School of Pharmacy and Biomedical Sciences

Platelet-cancer Crosstalk: Mechanisms and Therapeutic Implications

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Declaration

To the best of my knowledge and belief, this thesis titled '**Platelet-cancer crosstalk: mechanisms and therapeutic implications**', contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

The animal research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number ARE2018-34

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007). The research study received human research ethics approval from the Curtin University Human Research Ethics Committee, Approval Number HR54/2014.

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General Introduction

Throughout life, human cells are subjected to damage from radiation, smoking, pollution, obesity, lack of adequate physical activity and ageing. In addition to these factors, an individual's genetic susceptibility can predispose cells to harmful mutations through activation of an oncogene or inactivation of a tumour suppressor gene [1-5]. Considering the human body is made up of approximately ten trillion cells, the initiation of a tumour within an organ is inevitable. However, experimental data suggest that the tumour microenvironment (TME) can be either restrictive or supportive of tumour progression to malignancy [8]. Although the role of TME in restraining malignancy is understudied, its role in promoting cancer growth and metastasis has recently been recognised.

Tumour stroma is an integral part of the TME. All solid tumours, regardless of their histological origin, require stroma to grow. Stroma acts as a lifeline to the tumours by providing the blood and lymphatic vascular supply [9]. Additionally, tumour stroma also contains inflammatory cells and connective tissue [10]. There is a striking difference between the amounts of stroma from one tumour to another. For example, pancreatic cancer mass is 90% stroma whereas; melanoma contains a minimal amount of stroma [11,12]. The process of stromal generation is often described as an untoward wound healing. A normal wound healing is typically triggered by an injury to and a subsequent leakage of the blood vessels. Exposure of the tissue factor in the subendothelium to the coagulation factors in the blood generates a cascade of reactions that lead to clot formation to stop bleeding (haemostasis). The second step in the wound healing process, inflammation, involves an influx of inflammatory cells (e.g. neutrophils and monocytes) to the clot site to ingest and degrade the debris. This is followed by a proliferation phase which is characterised by angiogenesis (formation of new blood vessels), fibroblast ingrowth and tissue granulation. The wound healing eventually ends with a maturation and remodelling stage, leaving a scar of connective tissues and blood vessels [13]. In the context of cancer, the vasculature is leaky due to the ability of tumours to produce large quantities of vascular endothelial growth factor (VEGF) constitutively [14,15]. Moreover, tumours tend to harbour a procoagulant surface through the expression of tissue factor [16].

Platelets, the key players in thrombosis, are instrumental in wound healing through their contribution to clot formation, and the secretion of growth factors and cytokines which mediate the inflammation, proliferation and remodelling steps of wound healing [17]. However, the role of platelets in the growth of solid tumours was initially believed to be insignificant since earlier studies failed to show platelet deposition in solid tumours [12]. However, with the recent advancement in the

development of monoclonal antibodies, platelet migration into solid tumours is now clearly-documented [18-22].

It is well-known for more than 100 years that high platelet count or thrombocytosis occurs in patients with solid tumours. Thrombocytosis can be a sign of a malignant neoplasm in more than one-third of persons who do not have iron deficiency anaemia or benign inflammatory conditions [23]. Moreover, abnormal blood clotting or thrombosis can be a harbinger of cancer. Armand Trousseau was the first to establish the link between cancer and thrombosis in 1865, describing a migratory blood clot that heralds visceral cancer in some of his patients. Trousseau himself developed gastric cancer, and his self-diagnosis was confirmed when he noticed painful oedema along his veins, among other symptoms of gastric cancer [24]. Patients with cancer have 5-7 fold greater risk of developing venous thromboembolism (VTE) compared to persons without cancer [25].

Complications arising from cancer-associated thrombosis (CAT) are associated with considerable mortality, morbidity and health care expense. The risk of developing CAT depends on the primary origin of the tumours, with several studies showing that cancers of the pancreas, lung, brain, ovary, stomach and breast have the most association with CAT. Chemotherapy, radiotherapy, antiangiogenic drugs can also increase the risk of CAT. Other risk factors include obesity, age, immobilisation, history of VTE, major surgery, race (lower in Asians), sex (higher in female), and comorbidities (infection, renal diseases) [25,26].

It is often emphasized that pancreatic cancer patients have the highest risk of developing CAT, reported to be between 8-35% [27-31]. It is still unclear whether the numerous thrombotic events are unique to this type of cancer or the result of the advanced-stage diagnosis. Indeed, pancreatic cancer is highly aggressive and has the worst prognosis among all types of cancers, which has not improved in the last 50 years. In addition to the high metastatic rate and late diagnosis, sufferers can acquire chemotherapy resistance, which contributes to the low survival rate of pancreatic cancer [32]. Pancreatic cancer cells are known to express and release a high level of tissue factor, the major effector of the coagulation cascade [33-35]. Tissue factor and coagulation proteins have been shown to promote oncogenic signals in cancer cells, supporting cancer invasion and metastasis [36]. Additionally, tissue factor can indirectly activate platelets by forming a complex with the coagulation factor VIIa to activate factor X, which then mediates the conversion of prothrombin to thrombin, a potent platelet agonist. Thrombin is also the principal enzyme that catalyses the conversion of fibrinogen to fibrin, a process essential in forming blood clots [37].

In addition to the expression of the procoagulant and prothrombotic tissue factor, pancreatic cancer cells can activate blood clots via their interaction with neutrophils to generate neutrophil

extracellular traps (NETs); a mesh-like structure of DNA enriched with histones and neutrophil proteases [6,38-40]. Initially described as pathogen killing mechanism, the release of NETs is now well established in several pathological conditions, including cancer [38,41]. Our research group was one of the first to demonstrate a potential role of NETs in pancreatic cancer-associated thrombosis [6]. The pancreatic cancer cells (AsPC-1), but not primary cells control (mesenchymal stem cells MSC) were able to activate neutrophils to release NETs. Furthermore, we showed that purified NETs could induce platelet aggregation, independently of plasma proteins (i.e. coagulation factors), and that, in an ex vivo microfluidic assay, purified NETs served as a surface for platelet adhesion and thrombus formation [6]. Most recently, Boone et al., showed a reduction in cancer-induced platelet activation and thrombosis in an orthotopic pancreatic cancer mouse model that was unable to form NETs, underlying the functional importance of NETs in CAT [40].

Beyond thrombosis, recent lines of evidence have established the role of platelets in cancer metastasis. Activated platelets, regardless of the agonist, can secrete several biological factors that can modulate cancer activity and favour a more aggressive phenotype [42-44]. Specifically, platelets promote epithelial to mesenchymal transition (EMT) changes in cancer cells, which are described as a cell developmental programme towards a more invasive, and migratory phenotype [44,45]. Recently, it has been shown that EMT can also impart chemotherapy resistance in pancreatic cancer [46]. Therefore, platelets as a central player in EMT as well as CAT could be a promising target in pancreatic cancer treatment. In this regard, this thesis aims to investigate if targeting platelets in pancreatic cancer can reduce chemotherapy resistance and risk of thrombosis.

This thesis is a collection of the author's published and unpublished works conducted during the PhD programme. These reviews and experimental studies were executed to identify the mechanisms and therapeutic implications of the interplay between pancreatic cancer cells and platelets. The work resulted in six peer-reviewed articles (three reviews and three research articles), and one research article that is currently under preparation for submission. A brief summary of each chapter is described below.

Chapter one is a comprehensive literature review of the role of platelets in cancer growth and metastasis. In order to gain more insight into the possible risk of targeting platelets in cancer, the chapter reviewed the most common chemotherapies that are associated with thrombocytopenia (low platelet count). The manuscript also evaluated the therapeutic benefit of using antiplatelet medications in cancer treatment and concluded that a strategic targeting of platelets in solid tumours with associated thrombotic risks may be beneficial.

Chapter two is a study of the mechanisms of NET-induced platelet activation. Our team, along with other research groups, has demonstrated the ability of cancer cells to directly induce neutrophils to expel NETs [6,39,40]. In addition to their role in supporting cancer progression, NETs are suggested to be capable of activating thrombosis [47,48]. However, it has been demonstrated that cell-free DNA and histones, but not intact NETs, can activate coagulation [49]. Additionally, it was unclear whether NETs could activate platelets directly. Therefore, the study aimed to investigate the underlying mechanism of NET-induced platelet activation, and whether drugs targeting components of NETs or platelet function could be used to inhibit NET-dependent platelet activation, thus providing a potential strategy to prevent thrombotic complications associated with NET production in cancer and other diseases.

Chapter three examined the effects of platelet-derived factors, i.e. platelet releasate (PR), on pancreatic cancer cell proliferation and acquired resistance to gemcitabine, the most clinically used drug in the treatment of pancreatic cancer [32]. The study showed that PR effectively promoted cell proliferation, AKT and ERK phosphorylation and SLUG upregulation in pancreatic cancer cells in the presence of gemcitabine. Retained activation status or expression of these effector molecules are vital in the progression of cancer as AKT, and ERK activation is required for cell proliferation, and SLUG is a mesenchymal transcription factor that has been associated with metastasis and chemotherapy resistance [32]. Importantly, the article showed for the first time that PR modulated the expression of cytidine deaminase (CDD) and equiliberative nucleoside transporter (ENT1), two important proteins involved in drug metabolism. CDD is responsible for cellular gemcitabine deactivation, while ENT1 mediates gemcitabine uptake [32].

Moreover, the article showed for the first time that platelet-derived purine nucleotides (ADP and ATP) were the major regulators of SLUG and CDD upregulation in cancer cells. Interestingly, the study also discovered the expression of P2Y12, an ADP receptor primarily found in platelets and the brain, in pancreatic cancer cells, suggesting clinically available P2Y12 inhibitors such as ticagrelor could be employed to target pancreatic cancer cells.

Chapter four is a direct follow-up of the research article described in chapter three. Here, the study examined whether the antiplatelet P2Y12 inhibitor, ticagrelor, could affect cancer cells directly and whether it could potentiate the efficacy of chemotherapy medications *in vitro* and *in vivo*. The thesis shows for the first time that ticagrelor, used at clinically relevant concentrations, could effectively reduce pancreatic cancer cell proliferation *in vitro*. Moreover, ticagrelor activated apoptosis and reduced ADP and EGF-induced AKT activation in cancer cells. In combination studies, ticagrelor synergised with cisplatin, gemcitabine, paclitaxel and erlotinib *in vitro* to reduce cancer cell

proliferation. Mechanistically, siRNA knockdown of P2Y12 resulted in a significant reduction of EMT markers; ZEB1 and SLUG expression and basal EGFR activation. The study also found striking similarities between ADP P2Y12 signalling in platelets and cancer cells. Inhibition of P2Y12, but not P2Y1 (another major ADP receptor) in platelets can attenuate AKT activation in response to different physiological agonists (e.g. ADP, collagen, thrombin) [50]. Similarly, in pancreatic cancer cells, inhibition of P2Y12, but not P2Y1, reduced AKT activation in response to ADP, and EGF. This suggests that there is a positive ADP signalling loop in cancer cells that can lead to P2Y12/AKT activation, and inhibition of P2Y12 results in decreased AKT-mediated survival signalling, leading to apoptosis. In a pancreatic cancer xenograft mouse model, the combination of ticagrelor and gemcitabine significantly reduced tumour growth, whereas ticagrelor or gemcitabine as single agents had minimal effect. This study unravels a novel effect and mechanism of action of ticagrelor, and suggests that inhibition of P2Y12 in pancreatic cancer could be a therapeutic option.

In conclusion, detailed in **chapter five**, this thesis report several novel results, including the following:

I.   NETs can directly induce platelet aggregation in the absence of plasma components and independent of NET-associated DNA and histones.

II.   Platelets enhance cytidine deaminase expression (a marker associated with poor survival) in pancreatic cancer cells, which can impart gemcitabine resistance.

III.   Platelet-derived ATP and ADP can upregulate pro-survival signalling and chemo-resistance pathways in cancer cells

IV.   P2Y12 is expressed and is functionally active in pancreatic cancer cells. The receptor is required for cancer growth and contributes to the regulation of EMT-related transcriptional factors SLUG and ZEB1.

