saline.

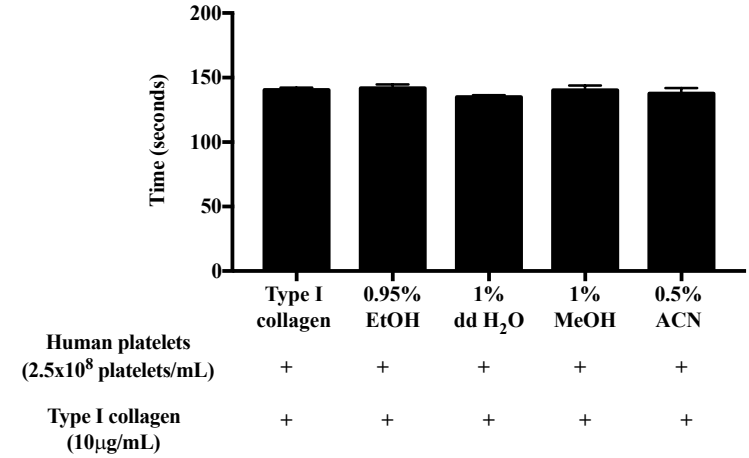
Appendix 3. Solvents of pharmacological reagents have no significant effect on type I collagen induced platelet aggregation.

Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to solvents of pharmacological reagents such as 0.95% ethanol, 1% double distilled H_2O, 1% methanol, and 0.5% acetonitrile demonstrate no significant effect on type I collagen induced % aggregation and time to initiation of platelet aggregation compared to type I collagen stimulated isolated platelets alone; 10 µg/mL type I collagen (83.90±1.48%, 140.40±1.80 seconds), 0.95% ethanol (76.67±1.66%, 141.70±3.07 seconds), 1% double distilled H_2O (71.67±4.41%, 134.80±1.62 seconds), 1% methanol (76.67±4.41%, 140.20±3.79 seconds), and 0.5% acetonitrile (75.00±2.88%, 137.50±4.42 seconds) (Appendix figure 3A-B).

A.



B.



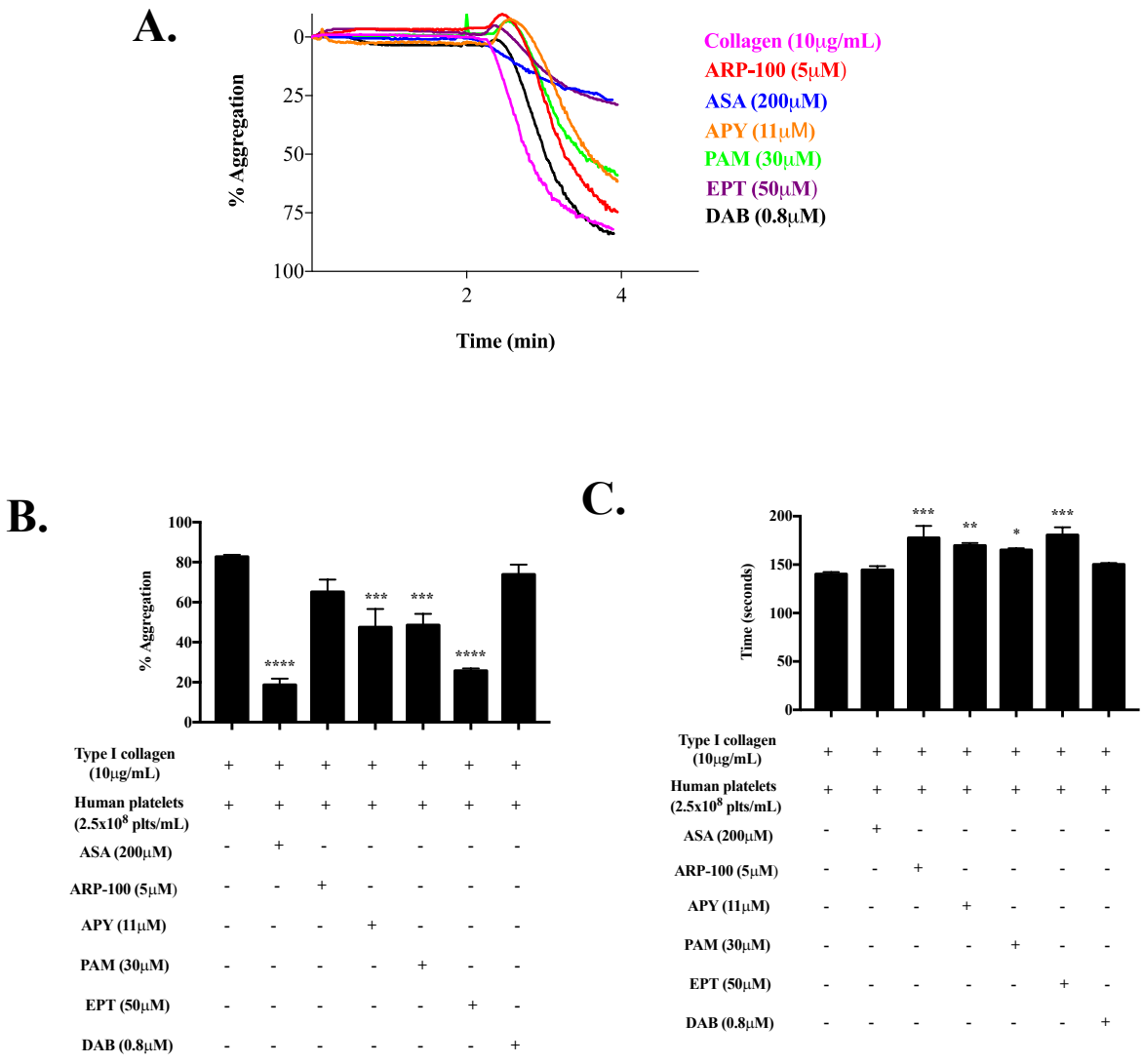
Appendix figure 3: The effect of solvents of pharmacological reagents on type I collagen induced isolated human platelet activation. (A) The solvents of pharmacological agents used demonstrate no significant effect on type I collagen induced % platelet aggregation compared to isolated platelets stimulated with type I collagen alone. **(B)** The solvents of pharmacological agents used demonstrate no significant effect on type I collaged induced time to initiation of platelet aggregation compared to isolated platelets stimulated with type I collagen alone. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=3, $p>0.05$. (EtOH - ethanol, dd H_2O - double distilled water, MeOH - methanol, and ACN - acetonitrile).