V.   P2Y12 is required for EGFR oncogenic signalling in pancreatic cancer cells

VI.   The P2Y12 inhibitor ticagrelor increased the anticancer activities of erlotinib, gemcitabine, paclitaxel and cisplatin *in vitro*

VII.   The combination of ticagrelor and gemcitabine significantly reduced tumour growth *in vivo*, whereas gemcitabine or ticagrelor as single agents had minimal effect.

These findings shed more light into the potential of targeting platelets in solid tumours associated with a high risk of thrombosis.

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List of primary publications

This PhD-by publication includes five first author peer-reviewed publications and one publication that is currently under preparation for submission. The following articles have been published and are attached under the related chapters. Author contributions and copyright authorisation for each publication are detailed in Appendix II.

1. **Elaskalani O**, Berndt MC, Falasca M, Metharom P. *Targeting Platelets for the Treatment of Cancer.* Cancers (Basel). 2017 Jul 22;9(7). pii: E94. doi:10.3390/cancers9070094. Review.

    {Q1 Cancer research, Impact Factor 5.326}

2. **Elaskalani O**, Abdol Razak NB, Metharom P. Neutrophil extracellular traps induce aggregation of washed human platelets independently of extracellular DNA and histones. Cell Commun Signal. 2018 May 29;16(1):24. doi: 10.1186/s12964-018-0235-0

    {Q1 Biochemistry and Cell Biology, Impact Factor 5.324}

3. **Elaskalani O**, Abdol Razak NB, Falasca M and Metharom P. *Epithelial-mesenchymal transition as a therapeutic target for overcoming chemoresistance in pancreatic cancer*. World Journal of Gastrointestinal Oncology. 2017 Jan 15;9(1):37-41. doi:10.4251/wjgo.v9.i1.37. Review.

    {Impact Factor 3.140}

4. **Elaskalani O**, Falasca M, Moran N, Berndt MC, Metharom P. *The Role of Platelet-Derived ADP and ATP in Promoting Pancreatic Cancer Cell Survival and Gemcitabine Resistance*. Cancers (Basel). 2017 Oct 24;9(10). pii: E142. doi:10.3390/cancers9100142.

    {Q1 Cancer research, Impact Factor 5.326}

5. Adamska A, **Elaskalani O**, Emmanouilidi A, Kim M, Abdol Razak NB, Metharom P, Falasca M

   Molecular and cellular mechanisms of chemoresistance in pancreatic cancer. Review. Advances in Biological Regulation. 2017 Nov 22. pii: S2212-4926(17)30176-8. doi: 10.1016/j.jbior.2017.11.007

{Q1 Cancer research}

List of secondary publications

The following publication is complimentary to the candidacy objectives and is attached in Appendix I. Author contribution and copyright authorisation is detailed in Appendix II

1. Abdol Razak N, **Elaskalani O**, Metharom P. Pancreatic Cancer-Induced Neutrophil Extracellular Traps: A Potential Contributor to Cancer-Associated Thrombosis. *Int J Mol Sci*. 2017;18(3).

{Q1 Medicine (miscellaneous), Impact Factor 3.687}

List of conference publications

The following studies were presented as posters in conferences, however they are not included in the thesis.

1. **Elaskalani O**, Falasca M, Metharom P. Crosstalk between pancreatic cancer cells and platelets (Poster). *Science on the Swan Conference*, Perth, Australia, May / 2016.

2. **Elaskalani O**, Falasca M, Metharom P. Platelets modulate MAPK, EMT pathways and migration in pancreatic cancer cells (Poster). *The Annual Perth Combined Biological Sciences Meeting*, Perth, Australia, July / 2016

3. **Elaskalani O**, Abdol Razak N, Metharom P. Extracellular DNA and histones are dispensable for neutrophil extracellular trap-induced aggregation of human washed platelets (Poster). The International conference on thrombosis and haemostasis issues in cancer (ICTHIC), Bergamo, Italy, April / 2018

Abbreviations

ACD	Acid-Citrate-Dextrose
ADP	Adenosine diphosphate
Ang-1	Angiopoietin-1
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATX	Autotaxin
CAT	Cancer-associated thrombosis
CDC42	Cell division control protein 42
CDD	Cytidine deaminase
CG	Cathepsin G
CLEC-2	C-type lectin-like receptor 2
COX-1	Cyclooxygenase-1
CTCs	Circulating tumour cells
CTH	Calf thymus histones
CXCL-5	C-X-C motif chemokine 5
dCK	Deoxycytidine kinase
DG Plt	Degranulated platelets
DNA	Deoxyribonucleic acid
DVT	Deep venous thrombosis
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ENT1	Equilibrative nucleoside transporter
ERK	Extracellular signal–regulated kinases
FBS	Fetal bovine serum
GARP	Glycoprotein-A Repetitions Predominant Protein
GEM	Gemcitabine
GEPIA	Gene Expression Profiling Interactive Analysis

GPCRs	G-protein coupled receptors
GPVI	Glycoprotein VI
GR	Gemcitabine resistant
GS	Gemcitabine sensitive
GTEx	Genotype-Tissue Expression
HB-EGF	Heparin-binding EGF-like growth factor
HMGB1	High-mobility group box 1 protein
IL-6	Interleukin-6
IL-8	Interleukin-8
KO	Knockout
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
MET	Mesenchymal-epithelial transition
miRNAs	microRNA
MK	Midkine
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MRP-2	Multidrug resistance protein 2
MSC	Mesenchymal stem cells
NE	Neutrophil elastase
NETs	Neutrophil-extracellular traps
NOX	NADPH oxidase
PaCa	Pancreatic cancer
PAD4	Peptidyl arginine deiminase
PAR1	Protease-activated receptor-1
PAR4	Protease-activated receptor-4
PDAC	Pancreatic ductal adenocarcinoma
PDE5	Phosphodiesterase-5
PDGF	Platelet-derived growth factor
PDPN	Podoplanin
PEG1	Prostaglandin E1
PF4	Platelet factor-4
PKB (AKT)	Protein kinase B

Plt	Platelet
PMA	Phorbol myristate acetate
PMPs	Platelet microparticles
PR	Platelet releasate
PRP	Plasma rich platelet
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
SEM	The standard error of the mean
siRNA	Small interfering RNA
SLUG	Zinc finger protein SNAI 2
Snail	Zinc finger protein SNAI 1
Syk	Spleen tyrosine *kinase*
TCGA	*The Cancer Genome Atlas*
TF	Tissue factor
TGF-β1	Tumour growth factor-beta1
Tica	Ticagrelor
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
TxA2	Thromboxane A2
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism
xl-CRP	Cross linked-collagen related peptide
YAP	Yes-associated protein 1
ZEB-1	Zinc finger E-box-binding homeobox 1

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Bibliography

Thesis hypothesis

Targeting platelets can reduce tumour growth and lower the risk of cancer-associated thrombosis in pancreatic cancer

The above hypothesis was investigated via the following objectives:

Objective 1:

The tumour microenvironment is well equipped to induce platelet activation, mainly through the expression of tissue factor. Recently, however, a novel mechanism of cancer-associated thrombosis that is initiated by activated neutrophils through neutrophil extracellular traps (NETs) was uncovered. Objective 1 of this thesis determined whether NETs could directly interact with platelets independently of tissue factor. Through the use of several pharmacological inhibitors, the study explored various pathways to prevent NET-induced platelet activation.

Objective 2:

Since platelets are activated in the context of pancreatic cancer, objective 2 explored how activated platelets can affect cancer cell survival and chemotherapy resistance. Using different *in vitro* models, the effect of platelets on cancer progress (e.g. epithelial-mesenchymal transition or EMT) and chemotherapy resistance were examined. Lastly, the therapeutic implication of targeting platelets in cancer therapy was investigated in vivo, using a xenograft pancreatic cancer mouse model.

Chapter 1:

Literature Review

Platelets as a therapeutic target for anti-cancer therapy

1.1. Publications

1. **Elaskalani O**, Berndt MC, Falasca M, Metharom P. *Targeting Platelets for the Treatment of Cancer.* Cancers (Basel). 2017 Jul 22;9(7). pii: E94. doi:10.3390/cancers9070094. Review.

1.2. Introduction

Cancer progression is a multi-staged dynamic process that involves complex interactions with numerous cell types and elaborate molecular signalling crosstalks. Several complications that are associated with cancers, including their treatment, can drastically increase morbidity and mortality of sufferers. Of note, cancer-associated thrombosis is a major complication that is frequently observed in patients with solid tumours and is significantly correlated with poor prognosis. Cancer cells can induce thrombotic events by directly activating platelets, the key regulator of thrombosis, or indirectly through various other mechanisms, including activation of the vascular endothelium, blood coagulation cascade, complement and the immune system, which contribute to heighten the prothrombotic state commonly detected in many cancers [51]. Whether targeting platelets can improve survival of cancer patients with a high risk of thrombosis (e.g. pancreatic, brain, lung and ovarian cancer) requires further investigation.

The ability of cancer cells to detach from an initial tumour site and spread to distant organs is the primary cause of death in cancer patients. Metastasis cascade begins with an entry of cancer cells into blood vessels (intravasation), dissemination into the blood circulation, extravasation from the vasculature, and finally, an establishment of secondary growth at distant sites. Cellular migration, as

well as a pro-tumour microenvironment, are crucial for the successful metastasis. Epithelial-mesenchymal transition (EMT) is a cellular developmental programme that is considered important for cancer cells to accomplish metastasis [52]. During EMT, cancer cells exhibit enhanced cell invasion, migration and resistance to inhibitory signals, including chemotherapy [46]. Platelets, which are abundant in the surrounding tumour microenvironment, have been shown by several studies, including ours, to contribute to EMT in cancer cells *in vitro* and in mouse tumour models (chapter two) [42,53,54]. Although this area of research is currently under active investigation, it remains to be validated whether a target inhibition of platelets could affect cancer cell EMT, reduce metastasis and subsequently prolong survival in patients. Platelets have recently emerged as a source of biomarkers, since tumour-educated platelets have been found to carry cancer-specific mRNA which can distinguish cancer patients from healthy controls with high accuracy [425].

In our 2017 review indicated above (a copy is included at the end of the chapter), we provided an overview of the latest mechanistic and clinical evidence in regards to the potential benefits of targeting platelets in cancer. However, there have been several exciting developments made in the field since the publication of our article. Thus, the following section will provide a summary of the most recent and significant research and difficulties concerning the role of platelets in cancer therapy.

1.2.1. Current clinical trials and the significance of G-Protein Coupled Receptor P2Y12

Two notable clinical trials are underway to test the efficacy of using antiplatelet drugs in combination with cancer treatment. The first clinical trial (NCT02404363) is set out to examine tumour progression and risk of thrombosis with or without clopidogrel in combination with chemotherapy in patients with advanced metastatic pancreatic cancer. Clopidogrel (also known as Plavix) is an antiplatelet drug that is used to reduce the risk of heart disease and stroke. Specifically, it functions by irreversibly blocking the P2Y12 receptor on platelets [55]. A previous study showed that clopidogrel could reduce cancer growth, metastasis, and coagulation in an orthotopic pancreatic cancer mouse model through its inhibitory effect on platelet [56].

Since its discovery, the P2Y12 receptor is often indicated to be predominantly expressed on platelets and brain tissues [57-60]. However, there are inconsistencies findings among the available literature in regards to the expression profile of P2Y12 in various tissues outside of platelets and the brain [53,61-64]. It must be noted that the Human Protein Atlas and several protein databases have demonstrated P2Y12 receptor expression in many normal and cancer tissues and cell lines [53,61,64-68]. In line with the above reports, the results from our studies indicated the presence of the receptor in multiple cancer

cell lines, and more importantly, we discovered P2Y12 to have a functional role in pancreatic cancer cells (Chapters three and four), specifically in cellular proliferation and chemotherapy resistance[53].

The second clinical trial (NCT03245489) examines whether a combination of antiplatelet drugs, clopidogrel and aspirin, could improve the immunological response to Pembrolizumab; a therapeutic antibody that blocks programmed cell death protein-1 in cancer cells, thus allowing the host immune cells to destroy them [69]. The clinical trial is currently active and recruiting patients with recurrent or metastatic squamous cell carcinoma of head and neck. This trial is supported by recent evidence indicating platelets as capable of restraining effective cancer immune response from the host [70].

Antiplatelets are commonly used in cardiovascular patients. Similarly to cancer, cardiovascular diseases are more closely associated with the elderly population. Thus, several cardiovascular clinical studies have been reanalysed to examine links between antiplatelet usage and the incidence of cancer. TRITON TIMI 38 trial in 2007 showed a small but statistically significant association between antiplatelet prasugrel (P2Y12 inhibitor) and increased incidence of solid tumours [71]. This observation led to a recent and widely spread "cancer follows bleeding" hypothesis. The authors proposed that aggressive inhibition of platelets (i.e. increased risk of bleeding) can lead to instability of platelet-cancer cells aggregate, and failure to keep cancer cells locally in situ [72,73]. The following section will discuss this view in light of recent clinical results and FDA recommendation.

Cancer follows bleeding: controversies and challenges of antiplatelet therapy

Platelets are bioactive cellular fragments that are released from large, multinucleated cells of the bone marrow called megakaryocytes. Platelets are the second most abundant cells in the circulation after red blood cells. They are devoid of any nuclei, yet they possess the necessary machinery for de novo protein synthesis; a process often initiated after activation [74].

Aside from their principal role in haemostasis and thrombosis, platelets are regarded as essential contributors to various physiological processes and are involved in the pathology of many diseases. For example, in angiogenesis, platelets secrete pro-angiogenic factors that stimulate the formation of new blood vessels from pre-existing one. This process is fundamental in vascular development, lymphangiogenesis, and wound healing and also in pathological conditions such as atherosclerosis and diabetes retinopathy [7]. Cancer cells can also benefit from platelets pro-angiogenic activity. Since tumours outgrow their blood supply, platelet-mediated angiogenesis is a crucial step for cancer cells survival [75,76]. Moreover, platelets play a significant role in the haemostatic dissemination of cancer cells (Fig. 1). The overwhelming experimental and clinical evidence show that targeting platelet

reduces cancer cell seeding at metastatic sites. For example, depletion of platelets, knockout of specific platelet receptors or using clinically available antiplatelets decreased cancer growth and metastasis in mice [42,44,54,56,62,63,70,77-82]. Recently, a large prospective study that included more than 200 000 patients concluded that long-term use of a low dose of aspirin (2-4 years) is associated with a 23% reduction in the risk of ovarian cancer [83].
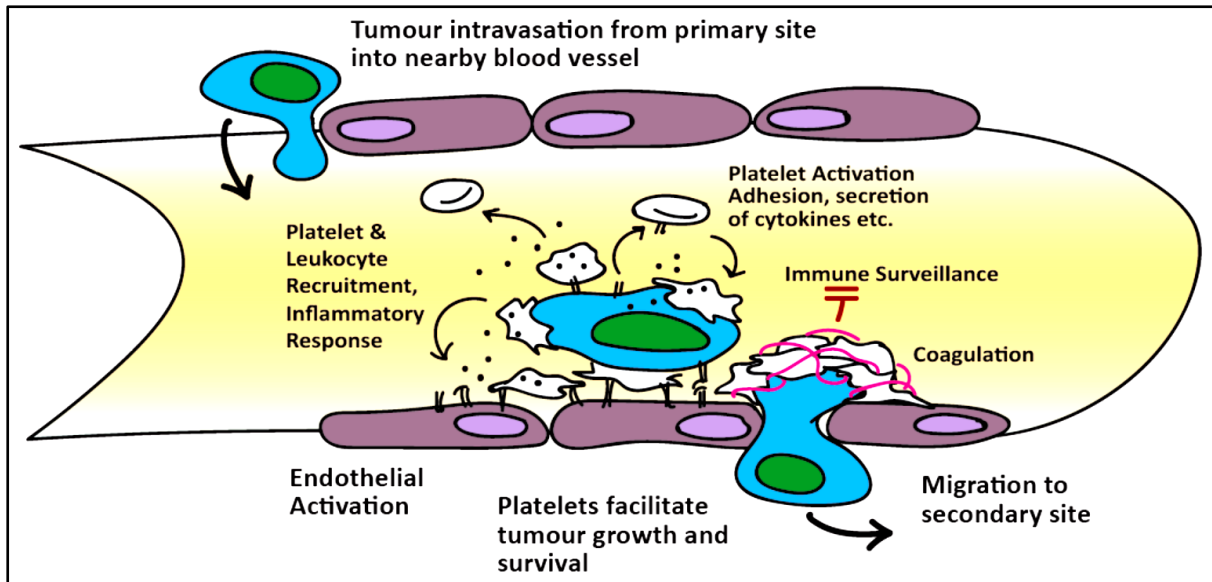

Figure 1*. **The role of platelet in haemostatic dissemination of cancer cells**. Cancer cells induce platelet activation and secretion of cytokines. Activated platelets protect cancer cells from natural killer cells-mediated cytotoxicity by forming a protective shield around cancer cells. Platelet-derived cytokines stimulate leukocyte recruitment, suppress cancer immune response and activate endothelial cells, ultimately leading to cancer cell escape to secondary sites.

*From poster presented by Elaskalani & Metharom, at Science on the Swan conference, Perth, Western Australia, 2017

However, recently Serebruany [72] proposed a theory that contradicted the majority of experimental data and clinical observations supporting the therapeutic benefits of antiplatelet therapy in cancer treatment. Serebruany suggested that cancer may, in fact, follows bleeding. Therefore, effective platelet inhibition would accelerate rather than slow down metastasis [72]. This theory stemmed from the analysis of the TRITON-TIMI 38 trial that compared two antiplatelet drugs; prasugrel to clopidogrel (P2Y12 inhibitors) in patients with acute coronary syndrome undergoing percutaneous coronary intervention [71]. Bleeding in Triton-TIMI 38 trial was classified as major (e.g. intracranial bleeding), minor (spontaneous gross haematuria), and insignificant bleeding [84,85]. The use of prasugrel was associated with an increased incidence of solid cancers compared to clopidogrel. The theory asserted that the difference in cancer incidence between prasugrel-treated patients and

clopidogrel-treated patients was due to the level of platelet inhibition by the two drugs (almost 100% in prasugrel-treated compared to 75% in clopidogrel-treated patients). Major bleeding was higher in the prasugrel-treated group (2.4%), compared to 1.8% in patients who received clopidogrel. However, the overall mortality was not significantly different between the two groups despite the statistical difference between the two groups regarding major bleeding and cancer incidence [71].

In support of Serebruany [72] proposal, PEGASUS-TIMI 54 trial showed an increased incidence of solid tumours in patients receiving ticagrelor (1.1% vs placebo 0.76%). The drug ticagrelor is similar to prasugrel as it inhibits platelet in almost all treated patients. The authors of TRITON-TIMI 38 and PEGASUS-TIMI 54 asserted that the higher rates of solid tumours occurring with prasugrel or ticagrelor therapy might have been the results of the increased medical scrutiny provided to the patients with more significant bleeding events. This increased medical attention would involve detailed imaging studies, usually performed in the case of major bleeding, which can lead to a detection of asymptomatic cancer [86,87].

In contrast to PEGASUS-TIMI 54, the PLATO trial that compared clopidogrel to ticagrelor showed a lower incidence of cancers in ticagrelor-treated patients (1.4%) compared to clopidogrel-treated patients (1.7%). Moreover, a meta-analysis of 14 randomised controlled clinical trials published before October 2014 comprising more than 60 000 subjects concluded that the use of P2Y12 inhibitors (clopidogrel, ticagrelor or prasugrel) in combination with aspirin was not associated with increased risk of non-cardiovascular death compared to aspirin alone [88].

Analyses of additional clinical trial results should shed more light on the association between the effects of antiplatelet use and cancers. Nonetheless, FDA review of the matter concluded that the chance of a false positive result is high and evidence of cancer causality is unlikely [89]. It is worth mentioning the ongoing competition for the market between the trademark manufacturers of clopidogrel, ticagrelor and prasugrel with advocates for both pro- and anti- "cancer follows bleeding" theory appearing to have a conflict of interest [90,91]. In conclusion, the explanation provided by the FDA is aligned with more than 100 years of literature, and there have been no published experimental data to show an association between aggressive platelet inhibition and solid tumour growth conclusively.

1.2.2. The influence of platelets on the anti-tumour response, cancer cell survival and metastasis

Transforming Growth Factor β1 (TGF-β1)

Cancer growth, invasion, angiogenesis and metastasis are accompanied by vascular endothelial damage and exposure to blood components, including platelets. During these processes, platelets can be stimulated directly by the tumour cells or indirectly via tumour-associated/activated endothelial cells, immune cells, or by several soluble factors and microparticles that are generated within the tumour microenvironment. It is well appreciated that activated platelets secrete several cytokines that can enhance tumour progression. However, platelet-derived TGF-β1 has received the most interest because platelets are the main source of serum active TGF-β1 [44,70,80]. Signalling through the TGF-β1 is one of the key pathways used by cancer cells to evade the immune response as TGF-β1 is an effective suppressor of T-cell proliferation and function. In several cancer mouse models, neutralisation of TGF- β1, or genetic modification of T-cells to become resistant to TGF-β1 resulted in a re-establishment of the cancer-immune response and eliminated tumours [92,93].

In addition to the secretion of pre-stored active TGF-β1, platelets express TGF-β-docking receptor Glycoprotein A Repetitions Predominant (GARP) which can bind to and activate latent TGF-β. A recent study by Rachidi et al. showed that serum active TGF-β1 was undetectable in platelet-GARP gene knockout (KO) mouse model. GARP KO platelets were unable to activate latent TGF- β1 secreted from platelets or non-platelet cells. Whereas, TGF-β1 KO platelets lacked TGF-β1 but expressed GARP and could still activate latent TGF-β1 from non-platelet sources. The study showed that platelet-GARP[KO], but not platelet-TGF-β1[KO], transgenic mice had an improved response to adoptive T cell therapy of melanoma compared to wildtype and TGF-β signalling was attenuated in tumours from platelet-GARP[KO] model [70].

Interestingly, the authors also showed that the treatment with antiplatelet drugs alone (aspirin and clopidogrel) after lymphocyte depletion did not affect cancer growth; however, they potentiated T-cell therapy, thus highlighting that platelets promote cancer growth via an immune-mediated mechanism. Further investigations are required to determine whether antiplatelet medications or cancer may affect GARP expression on platelets.

Anoikis

Pre-clinical data have demonstrated that platelet-associated factors can directly induce epithelial-mesenchymal transition (EMT) in cancer cells. EMT is a latent embryonic programme and a dynamic process in which cancer cells acquire mesenchymal (MSC)-like characteristics. During this process, the cells exhibit a reduced expression of the cell-cell adhesion proteins (e.g. E-cadherin, claudin-1) and an increased expression of proteins and transcriptional factors associated with promoting invasion and migration of cancer cells (e.g. N-cadherin, SLUG, SNAIL, TWIST and ZEB-1) [44]. EMT is not only required for metastasis but allows cells to overcome anoikis, which is a type of programmed cell death in anchorage-dependent cells[94]. Anoikis prevents normal cells from seeding into ectopic sites; however, cancer cells develop resistance towards anoikis through activation of cell survival signals (PI3K/AKT) and EMT. Since platelets can promote EMT and activation of survival signals in cancer cells, Haemmerle et al., recently showed that platelets also contribute to anoikis resistance through modulation of YAP1 signalling in cancer cells. YAP1 was significantly dephosphorylated in ovarian cancer cells treated with platelets in low attachment conditions. YAP1 is a transcriptional factor that translocates into the nucleus after dephosphorylation to promote cell proliferation and inhibit anoikis. Indeed, increased nuclear expression of YAP1 is associated with metastasis, thrombocytosis and poor prognosis in ovarian cancer patients. Platelet transfusion in mice to induce thrombocytosis resulted in an increase in metastasis in an ovarian cancer mouse model. Interestingly, this effect was drastically reduced after depletion of YAP1. This indicated YAP-1 signalling as the primary target of platelet in ovarian cancer cells under detached conditions, and its inhibition reduced platelet-assisted metastasis [95].