Appendix 4. Pharmacological inhibition of TXA_2, ADP, MMP-2, and GPIIb/IIIa platelet pathways attenuate type I collagen induced isolated human platelet activation.

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 200μM acetylsalicylic acid, or 11μM apyrase, or 30μM presurgrel active metabolite, or 50μM eptifibatide then stimulated with 10μg/mL of type I collagen demonstrate significant reduction in % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with type I collagen alone; 10μg/mL type I collagen; 82.75±0.86%, 200μM ASA; 18.69±3.11%, 11μM APY; 47.56±9.03%, 30μM PAM; 48.57±5.58%, and 50μM EPT; 25.77±1.14%. On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) treated with 5μM ARP-100 or 0.8μM dabigatran then stimulated with 10μg/mL of type I collagen demonstrate no significant effect on % platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with type I collagen alone; 10μg/mL type I collagen; 82.75±0.86%, 5μM ARP-100; 65.13±6.18%, 0.8μM DABI; 73.93±4.87% (Figure 23B).

Isolated human platelets ($2.5x10^8$ platelets/mL) treated with 5μM ARP-100, or 11μM apyrase, or 30μM Prasugrel active metabolite, or 50μM eptifibatide then stimulated with 10μg/mL of type I collagen demonstrate significant prolongation in time to initiation of platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with type I collagen alone; 10μg/mL type I collagen; 140.40±1.80 seconds, 5μM ARP-100; 177.70±12.40 seconds, 11μM APY; 169.70±2.57 seconds, 30μM PAM 165.20±1.74 seconds, 50μM EPT; 180.70±7.81 seconds).

On the other hand, isolated human platelets ($2.5x10^8$ platelets/mL) exposed to 200µM acetylsalicylic acid and 0.8µM dabigatran and stimulated with 10µg/mL of type I collagen demonstrate no significant effect on initiation of platelet aggregation compared to isolated human platelets ($2.5x10^8$ platelets/mL) stimulated with type I collagen alone; 200µM ASA; 144.6±3.88 seconds, 0.8µM DABI; 150.20±1.57 seconds (Appendix figure 4).