Neutrophil extracellular traps

Recent studies have shown that neutrophil extracellular traps (NETs) can serve as a platform for platelet adhesion, promoting thrombosis [48]. NETs are web-like structures made up of DNA and proteins that are released from activated neutrophils during inflammation and infection, and they are considered as a possible contributing factor in the formation of cancer-association thrombosis [6,38,96,97]. Although platelets and neutrophils are co-contributors in the pathogenesis of several conditions [98,99], little is understood in regards to their crosstalk in the context of thrombotic complications in cancer. Only recently, our research group showed for the first time that pancreatic cancer cells could directly induce NETs release from neutrophils, and cancer-primed platelets could stimulate neutrophils to generate NETs [6].

Although NETs are increasingly being recognised to have a role in facilitating cancer-associated thrombosis [56,96,97,100,101], it is unclear how NETs, at the molecular level, affect the cascades of coagulation and thrombosis. A recent paper by Noubouossie et al. showed that neutrophil-derived DNA and histones, but not intact NETs, directly activated coagulation [49]. Our work, in chapter 2, further added to the current understanding by showing that NETs could directly induce platelet aggregation independently of DNA, histones and plasma component. Therefore, targeting platelets rather than the NET-scaffold may be a more feasible strategy to moderate or inhibit NET-dependent cancer-associated thrombosis.

Purinergic signalling

In addition to several growth factors, platelet release purine nucleotides from dense granules after activation. There is a growing body of evidence highlighting the role of purinergic signalling in cancer growth and metastasis. Purinergic signalling refers to extracellular purine nucleotide or nucleoside interaction with their corresponding receptors to mediate cellular functions. Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are examples of extracellular purines that are released from platelets that can activate cancer growth [102].

Additionally, platelets express purinergic receptors (P2X1 for ATP and, P2Y1 and P2Y12 for ADP) which are involved in platelet activation [50]. Solid tumours are known to secrete more ADP compared to normal tissues, especially under hypoxic condition. ADP released by tumours into the microenvironment stimulate nearby platelets, which in turn, release soluble factors to support tumour growth, angiogenesis and metastasis [103].

Our work (Chapter 3) and the study by Cho et al. were one of the first studies to investigate the role of the ADP/P2Y12 receptor in cancer progression [53,63]. We showed that platelet-derived ADP could activate P2Y12 receptor on cancer cells, and induce upregulation of a transcription factor protein, SLUG; an EMT marker vital for cancer cells to acquire chemotherapy resistance and metastasis. Moreover, we showed that inhibition of P2Y12 on cancer cells significantly attenuated pro-tumour signalling conferred by platelets. This study was the first to underscore the importance of platelet-derived ADP on cancer cell survival. On the other hand, the study by Cho et al. showed that inhibition of platelet-P2Y12 receptor reduced cancer cell-induced platelet activation, and subsequently negated platelet-assisted cancer growth.

Furthermore, in Chapter 4, we provide the first demonstration of P2Y12-EGFR signalling pathway and showed that the P2Y12 inhibitor drug, ticagrelor, synergised with chemotherapy in pancreatic cancer cells, suggesting a novel combination strategy for the treatment of cancer.

Platelets, pro- or anti-tumour?

In 2017, Michael et al. demonstrated an inhibitory effect of platelet-derived microparticles (PMPs) on cancer progression [104]. Using patient-derived cancer tissues and allograft mouse models, the study showed that PMPs could transfer platelet microRNAs (miRNAs; small non-coding RNA) to solid tumours. Injection of purified PMPs into mice attenuated tumour growth, whereas treating cancer cells *in vitro* with PMPs increased cancer cell apoptosis. The authors identify MiR-24 to be the main microRNAs species in PMPs to negatively regulate cancer cell proliferation.

A recent paper in 2018 has also claimed an anti-tumour effect of platelets on primary cancer growth. The study by Smeda et al., have shown that inhibition of platelet function with clopidogrel and aspirin increased primary tumour growth in an orthotopic breast cancer mouse model. However, in the same study, the authors showed a reduction in pulmonary metastasis when clopidogrel and aspirin were given to mice that had cancer cells injected intravenously [105].

Despite the well-established positive contribution of platelet to cancer growth in the literature, these two studies were the first to show a tumour suppressive role of platelets *in* vivo and suggested that the effects of platelets may be cancer-type or cancer-stage specific.

1.3. Summary

It is generally accepted that platelets can modulate cancer cell behaviour as well as cancer-immune response to favour a more aggressive cancer phenotype. However, recently the risk of a potential association between platelet inhibition and solid tumours has attracted increased attention in basic and clinical research. The claimed risk arose primarily from the analysis of TRITON-TIMI 38 and PEGASUS-TIMI 54 randomised clinical trials, in which the incidence of solid tumours was higher with the more potent P2Y12 antiplatelets. However, these studies were not appropriately designed or powered to detect a cancer incidence rate. Since then, meta-analyses and FDA review have rejected the claim of an association between platelet inhibition and cancer incidence.

The overwhelming experimental evidence has indicated that platelets are instrumental in cancer growth, angiogenesis, metastasis and cancer-associated thrombosis. Although NETs released in the circulation during cancer are known to promote coagulation and thrombosis, it is unclear how these responses are mediated. Furthermore, the contribution of platelets to chemotherapy resistance and potential benefits of using antiplatelets in combination with chemotherapy are not well understood. Understanding these mechanisms could demonstrate the significance of targeting platelets in cancer treatment, especially in metastatic tumours with a high risk of thrombosis.

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Targeting Platelets for the Treatment of Cancer

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Abstract: The majority of cancer-associated mortality results from the ability of tumour cells to metastasise leading to multifunctional organ failure and death. Disseminated tumour cells in the blood circulation are faced with major challenges such as rheological shear stresses and cell-mediated cytotoxicity mediated by natural killer cells. Nevertheless, circulating tumour cells with metastatic ability appear equipped to exploit host cells to aid their survival. Despite the long interest in targeting tumour-associated host cells such as platelets for cancer treatment, the clinical benefit of this strategy is still under question. In this review, we provide a summary of the latest mechanistic and clinical evidence to evaluate the validity of targeting platelets in cancer.

Keywords: cancer; cancer therapy; platelet; antiplatelet

1. Introduction

Metastasis and acquired chemotherapy resistance are major obstacles in the treatment of patients with cancer. Recent seminal studies by Erpenbek and Schon [1] and Labelle et al. [2] have rekindled renewed interest into the role of platelets in cancer survival and metastasis. Using a mouse pulmonary melanoma metastasis model, Erpenbeck and Schon [1] demonstrated a critical role for platelets in metastasis since metastasis was totally blocked by platelet depletion. Potential adhesion receptors involved in mediating the augmentation of metastasis included the platelet-specific integrin, αIIbβ3, and P-selectin. The seminal paper by Labelle M et al. [2] identified a key role for platelets in mediating epithelial-mesenchymal transition (EMT) in circulating cancer cells.

Apart from their role as a key regulator in haemostasis [3], platelets can also mediate host immune and inflammatory responses [4,5]. A large number of experimental and clinical data underpin a pro-metastatic role of platelets in cancer [2,6–8]. It is well accepted that some tumour cells can stimulate platelets [9,10]. Once activated, platelets release an array of biologically active molecules which can modulate tumour growth and metastasis [11–13]. Within the blood compartment, tumour cells can form aggregates with platelets and thus avoid natural killer cell mediated cytotoxicity [14–16]. Therefore, tumour cell adhesion of platelets and their activation is a crucial step for tumour cell survival within the blood circulation [17,18].

To facilitate adhesion to platelets, some cancer cells can upregulate aberrant surface proteins. For example, lung cancer cells express P-selectin glycoprotein ligand 1 (PSGL-1), a protein commonly found on white blood cells, that binds with high affinity to P-selectin on the surface of activated platelets [19]. Selected cancer cells can also express podoplanin, a protein which can elicit platelet activation and aggregation through interaction with the C-type lectin-like receptor-2 (CLEC-2) on platelets [20]. In addition to direct physical interaction with tumour cells, platelets support cancer

progression by regulating tumour angiogenesis through a variety of secreted biological factors, e.g., vascular endothelial growth factor (VEGF), dopamine, serotonin and endostatin [21–25].

Additionally, platelets can modulate the behaviour of tumour cells. Platelets are the major storage site for transforming growth factor beta 1 (TGFβ1) within the blood circulation, which is released from α-granules upon activation [26]. Platelet-derived TGFβ1 can promote an epithelial-mesenchymal transition in cancer cells, an essential step in cancer invasion and metastasis [2]. While the majority of research so far has focused on the role of platelets in cancer metastasis and angiogenesis, recent data suggest an expanding role of platelets in tumour development, particularly their potential contribution to chemoresistance and cancer growth [27,28]. In this regard, lyso-phosphatidic acid (LPA) and platelet factor 4 (PF4) are also released from activated platelets and been shown to positively enhance tumour growth [29,30].

The study of platelet function in cancer patients has many challenges. In addition to disturbances of blood physiology due to cancer and the patient's clinical state [31–33], many chemotherapeutic agents themselves can directly affect the behaviour of platelets [34,35]. Moreover, some cancers and certain therapeutic regimens can lower the number of blood cells and platelets in the circulation, resulting in adverse side-effects such as anaemia and bleeding issues that raise ethical and practical concerns in acquiring samples for research purposes. The majority of the literature on platelet-tumour crosstalk has used platelets from healthy volunteers or mouse tumour models for experimental research which may not accurately mirror the disease in human. Although many aspects of the interactions can be delineated via these approaches, whether these models are informative for clinical trial development is still under investigation. This review highlights current literature in regard to the benefit of targeting platelets in cancer therapy.

2. The Role of Platelets in Cancer Metastasis

Cancer metastasis requires changes in cancer cells that lead to a more aggressive phenotype, characterised by an elongated shape, high motility and invasive capacity [36–39]. In fact, the majority of cancer-associated mortality results from the ability of tumour cells to invade secondary sites, leading to multifunctional organ failure and death. Inside the primary tumour, cancer cells utilise autocrine and paracrine growth signals provided by other tumour cells and stroma cells [40,41]. Furthermore, within the blood circulation, platelet-cloaked tumour cells can bypass natural killer cell-mediated cytotoxicity [15].

Platelets are a major storage site for TGFβ1 [26]. Activated platelets can supply sufficient TGFβ1 to enable successful metastasis of tumour cells. Tumour cells primed with platelets in vitro showed increased metastasis after injection into mice [2,8]. Mechanistically, platelet-derived TGFβ1 acts via the p-Smad pathway to induce a phenotypic conversion in cancer cells, from epithelial to mesenchymal-like cells, capable of invading extracellular matrices, migrating and surviving in the blood circulation [42,43]. Epithelial-mesenchymal transition (EMT) is characterised by upregulation of mesenchymal-like proteins such as N-cadherin, Slug, Snail, vimentin and either downregulation, translocation or loss of function of epithelial-like proteins such as E-cadherin and claudin-1 (reviewed by Xu et al.) [44]. Soluble platelet-derived factors (mainly TGFβ1) and direct physical contact with tumour cells (activating NF-κB pathway) work synergistically to induce EMT and subsequent migration and metastasis [2,45].

In addition to their pro-EMT role, Labelle et al. [46] provided evidence for the prominent role of platelets in early metastatic niche formation. Platelet-derived, but not tumour-derived signals, as well as platelet aggregates around tumour cells, are essential for granulocyte recruitment to the early metastatic niche [46]. Platelet-derived chemoattractants such as C-X-C motif ligands (CXCL5/7) specifically induce deployment of granulocytes and not monocytes, lymphocytes or NK cells to the early metastatic niches [46]. Similarly, Orellana et al., recently demonstrated the chemotactic effect of platelets on ovarian cancer cells, with the subsequent phenotypic change favouring a mesenchymal

phenotype [47]. Therefore, platelets not only provide survival signals for tumour cells but also recruit host cells to the disseminated tumour foci.