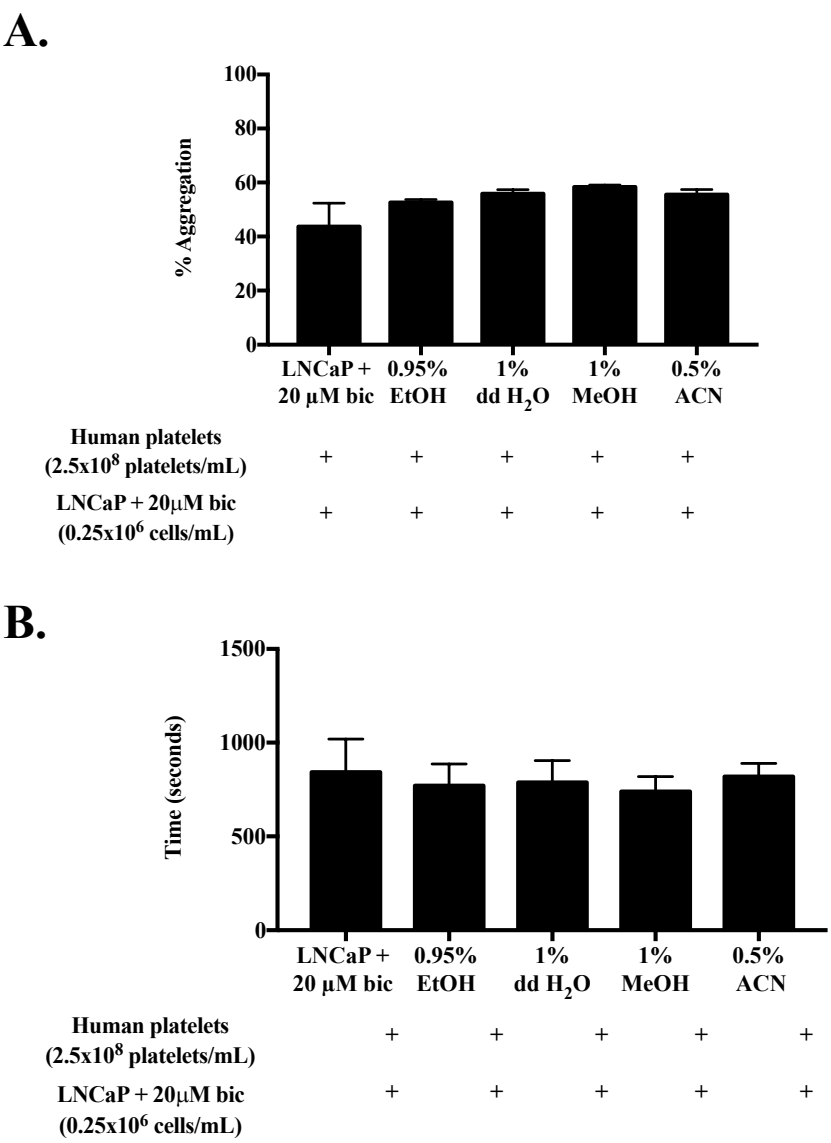
Appendix figure 4: Contribution of platelet pathways to type I collagen induced isolated platelet aggregation. (A) Representative platelet aggregation traces of human platelets stimulated with 10µg/mL of type I collagen in the presence of acetylsalicylic acid (ASA) (200µM), ARP-100 (5µM), apyrase (APY) (11 µM), Prasurgrel active metabolite (PAM) (30µM), eptifibatide (EPT) (50µM), and dabigatran (8 µM). **(B)** Isolated human platelets treated with ASA, APY, PAM, and EPT then stimulated with 10µg/mL of type I collagen demonstrate significant reduction in % aggregation compared to platelets stimulated with type I collagen alone. (C) Isolated human platelets incubated with ARP-100, APY, PAM, and EPT then stimulated with 10µg/mL of type I collagen demonstrate significant prolongation in time to initiation of platelet aggregation compared to platelets stimulated with type I collagen alone. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6, *p<0.05, **p<0.01,***p<0.001, ****p<0.0001.

Appendix 5. The solvents of pharmacological reagents demonstrate no significant effect on LNCaP + 20μM bic, DU145 or PC3 induced TCIPA.