Besides TGFβ1 and chemo-attractants, activated platelets also secrete autotaxin (ATX); an enzyme with phospholipase D activity that generates LPA from lysophosphatidylcholine and contributes to cancer progression by promoting cancer proliferation, angiogenesis and metastasis [48–52]. Moreover, platelet-derived microvesicles contain micro-RNA-233, which can enhance lung cancer cell invasion by directly targeting the tumour suppressor EPB41L3 [13]. Platelets appear to be integral to the cancer metastasis processes either by directly interacting with cancer cells, attracting other host cells to the hetero-aggregate site, or even by attracting tumour cells to a location rich with survival factors (Figures 1 and 2).
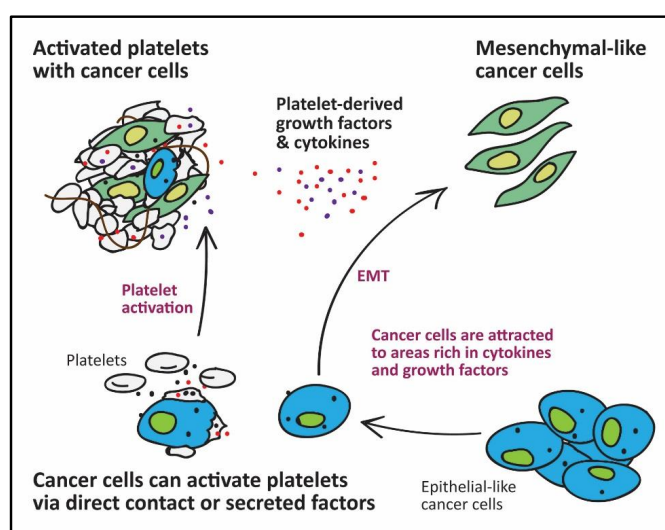
Figure 1. Platelets in Metastasis: Cancer cells can activate platelets. Activated platelets secrete growth factors and chemokines to attract other cancer cells to areas rich in survival factors. Platelet TGFβ1 induces EMT in cancer cells, which are characterised by an elongated shape and improved metastatic ability.
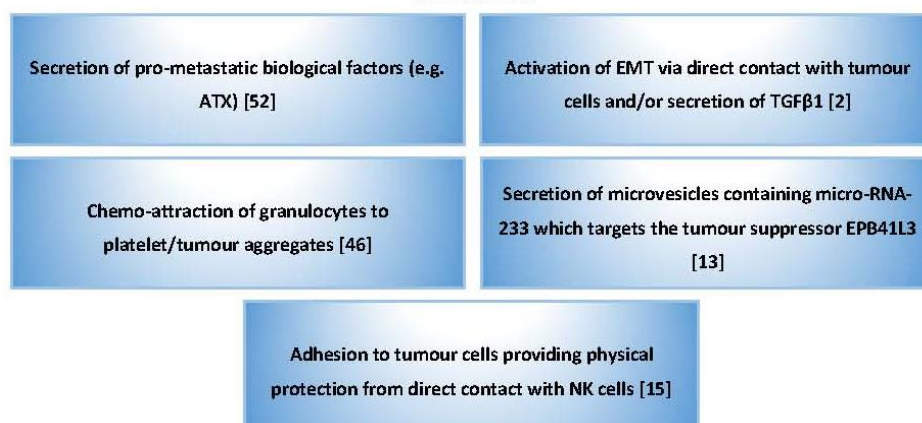

Figure 2. Summary of the pro-metastatic properties of platelets in cancer. Platelets promote cancer progression by releasing an array of pro-metastatic biological factors and by shielding cancer cells from NK-mediated cytotoxicity.

3. The Role of Platelets in Tumour Angiogenesis

The tumour microenvironment plays a major role in cancer progression. In 1971, Folkman [53] described the requirement of endothelial cell-mediated neovascularization for tumour growth and survival. Tumour cells release growth factors that stimulate angiogenesis, defined by the regeneration of endothelial cells to form new blood vessels from pre-existing ones. Newly formed blood vessels supply the dividing tumour cells with blood and oxygen. Thus, angiogenesis is a fundamental process for tumour growth and survival, which is governed by a balance between pro- and anti-angiogenic factors [53].

Platelets contain a diverse range of biological molecules that can regulate angiogenesis [25], for example, dopamine and serotonin, neurotransmitters synthesised in the central nervous system with a well-established role in mediating numerous neurological and psychological processes. Drugs that target their synthesis are clinically available for controlling several pathological conditions [54,55]. In the blood circulation, dopamine and serotonin are predominantly stored in the dense granules of platelets and released upon platelet activation [21,56]. Dopamine plays a significant role in inhibiting angiogenesis. Intraperitoneal injection of dopamine was able to block angiogenesis and tumour growth in an in vivo mouse model where it inhibited VEGF/vascular permeability factor (VPA)-mediated proliferation and migration of human umbilical vein endothelial cells (HUVEC) [22]. Interestingly, daily dopamine use blocks stress-mediated tumour growth and angiogenesis in vivo [57]. The activity of dopamine was attributed to its action on the dopamine-2-receptor on endothelial cells, resulting in impaired VEGF-mediated phosphorylation of VEGF receptor-2 (VEGF-R2) [22,58,59]. It has been hypothesised that dopamine induces endocytosis of VEGF-R2, resulting in fewer receptors available for VEGF binding. Furthermore, pretreatment of HUVEC with dopamine hampers the disruptive effect of VEGF on zonula occuldens (ZO-1) protein, a tight junction protein that preserves endothelial cell-cell adhesion [22,60].

In addition to its role in promoting cancer cell proliferation [61–63], serotonin can activate angiogenesis by promoting proliferation of endothelial cells through the activation of several signalling kinases; for example, Src, PI3K, AKT, ERK, and mTOR. Interestingly, the downstream signalling pathway mediated by serotonin is shared with VEGF [23].

VEGF is a chemotactic vascular permeability factor stored in α-granules and released from activated platelets. It promotes angiogenesis through VEGF-R2 on endothelial cells [64,65]. Tumour VEGF stimulates the release of von Willebrand factor from endothelial cells. Together with increased permeability of endothelial cells and exposure of subendothelial proteins like collagen, platelets are attracted, activated and adherent to the tumour-activated endothelial cells (hypothesis by Pinedo et al. [66]). VEGF action is transduced via tyrosine kinases and ultimately leads to endothelial cell proliferation and formation of new blood vessels to boost tumour growth and survival [67]. Activated platelets can secrete either pro- or anti-angiogenic factors, depending on the nature of the stimulant [24,68]. In addition to their ability to release angiogenic factors upon activation, platelets can also sequester VEGF, as evidenced by the preferential accumulation of VEGF in platelets compared to plasma or other cells after subcutaneous injection of radiolabeled VEGF into mice [69,70]. Wu et al., have recently demonstrated that non-small cell lung cancer (A549)-activated platelet releasate can stimulate angiogenesis even in the presence of VEGF neutralising antibody [71]. Therefore, platelet-derived VEGF contributes to tumour angiogenesis but is not essential as activated platelets can also release microvesicles and exosomes to induce expression of angiogenic factors in cancer cells [11].

Platelet-derived angiogenic factors are also relevant as useful prognostic markers. For example, platelets isolated from breast cancer patients contain a higher level of VEGF and angiopoietin 1 (Ang-1), while platelets from prostate cancer patients show a greater level of VEGF but not Ang-1 [72]. Similarly, platelets isolated from breast cancer patients display a higher degree of pro-angiogenic and metastatic growth factors; for example, TGFβ1, VEGF, and platelet-derived growth factor (PDGF), compared to a control group [73]. Platelet VEGF levels correlate with angiogenesis and staging in non-Hodgkin lymphoma [74]. Peterson et al., have evaluated the normal physiological ranges of angiogenesis

regulators relevant to monitoring cancer prognosis and response to anti-angiogenesis therapy [75] (see Table 1).

Table 1. Normal physiological levels of angiogenic factors in platelets.

Angiogenic Factor	Normal Physiological Level in 10^6 Platelets (Median (Range))
VEGF	0.68 (0.02–1.47) pg [75], 0.9 (0.1–2.3) pg [73]
PDGF	21 (12–33) pg [75], 19.1 (9.3–48.9) pg [73]
PF4	10 (2.4–22) ng [75], 10.2 (4.2–20.5) ng [73]
TSP-1	27 (7–54) ng [75]
bFGF	0.42 (0.15–0.75) pg [75]

4. The Role of Platelets in Tumour Growth

The ability of cancer cells to trigger secretion of growth factors from platelets has attracted a widespread interest of a potential role of platelets in cancer cell growth and proliferation. Platelets are a rich source of biologically active molecules, and there is evidence that different platelet agonists can elicit different patterns of release from platelets [24,76,77]. Accordingly, various cancer cells could potentially induce different patterns of platelet secretion. Accumulating evidence has established a pro-metastatic role of platelets in cancer. However, the impact of platelets on cancer cell proliferation is controversial, confounded by variable findings in different cancer cell types.

More than 30 years ago, Ibele et al. [78] suggested that platelets may play a role in host defence against malignant tumours. Moreover, monocytes in the presence of platelets showed higher tumour killing capacity compared to monocytes alone. Surprisingly, aspirin decreased the cytotoxic effect of platelets on tumour cells, highlighting a potential toxic effect of platelet arachidonate metabolites on cancer cells [78,79]. Similarly, unstimulated and thrombin-activated platelets showed tumoricidal activity with the chronic myelogenous leukaemia cell line, K562, an effect that was completely blocked by esterase inhibitors in unstimulated platelets but not in thrombin-activated platelets. In contrast, some cancer cell lines were resistant to the cytotoxicity of platelets. The authors proposed an explanation for their findings in concordance with emerging studies that established a pro-metastatic role of platelets; the cytotoxic effect of platelets being mainly relevant to sensitive cancer cells while resistant cells were not affected. The formation of hetero-aggregates of platelets and resistant tumour cells on the endothelial surface would trigger the release of cytotoxic factors from platelets, causing injury to the endothelium, thus creating pores for resistant cancer cells to penetrate and metastasize [80]. Therefore, the cytotoxic effect of platelets may promote the survival of aggressive cancer cells.

Platelets contain immune defence factors such as pro-apoptotic members of the tumour necrosis factor (TNF) family, including tumour related apoptosis inducing ligand (TRAIL), TNFα, CD154 and Fas ligand (Fas-L) [81]. Fas-L is expressed and released by platelets after activation with ADP or thrombin. The interaction between Fas-L/Fas receptor leads to activation of the caspase-mediated apoptosis pathway in tumour cells that express Fas-R, such as adult T-cell leukaemia (CEM) [82]. Using mouse cancer cell lines and platelets, Wang et al., also examined the impact of platelets on cancer cell proliferation. Although platelets did not activate apoptosis in various cancer cell lines, they decreased proliferation by inducing cell cycle arrest [83].

In contrast to the reports of tumoricidal activity mentioned above, platelets augment the proliferation of ovarian cancer cells in vitro and in vivo, independent of platelet adhesion to cancer cells, an action mediated mainly through TGFβ1 [84,85]. Additionally, a recent paper by Haemmerle et al. also highlights an important role of platelet's protein, focal adhesion kinase (FAK), in mediating platelet infiltration and tumour growth in ovarian cancer mouse model [85]. Thrombocytosis, commonly referred to a platelet count >400–450,000 per millilitre of blood, is observed in approximately one-third of women who have been recently diagnosed with ovarian cancer [86–88]. In addition to thrombocytosis, reports of thrombophilia (a hypercoagulable state) and tumour-infiltrating platelets

are closely associated with an advanced-stage disease, and a poor prognosis [86,89]. It has been proposed that the high platelet count is the result of tumour-derived plasma interleukin-6 (IL-6), which can mediate the synthesis of thrombopoietin (a hormone responsible for regulating platelet production) in the liver to stimulate platelet production [86].

Infiltration of platelets into solid tumours has also been demonstrated in colorectal cancer, hepatocellular carcinoma, breast cancer and gastric cancer, and their presence was associated with tumour growth in insulinoma and melanoma mouse models [90]. More recently, Pucci et al., have delineated the impact of PF4 on cancer progression; PF4 enhanced platelet production and accumulation at the tumour site, which accelerated lung adenocarcinogenesis in a genetically modified mouse model. Similarly, platelets exert a pro-proliferative effect on a panel of hepatocellular carcinoma cell lines by activating the MAPK pathway and decreasing apoptotic effectors. However, the nature of the growth factor(s) responsible for this effect was not defined by the authors [91].

In 1984, Tucker and colleagues demonstrated a role for platelet TGFβ1 in the proliferation of different cell lines (mouse embryo-derived cells (AKR-2B), rat kidney-derived cells (NRK), African green monkey kidney cells (BSC-1) and mink lung cells (CCL-64). Interestingly, platelet TGFβ1 showed different (stimulatory or inhibitory) effects on proliferation based on the experimental conditions (cells growing as unattached rounded cells in soft agar or as an adherent monolayer) [92]. Likewise, Roberts et al., demonstrated a bifunctional role of TGFβ1 on different human cancer cell lines. In their study, TGFβ1 significantly reduced colony formation with the lung cancer cell line (A549) and breast cancer cells (MCF-7), while it had no effect on colon cancer cells (HT-29). Also, TGFβ1 could either potentiate or antagonise the effect of other growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [93].

Table 2. Commonly used anticancer drugs and risk of thrombocytopenia [94].

Drug	Side Effects
Alkylating Agents	
Cyclophosphamide	Lesser effect on peripheral platelet count compared to other alkylating agents.
Ifosfamide	Greater suppression of platelet count than cyclophosphamide
Carmustine	Delayed and prolonged suppression of platelet count, reaching a nadir at 4–6 weeks after administration, with slow reversal
Busulfan	Prolonged and cumulative effect lasting months or years
Thiotepa	Delayed effect compared to cyclophosphamide with platelet nadir at 3 weeks
Streptozocin	Suppression of platelet count in 20% of patients
Dacarbazine	Mild suppression of platelet count which is reversible within 1.2 weeks
Temozolomide	Similar to dacarbazine
Procarbazine	Suppression of platelet count after one week of initiating treatment and reversed within two weeks off treatment
Platinum analogues	
Cisplatin	Transient thrombocytopenia
Antimetabolites	
Methotrexate	Effect on platelets is completely reversed within two weeks. However, prolonged suppression may occur in patients with compromised renal function.
5-Florouracil	Thrombocytopenia, less often with infusion compared to bolus regimen
Cytarabine	Potent myelosuppression with severe thrombocytopenia
Gemcitabine	Mild haematological toxicities [95]. Myelosuppression is more prominent with longer duration infusion.
6-mercaptopurine	Gradual thrombocytopenia
Cladribine	Cumulative thrombocytopenia with repeated administration.
Others	
Topotecan	Neutropenia with or without thrombocytopenia.
Etoposide	Infrequent thrombocytopenia, which is usually, not severe.
Bleomycin	Minor myelosuppression
Mitomycin	Marked thrombocytopenia
Hydroxyurea	Occasional thrombocytopenia
Vorinostat	Thrombocytopenia is more prominent with intravenous administration.

Overall, the secretome of platelets contains a multitude of biologically active factors, with a net effect dependent on the interactions between platelet-derived factors, tumour-derived factors and

tumour receptors. In addition, the role of platelets in tumour growth is highly dependent on tumour type; as different platelet stimuli have been suggested to trigger a different pattern of platelet release, cancer cell-induced platelet secretion may function in a similar way [24,96]. One of the possible reasons for the contradictory results of some of the early in vitro studies is the variable ratio of platelets to tumour cells, which may differ from the expected ratio in cancer patients. For example, thrombocytosis is often found in cancer patients before treatment (surgery, chemotherapy or radiotherapy) [97,98]. However, use of some chemotherapeutic agents is associated with low platelet count (Table 2). Therefore, the platelet to cancer cell ratio varies through the course of the disease and is highly affected by treatment. Nonetheless, studies that have utilised genetically modified mouse models indicate a positive influence of platelets on tumour growth (Table 3). Moreover, some of the platelet-derived biological factors (e.g., PDGF, TGFβ1, ATX) have been studied independently of platelets and showed an active role in cancer progression [44,48,50,99].

Table 3 summarises the current understanding of the role of platelets on tumour growth. Unlike numerous in vitro studies which suggest an anti-tumour effect of platelets, the pro-tumour role of platelets is well established in several mouse models [100].

Table 3. Summary of reported platelet effects on tumour growth.

Platelets Decrease Tumour Growth	Platelets Enhance Tumour Growth
In in vitro experiments, platelets showed a cytotoxic effect on cancer cells (Malme, a melanoma cell line, and 786, a renal carcinoma cwll line). The platelet effect was abrogated by aspirin [78]	Platelet-derived TGFβ1 enhances ovarian cancer growth in vitro and in vivo [84].
Platelets kill tumour cells (LU99A, a lung cell line, and K562, a chronic myeloid leukaemia cell line) via cyclooxygenase or nitric oxide-dependent pathways [79].	Platelets promoted proliferation of cancer cells (PLC/PRF/5, Hep3B and HepG2 cells hepatocellular carcinoma cell lines) in vitro via activation of the MAPK pathway [91].
Platelets kill tumour cells via activation of an apoptosis pathway in cancer cells (CEM, leukaemia cell line) through interaction between platelet-derived FAS-L and FAS receptor on the cancer cell [82].	Platelets enhance the growth of an SKOV3 human ovarian cancer xenograft [101].
Platelets prevented murine cancer cell growth (EG7 (H-2^b), L1210, YAC-1 (H-2^a) lymphoma cell lines, B16 H-2^b, a melanoma cell line, and RM1 (H-2^b), a prostate cancer cell line) by inducing cell cycle arrest rather than activating apoptosis [83].	Deposition of platelets in a solid tumour, as well as tumour growth (pancreatic islet insulinoma), was significantly reduced in P-selectin deficient mice [90].
	In a genetically modified lung cancer mouse model, PF4 enhanced platelet production and accumulation in the lung, which accelerated cancer progression [30].
	Platelets enhance the proliferation of colon and pancreatic cancer cells by upregulating the oncoprotein c-MYC [102].

5. The Role of Platelets in Chemotherapy Resistance

The ability of malignant tumours to grow despite chemotherapy is considered a significant contribution to treatment failure and low survival rates associated with highly resistant types of tumours, such as pancreatic cancer. Malignant tumours are usually made up of multiple populations of cancer cells which differ in their metastatic ability and response to chemotherapy. The more resistant cancer cells eventually dominate the tumour as the more sensitive cells are eradicated by chemotherapy. The principal mechanisms mediating chemotherapy resistance are enhanced proliferation of cancer cells through activation of the MAPK signalling pathway, activation of anti-apoptotic proteins, or phenotypic conversion in cancer cells through the epithelial-mesenchymal transition, all of which could potentially be affected by platelets. The platelet secretome is rich in growth factors and is used clinically to enhance tissue regeneration [103]. During the wound healing process, platelets display a pro-proliferative role through the secretion of various growth factors. Platelets thus possess the ability to counter the anti-proliferative effect of chemotherapeutic agents.

Currently, only a few studies have examined the contribution of platelets to chemotherapy resistance. In 2012, Balicka et al. demonstrated a role for platelets in paclitaxel and 5-fluorouracil resistance in colon (Caco-2) and ovarian (59 M) cancer cells [27]. Platelets and their releasate

antagonised the cytotoxic effect of paclitaxel and 5-fluorouracil via several complementary mechanisms. First, by shifting the balance between anti-apoptotic and pro-apoptotic genes towards cell survival through upregulation of anti-apoptotic proteins such as NFκB1. Second, by blocking cell cycle arrest caused by the anticancer drugs. This occurred through upregulation of cyclins, the principal regulators of cell cycle progression. Third, platelets also enhanced the phosphorylation of DNA repair proteins, for example, Chk1, BRCA1, and Mre11. Moreover, platelets upregulated the MAPK signalling pathway, which is involved in cell growth, invasion, and migration [27]. Similarly, D'Alessandro et al. recently demonstrated the ability of platelet factors to hinder the cytotoxicity of the chemotherapy drugs, sorafenib and regorafenib, in hepatocellular carcinoma by increasing the phosphorylation of ERK, p38 and by inhibiting the induction of apoptosis Moreover, platelets also counteracted the efficiency of both drugs in halting cancer cell migration and invasion [104]. Clinically, chemotherapy resistance has been correlated with high platelet count [105].

Platelets also drive EMT in cancer cells with a subsequent increase in migration, invasion and metastasis [2]. The presence of platelets around breast cancer primary tumour cells was associated with EMT morphological features and chemotherapy resistance [106]. Independent of platelet activity, EMT has been shown to impart chemotherapy resistance in lung [107], pancreatic [108], breast [109] and ovarian cancers [110]. Zheng et al., demonstrated using a pancreatic adenocarcinoma mouse model with deleted mesenchymal transcriptional factors, Snail and Twist, that EMT inhibition did not prevent metastasis but contributed significantly to enhanced gemcitabine sensitivity [111]. Pancreatic cancer is a highly metastatic type of cancer, and is known to trigger platelet activation, aggregation, and secretion [31,112,113]. Platelets are considered the primary source of TGFβ1 in the blood circulation, which is a primary inducer of EMT [2]. Therefore, targeting the activity of platelets in cancer may not only diminish cancer metastasis but also suppress chemotherapy resistance. Chemotherapy in combination with antiplatelet therapy may thus represent a potential approach to overcome tumour chemoresistance.

6. The Effects of Cancer Cells on Platelets

Cancer cells can directly trigger platelet activation by releasing factors that act as agonists or by direct physical contact [20,114,115]. One of the best characterised mechanisms of tumour cell-induced platelet activation is through podoplanin/CLEC-2 interaction. Podoplanin (PDPN) is a transmembrane sialoglycoprotein highly expressed on metastatic cancer cells. It is also found on tumour-initiating cells and is associated with poor prognosis in lung adenocarcinoma [116]. PDPN can trigger platelet activation, aggregation and secretion through interaction with the CLEC-2 receptor on the surface of platelets. MS-1, an anti-PDPN antibody, which blocks PDPN/CLEC-2 interaction, significantly reduced tumour metastasis and tumour growth in vivo [117]. Direct contact with platelets, however, is not always required to trigger activation as ADP released from cancer cells can activate platelet $P2Y_1$ and $P2Y_{12}$ receptors [9,118,119], while an unknown factor released from prostate cancer cells can instigate activation of platelets through the FcYRIIa receptor [115].

Moreover, cancer cells can activate platelets indirectly through the coagulation pathway. The procoagulant potential of different tumours is highly dependent on their expression of tissue factor (TF). TF mediates thrombin generation through activation of the extrinsic pathway of coagulation, which can directly activate platelets [120]. Tissue factor has also been found in tumour-derived microvesicles associated with enhanced venous thromboembolism in mice [121]. Cancer cells of different origin express varying levels of TF. For example, the pancreatic cancer cell line BXPC3 expresses a higher level of TF compared to the breast cancer cell line MCF7 [122]. Notably, pancreatic cancer is highly associated with venous thromboembolism, which can be related to TF either expressed or released by pancreatic cancer cells [31,121,123]. On the other hand, platelets can promote TF expression in cancer cells as shown with ovarian cancer cells co-cultured with platelets [47]. Ovarian cancer is also associated with a high risk of venous thromboembolism [124]. In addition to tissue factor, cancer cells can activate platelets indirectly through eliciting neutrophils to release

neutrophil extracellular trap (NET). NETs are an extracellular mesh of DNA associated with histones, elastases and myeloperoxidase (MPO), previously known for their antimicrobial function [125]. Recent studies have shown the ability of cancer cells to prime and induce neutrophils to generate NETs which are associated with thrombus formation [126,127]. Furthermore, NETs can instigate platelet activation and aggregation [128–130].