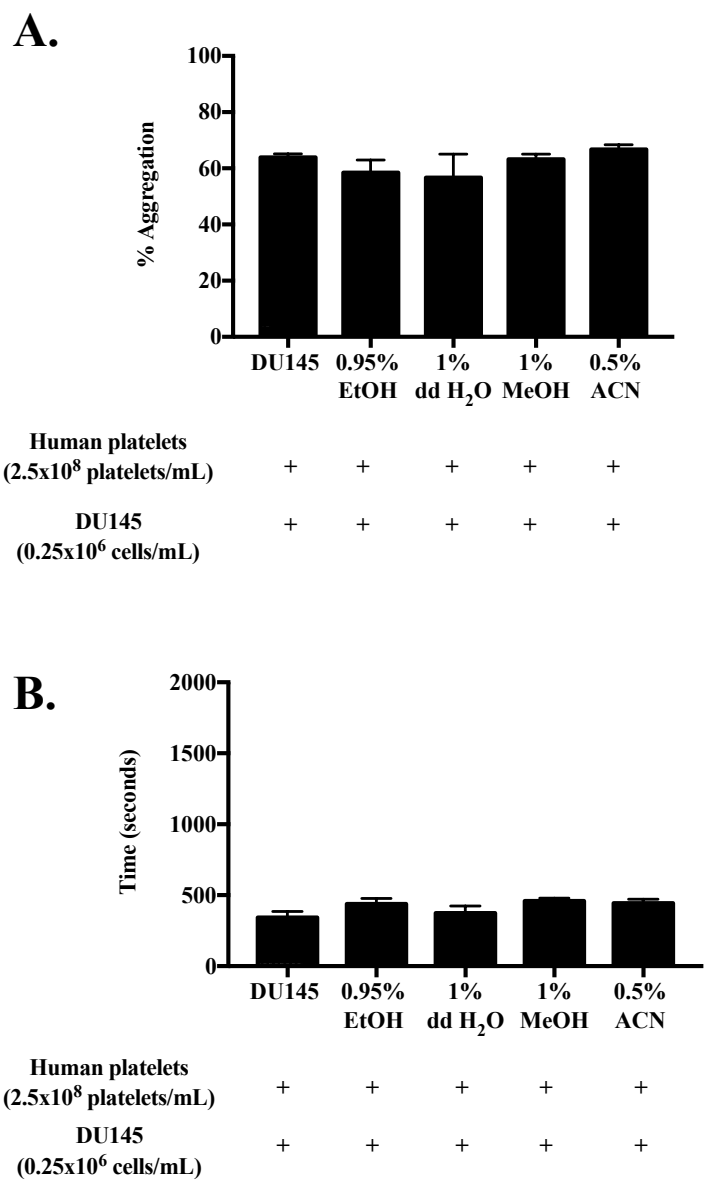
Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to solvents of pharmacological reagents demonstrate no significant effect on LNCaP + 20μM bic induced % aggregation and time to initiation of platelet aggregation compared to LNCaP + 20μM bic stimulated isolated platelets ($2.5x10^8$ platelets/mL) alone; LNCaP + 20μM bic (43.73±8.62%, 842.80±176.10 seconds), 0.95% ethanol (52.58±1.15%, 770.50±116.60 seconds), 1% double distilled H_2O (55.83±1.53%, 788.30±116.50seconds), 1% methanol (58.33±0.76%, 739.80±79.96 seconds), and 0.5% acetonitrile (55.53±1.90%, 819.20±70.12 seconds) (Appendix figure 5).

Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to solvents of pharmacological reagents demonstrate no significant effect on DU145 induced % aggregation and time to initiation of platelet aggregation compared to DU145 stimulated isolated platelets ($2.5x10^8$ platelets/mL) alone; DU145 (63.90±1.23%, 343.30±43.08 seconds), 0.95% ethanol (58.37±4.49%, 439.0±38.21 seconds), 1% double distilled H_2O (56.67±8.33%, 373.70±50.76 seconds), 1% methanol (63.17±1.83%, 458.3±20.84 seconds), and 0.5% acetonitrile (66.67±1.66%, 443.30±29.63 seconds) (Appendix figure 6).