As mentioned earlier, cancer cell adhesion to platelets is vital for successful metastasis, which can be mediated through surface proteins and predispose platelet activation. For example, interaction between integrins (transmembrane glycoproteins) expressed on platelets (e.g., αIIbβ3), and some types of tumour cells via ligands that are normally present in plasma such as fibrinogen and fibronectin can lead to activation of platelets. Integrins are involved in tumour-platelet adhesion and subsequent tumour arrest within the blood circulation [17,131]. In addition to integrins, tumour cells can adhere to platelets via the P-selectin ligands. PSGL-1 as acts as a P-selectin counter-receptor in non-small cell lung cancer cells, multiple myeloma cells, and prostate cancer cells [19,132,133]. Other P-selectin ligands, CD24 and CD44, are found on breast cancer cells and colon cancer cells, respectively [134,135]. In mice, engagement of P-selectin by PSGL-1 results in platelet activation and enhanced micro-aggregate formationwhile P-selectin null mice display inadequate thrombus formation [136]. Finally, podocalyxin is a membrane mucin protein expressed by testicular cancer cells that can also mediate platelet adhesion via P-selectin and integrins [137]. Figure 3 summarises the effects of cancer cells on platelets.
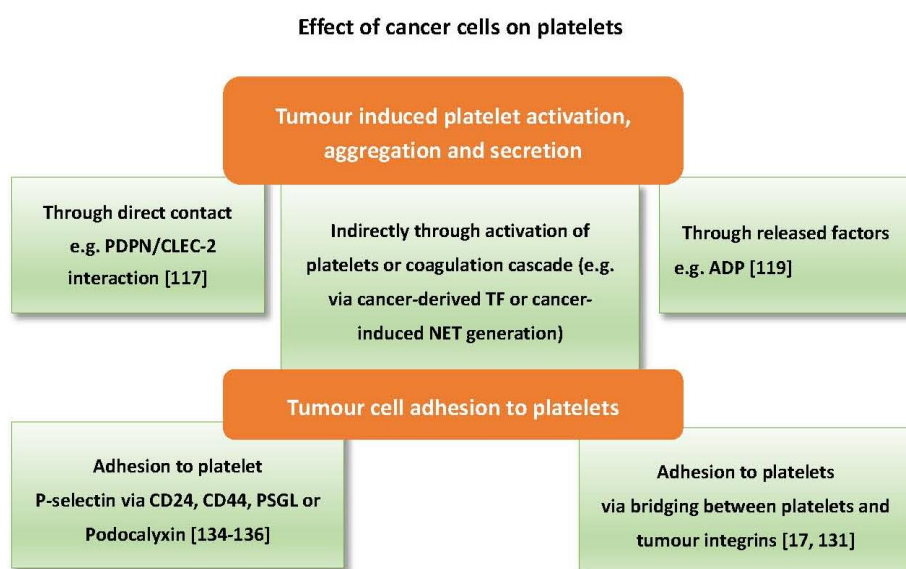
Figure 3. Summary of cancer cell-platelet interactions. Cancer cells can activate platelets through expression or release of platelet agonists (e.g., PDPN and ADP). Cancer cell-derived tissue factor (TF) can also indirectly activate platelets via the coagulation cascade and generation of thrombin. Cancer cells can also express ligands (e.g., CD24, PSGL, and integrins) that facilitate cancer cell-platelet adhesion.

7. Challenges to Antiplatelet Therapeutic Approaches in Cancer

There is increasing evidence supporting an active role of non-cancer cells within the tumour microenvironment in cancer progression, thus introducing additional strategies in cancer therapy in which different classes of drugs could be combined to target different cell types that collectively would impede tumour growth and metastasis. Available preclinical data provide examples of this approach; for instance, in a mouse tumour model, a treatment combining low dose cyclophosphamide with the thrombin inhibitor, Dabigatran etixulate, reduced tumour growth and metastasis through

potentially limiting tumour-platelet crosstalk [138]. In addition to its role in the direct activation of platelets, thrombin generates fibrin. Some cancer cells can also release thrombin [114].

The use of antiplatelets in cancer therapy may be confounded due to declining platelet function as a consequence of disease progression, myelosuppressive chemotherapy and/or radiotherapy (reviewed by Liebman) [139]. For example, platelets obtained from thrombocytopenic cancer patients before platelet transfusion have shown impaired responses to thrombin, collagen-related peptide, and ADP as measured by αIIbβ3 activation and P-selectin translocation [140]. Thus, bleeding risk needs to be carefully evaluated, especially in cancer patients with comorbidities such as cardiovascular disease, before use of any antiplatelet drug. Although there is a well-established pro-metastatic role of platelets in cancer, the effect of platelets on cancer progression could vary based on type and stage of the tumour. Thus, the addition of antiplatelet treatment to cancer therapy should be individualised based on the clinical and experimental evaluation. Many factors must be considered before deciding to administer antiplatelets in cancer therapy; these include the risk of bleeding, comorbidity, chemotherapy and radiotherapy dose and duration, drug interaction, and type and stage of the tumour. Table 2 highlights the effect of a group of cytotoxic drugs on the platelet count. Platelet studies and presence of specific markers of platelet activation are among the experimental factors that should be assessed before administering antiplatelet drugs to cancer patients. Figure 4 highlights potential challenges with targeting platelets in cancer therapy.
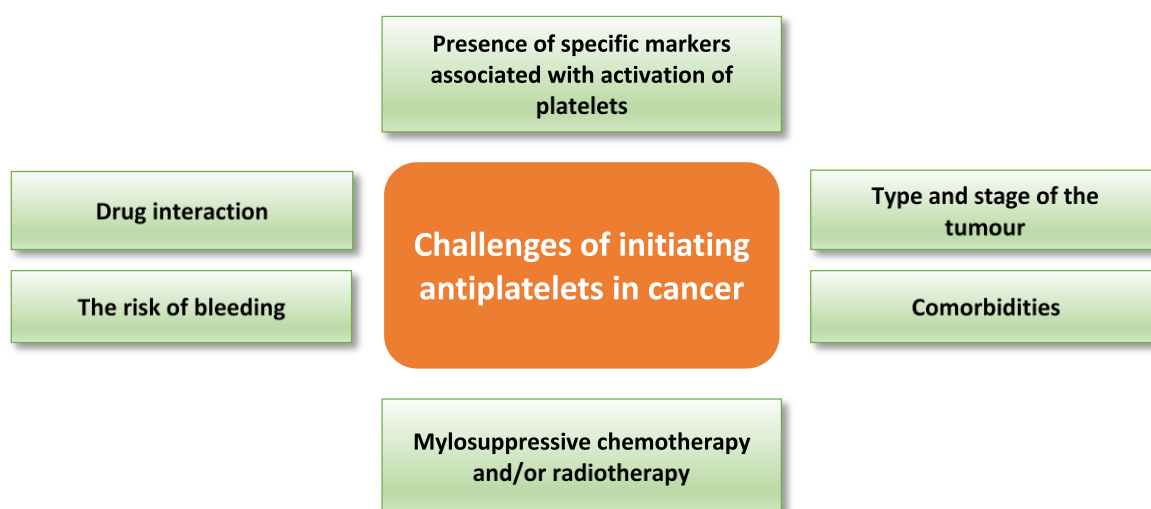
Figure 4. Potential challenges in targeting platelets during cancer therapy. Clinical implementation of antiplatelets in cancer may face several confounders which can be patient-related or therapy-related.

8. Clinical and Preclinical Use of Antiplatelet Therapies in Cancer

8.1. Aspirin in Cancer

The impact of a common household drug, aspirin, on cancer progression has attracted considerable interest. Here, we present an overview of the anti-platelet and anti-metastatic efficacy of aspirin. Despite the encouraging results from preclinical models and the molecular rationale, the results obtained in human trials are less clear.

8.1.1. Preclinical Studies

In 1962, Gasic et al. reported a reduction in metastasis of TA3 tumour cells in mice injected with *Vibrio* Cholera neuraminidase (VCN); a potent thrombocytopenic agent [141]. In a subsequent study by the same group, tumour cells that were able to aggregate platelets in vitro showed more lung metastasis compared to tumour cells devoid of this ability. Furthermore, platelet-deficient mice showed

reduced lung metastasis from tumours that aggregated platelets in vitro. In contrast, tumour cells that did not aggregate platelets in vitro still formed metastases in thrombocytopenic mice although the number of metastatic foci was fewer. Interestingly, aspirin significantly decreased lung metastasis without affecting the size of the primary tumour [6].

In another study, pre-incubating platelets with aspirin inhibited murine sarcoma cells (mFS6)-induced platelet aggregation [142]. Similarly, Bradley et al., demonstrated a pro-aggregation effect of uterine carcinosarcoma (Colo 562) cells on human washed platelets. However, aspirin did not prevent platelet adhesion to tumour cells, platelet secretion or micro-aggregate formation [143]. Further, in an in vivo model, aspirin reduced lung metastasis of rat mammary carcinoma (Mtln3) but did not provide an additive effect when combined with the fibrinolytic agent, streptokinase, which itself caused a significant reduction in metastasis [144]. In contrast, a combination of aspirin and ATP102 (an ADPase) significantly decreased breast cancer and melanoma bone metastasis in mice. However, each alone did not show an anti-metastatic effect [143].

Although aspirin is a potent inactivator of cyclooxygenase-1 (COX-1), thus an inhibitor of platelet function, its failure to demonstrate an anti-metastatic role in some studies may be due to the ability of the tumour to activate platelets efficiently without COX-1-dependent synthesis of thromboxane A2 (TxA2), a hormone responsible for promoting platelet activation and aggregation. For example, limited effect of aspirin has been observed on platelet activation, aggregation and adhesion with agonists such as ADP, thrombin, high-dose collagen and elevated shear stress [145–148].

8.1.2. Clinical Studies

The therapeutic use of aspirin has been extensively studied in colon cancer. In 1988, Kune and colleagues investigated the association of risk of colorectal cancer with medication use and found a statistically significant lower incidence of colorectal cancer cases among users of aspirin-containing medication [149]. In the subsequent APACC trial, 272 patients with a history of colorectal adenomas (an early sign of abnormal cell growth in the colon) were randomised to daily lysine acetylsalicylate (160 or 300 mg/day) or placebo for four years. The daily use of soluble aspirin showed a positive effect in reducing adenoma recurrence after one year of starting treatment as confirmed by colonoscopy [150]. In a larger double-blinded clinical trial involving 1121 patients with a recent history of adenomas, daily use of low-dose aspirin (81 mg) showed a moderate reduction in the incidence of one or more adenomas compared to placebo after one year [151]. A significant decrease in the size of polyps in patients with familial adenomatous polyposis was observed in the aspirin group compared to the placebo group in a separate randomised double blinded clinical trial [152]. In all the above studies, aspirin showed a positive effect, decreasing the very early stages of carcinogenesis. In addition to its protective effect, aspirin (81 to 325 mg once or more per day) use after diagnosis was associated with improved overall survival and decreased colorectal cancer-specific and overall mortality [153]. In the CAPP2 randomised controlled clinical trials in patients with Lynch syndrome (genetic mutations that increase the chance of developing cancer), regular use of aspirin (600 mg/day) also reduced the incidence of cancer [154]. Cao et al., have recently reported the results of a 32 years follow-up study which corroborated previous findings showing a positive effect of long-term aspirin use (81 to 325 mg at least two times a week) in reducing the incidence of cancer, especially gastrointestinal tumours [155]. Moreover, Frouws et al., have demonstrated that aspirin use (100 mg/day or lower) after diagnosis can significantly improve overall survival of gastrointestinal cancer [156]. Risch et al., have also documented a reduction in risk of pancreatic cancer by regular use of aspirin [157].

Since cancer and cardiovascular disorders are more prevalent in the elderly population, retrospective analysis of patient data pooled from large randomised clinical trials designed to examine daily aspirin in prevention of cardiovascular disease has been reviewed for an association between daily aspirin intake and incidence of cancer. In 2012, Rothwell and colleagues analysed data from five large randomised clinical trials of daily aspirin use ($\geq$75 mg) including the UK Thrombosis Prevention Trial (TPT). In the TPT trial, aspirin was formulated as slow release to inhibit platelet

function with minimal systemic bioavailability. In concordance with several animal studies, aspirin showed a reduction in cancer metastasis in the TPT trial consistent with a platelet-mediated effect [7]. Holmes and colleagues suggested a further reason for targeting platelets in cancer apart from it's effect on metastasis; they demonstrated a role of aspirin in decreasing VEGF levels released from thrombin-activated platelets and associated tamoxifen use. Selective oestrogen receptor modulators like tamoxifen are extensively used in hormonal responsive breast cancer and are associated with increased plasma and platelet-derived VEGF [158].