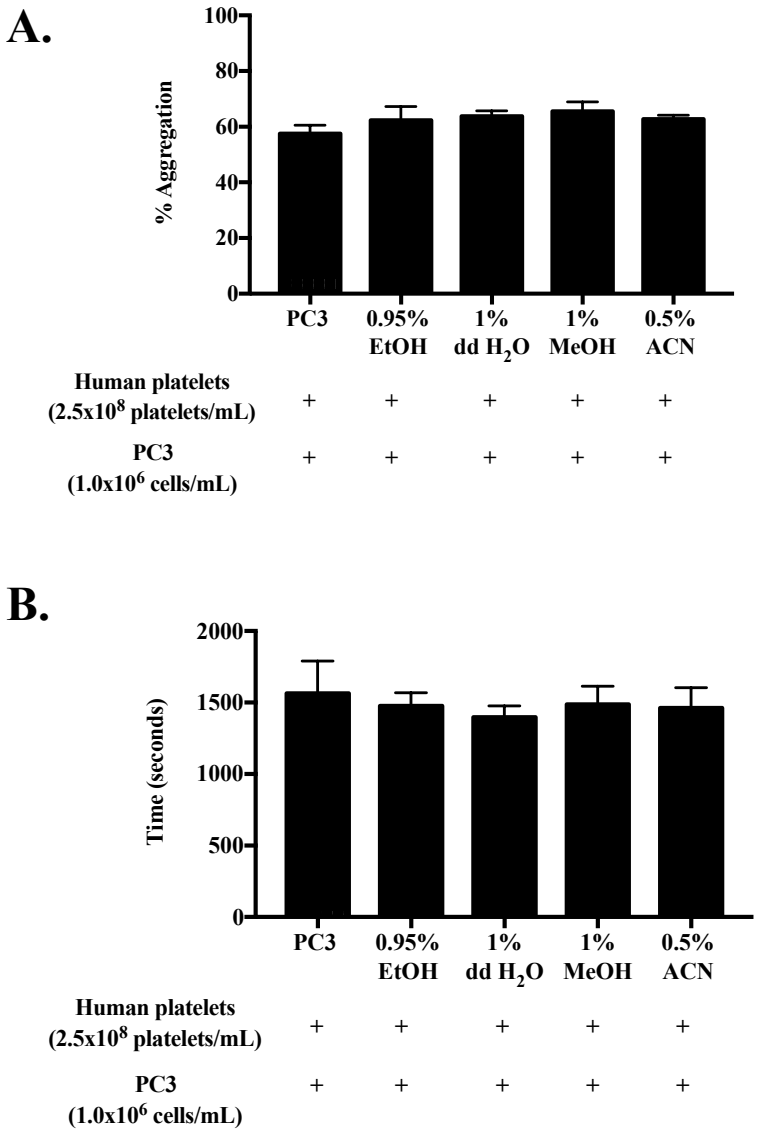
Exposure of isolated human platelets ($2.5x10^8$ platelets/mL) to solvents of pharmacological reagents demonstrate no significant effect on PC3 induced % aggregation and time to initiation of platelet aggregation compared to PC3 stimulated isolated platelets ($2.5x10^8$ platelets/mL) alone; PC3 (57.55±2.98%, 1564±227.50 seconds), 0.95% ethanol (62.27±5.04%, 1478.0±91.94 seconds), 1% double distilled H_2O (63.78±1.92%, 1398.0±79.92 seconds), 1% methanol (65.57±3.38%, 1487.0±126.9 seconds), and 0.5% acetonitrile (62.67±1.45%, 1462.0±141.60 seconds) (Appendix figure 7).



Appendix figure 5: The effect of solvents of pharmacological reagents on LNCaP + 20µM bic induced TCIPA. (A) The solvents of pharmacological agents used demonstrate no significant effect on LNCaP + 20µM bic induced % platelet aggregation compared to isolated platelets stimulated with LNCaP + 20µM bic alone. **(B)** The solvents of pharmacological agents used demonstrate no significant effect on LNCaP + 20µM bic induced time to initiation of platelet aggregation compared to isolated platelets stimulated with LNCaP + 20µM bic alone. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=6, $p>0.05$. (Bic – bicalutamide, EtOH - ethanol, dd H_2O - double distilled water, MeOH - methanol, and ACN - acetonitrile).



Appendix figure 6: The effect of solvents of pharmacological reagents on DU145 induced TCIPA. (A) The solvents of pharmacological agents demonstrate no significant effect on DU145 induced % platelet aggregation compared to isolated platelets stimulated with DU145 cells alone. **(B)** The solvents of pharmacological agents demonstrate no significant effect on DU145 induced time to initiation of platelet aggregation compared to isolated platelets stimulated DU145 cells alone. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=3, $p>0.05$. (EtOH - ethanol, dd H_2O - double distilled water, MeOH - methanol, and ACN - acetonitrile)



Appendix figure 7: The effect of solvents of pharmacological reagents on PC3 induced TCIPA. (A) The solvents of pharmacological agents used demonstrate no significant effect on PC3 induced % platelet aggregation compared to isolated platelets stimulated with PC3 cells alone. **(B)** The solvents of pharmacological agents used demonstrate no significant effect on PC3 induced time to initiation of platelet aggregation compared to isolated platelets stimulated PC3 cells alone. Statistics: One-way ANOVA, Dunnett's multiple comparison test, N=3, $p>0.05$. (EtOH - ethanol, dd H_2O - double distilled water, MeOH - methanol, and ACN - acetonitrile).