In breast cancer, aspirin use (75 mg/day) after diagnosis reduced all-cause mortality and breast cancer-specific mortality in an observational study. This study included 4627 patients with 22% of females prescribed aspirin after diagnosis [159]. More convincing evidence came from a large prospective observational study including 4164 females diagnosed with breast cancer between 1976 and 2002, who were followed up until 2006. Aspirin use was associated with decreased distant recurrence and death either from breast cancer or any other cause [160]. A recent meta-analysis has reviewed the association between aspirin use and mortality in breast cancer and concluded that there is a small positive effect of aspirin in improving survival in breast cancer patients [161].

Contrary to these studies, Murray et al., reported little evidence of an association between low-dose aspirin intake (75 mg/day in 97.1% of the cohort) after diagnosis and cancer-specific death in a cohort of breast cancer patients in the UK [162]. Furthermore, Holmes et al., described a non-association between low-dose aspirin use (75 or 160 mg once or more per day) and low risk of breast cancer-related mortality in a nested case-control study in Sweden [163].The reason for these conflicting results may be the lack of data on patient acquiescence (compliance) and non-prescription use of aspirin. A phase II randomised clinical trial failed to show any positive effect of dual antiplatelet therapy (aspirin (325 mg/day) and clopidogrel (75 mg/day after 300 mg loading dose)) in reducing the number of circulating tumour cells (CTCs) in patients with metastatic breast cancer, the dual antiplatelet therapy was well tolerated with significant platelet inhibition after one month. Small sample size and a small number (less than five) of CTCs at baseline in the majority of the study population precluded a clear finding on the effect of antiplatelet therapy [164].

Head and neck cancer patients with high platelet counts have more than two times higher death rate compared to patients with mid-normal platelet counts. Antiplatelet (including aspirin) use was associated with a higher overall survival rate, with a more pronounced effect in the group of patients with high platelet count [165]. A retrospective study by Furlan et al., had a similar finding, and the authors suggested a synergetic effect of aspirin with radiotherapy [166].

Overall, several studies have documented the positive effect of aspirin (low or standard dose) on cancer incidence and cancer associated mortality in gastrointestinal tumours. However, it is not clear whether the effect of aspirin is related to its direct effect on cancer, platelets, both or unidentified mechanism. Therefore, randomised clinical trials are required to assess the use of aspirin and/or other antiplatelet medications in types of cancers associated with high risk of thrombosis e.g., pancreatic and ovarian cancer.

8.2. Other Antiplatelet Strategies in Cancer Therapy

While there has been considerable interest on the use of aspirin in cancer due to its direct effect on tumour cells and also its antiplatelet activity other antiplatelet drugs have also been examined in the context of cancer. More detailed information on general antiplatelet strategies can be found in reviews elsewhere [167,168]. Table 4 highlights studies that examined antiplatelet drugs in combination with chemotherapy in animal models or analysis of cancer incidence in patients taking antiplatelet for non-cancer diseases.

In addition to using antiplatelets in cancer treatment, their use may offer additional benefits in controlling venous thromboembolism (VTE) associated with cancer. Cancer patients have a high risk of developing VTE, which is related to low survival rate [169]. The cancer site and type are among factors that determine the incidence and severity of VTE in cancer [170]. For example,

pancreatic cancer is associated with a high incidence of VTE [170–172]. Platelets are a key player in thrombosis, and several studies have shown a close interplay between pancreatic cancer cells and platelets [123,173]. Factors such as tumour cell induced platelet aggregation, and increased expression of pro-coagulant factors including tissue factor and thrombin, promote a pro-thrombotic state which ultimately contributes to the development of VTE [121]. Platelet count and activity may predict the risk of VTE in cancer patients [174,175]. The series of events that lead to cancer-associated VTE is still unclear; however recent studies indicate interactions between platelets, tumour and immune cells (especially neutrophils) can instigate the process. Neutrophil extracellular traps (NETs), generated from activated neutrophils, has been shown to act as a scaffold for platelet aggregation and thrombus formation [127,128]. Experimental evidence from infection and inflammation models suggests a role of activated platelets in promoting NET-derived thrombus formation [176–178]. Whether antiplatelet use may reduce the risk of NETs and VTE in cancer is still unknown.

Table 4. Examples of antiplatelet drugs investigated in the context of cancer.

Antiplatelet drugs	Study outcome	References
Dipyridamole and RA-233	In a pancreatic cancer mouse model, the combination of dipyridamole and RA-233 (cAMP-PDE inhibitor) reduced hepatic metastasis	[179]
Prasugrel	In the TRITON-TIMI 38 double-blinded randomised multicentre clinical trial of more than 13000 individuals assessing prasugrel versus clopidogrel in patients with acute coronary syndrome, prasugrel was associated with an increased incidence of gastrointestinal cancer. The exact mechanism is not entirely understood.	[180]
Clopidogrel	In prostate, breast and colorectal cancer patients, there was no increased risk of cancer-specific mortality among clopidogrel users. This study was in response to TRITON-TIMI 38 In a pancreatic cancer mouse model, clopidogrel reduced tumour growth, metastasis and thrombosis associated with cancer cell microparticle-derived tissue factor In a lung adenocarcinoma mouse model, clopidogrel reduced cancer growth and progression. [181]	[30,181,182]
Aspirin /Clopidogrel	In HBV transgenic mice, aspirin /clopidogrel delayed or prevented the development of hepatocellular carcinoma and improved the overall survival.	[183]
Clopidogrel with or without aspirin	In a large retrospective study involving 184,781 patients, use of clopidogrel with or without aspirin was associated with lower incidence of cancer	[184]
Ticagrelor	In melanoma and breast cancer mouse models, ticagrelor significantly reduced cancer metastasis and improved survival.	[185]

9. Conclusions

The past 50 years have witnessed considerable advancement in our understanding of the tumour microenvironment and its role in cancer progression. Gasic et al. [6] provided the first experimental data for a pro-metastatic role of platelets in cancer. The accumulating evidence has since established the experimental rationale for targeting platelets in cancer.

Concomitant use of antiplatelet therapy in cancer patients carries both benefits and risks. Further collaboration between clinicians and research scientists is needed to investigate side effects and antiplatelet drug interactions with chemotherapeutic medications. The presence of specific cancer-related biomarkers could potentially predict patient response or necessity for antiplatelet therapy. For example, podoplanin is a potent platelet agonist and has been shown to be upregulated in several types of tumours. Aspirin is both an inhibitor of podoplanin-induced platelet aggregation in vitro and metastasis in vivo. Hence, podoplanin expression on tumour cells could serve as a predictive biomarker for individualised therapy.

Whether the addition of antiplatelet treatment alongside chemotherapeutic medication could increase therapeutic efficacy by reducing resistance, needs to be addressed. Nevertheless, the available evidence suggests targeting platelets in cancers known to have a high risk of thrombotic events, e.g., pancreatic and ovarian cancers, is therapeutically beneficial.

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Chapter 2:

Neutrophil extracellular trap-induced platelet aggregation: mechanisms and therapeutic implications in cancer.

2.1.   Publication

1.   **Elaskalani O,** Abdol Razak NB, Metharom P. *Neutrophil extracellular traps induce aggregation of washed human platelets independently of extracellular DNA and histones.* Cell Commun Signal. 2018 May 29;16(1):24. doi: 10.1186/s12964-018-0235-0.

2.2.   Introduction

Cancer-associated thrombosis (CAT) is a serious complication in cancer patients. CAT can cause debilitating secondary diseases such as deep vein thrombosis (DVT) and pulmonary embolism (PE), as well as impairing the patient's response to chemotherapy and shorten overall survival [23,248]. Recently, it has been shown that platelet-derived cytokines are necessary for attracting inflammatory immune cells, such as neutrophils, to the early metastatic niche [42,249]. Platelets are capable of activating neutrophils to release neutrophil extracellular traps (NETs); mesh-like structure primarily composed of DNA and histones. Originally thought to be released mainly during infection to trap and kill the invading pathogens, NETs release is now documented in several pathological conditions [41]. NETs can form a platform for platelet adhesion and thrombi formation [48,250]. Mechanistically, it has been shown that neutrophils DNA and histones, but not intact NETs, are able to activate coagulation *in vitro*. This thesis demonstrated that NETs can directly activate platelet aggregation, which indicates that platelets are the primary target of NETs in thrombosis. Publication 1 in this chapter (a copy is attached) investigated the mechanisms underlying NETs-induced platelet aggregation. The following sections review the most recent literature in regards to the role of platelets in NET production in cancer, the role of NETs in cancer metastasis, and whether targeting platelets can negate the contribution of NETs to cancer progression.

2.2.1. Platelets, an accomplice in tumour-induced NETosis

During NETosis, activated neutrophils expel a net of DNA, histones and neutrophils proteases. Our laboratory has shown for the first time that human washed platelets exposed to cancer, but not normal cells, can directly induce NETosis *in vitro* [6]. An essential mechanism of platelet-induced NETosis is through the binding of platelet P-selectin to neutrophil P-selectin glycoprotein ligand-1 (PSGL1) [251]. Another mechanism is through platelet Toll-like receptor 4 (TLR4). Bacterial lipopolysaccharide (LPS), which can be present during an infection, can activate platelet TLR4, and this interaction has been reported to prime and trigger NETosis [252]. Recently, it has been demonstrated that platelet-derived high mobility group box 1 (HMGB1) protein can induce neutrophils to release NETs [99,253]. HMGB1 is a damage-associated molecular pattern which can induce autophagy in endothelial cells and neutrophils [254]. Autophagy is a process by which stressed cells digest their organelles to generate anabolic substrates, and is a known mechanism for NETosis [255].

The previous mechanisms of platelet-induced NETs might be disease-specific since our laboratory showed that platelets activated by cancer cells, but not by the physiological agonists thrombin or collagen, were able to induce NETs [6]. Both cancer cells and collagen or thrombin can induce platelet activation and P-selectin exposure on platelets [192]. Therefore, the difference between cancer-activated platelet and agonist-activated platelet in the induction of NETosis may be explained by the ability of platelets to uptake cancer-derived factors, and subsequently transfer these factors to neutrophils, resulting in heightened neutrophil activation and more NET release. This hypothesis is currently under investigation by another member of the research group. In summary, both NETs and platelets positively contribute to cancer progression and are significant drivers of cancer-associated thrombosis (Fig. 1). However, more studies are required to investigate the impact of NET-platelet interaction on cancer progression.

2.2.2. The role of NETs in cancer metastasis

Recent data suggest that NETs promote cancer progression [101,199,256-258]. Most recently, Lee et al. demonstrated that NETs are required to establish a pre-metastatic niche in the omentum; a fatty tissue that is preferentially colonised by ovarian cancer cells. In orthotopic ovarian cancer mouse models, the authors showed accumulation of neutrophils and formation of NETs in the omentum once the primary tumour became palpable and before metastasis. Intriguingly, omentum metastasis

was significantly decreased in mice with neutrophils deficient in peptidylargninie deaminase (PAD4); an enzyme required for NETosis. In clinical specimens, NETs were significantly higher in omental tissues from patients with early stage carcinoma that have not developed metastasis compared to control omental tissues from women with no history of cancer. The omentum is a highly vascularized tissue, and NETs were found in abundance near the vessels in omental tissues from ovarian cancer patients [249]. Therefore, it is possible that NET formation is partially mediated through platelets or endothelial cells primed and activated by tumour-derived factors. In a landmark study, Albrengues et al., showed the ability of LPS-induced NETs to activate proliferation and metastasis in dormant cancer cells [47]. The authors demonstrated that breast cancer cells injected intravenously in mice were trapped in the lung; however, they remain dormant, non-proliferating single cells. Induction of neutrophils to release NETs through nasal instillation of LPS resulted in significant and aggressive metastasis. Additional treatment with DNase, PAD4 inhibitor or neutrophil depletion effectively prevented LPS-mediated dormancy escape of cancer cells, suggesting there is a direct role for NETs in modulating cancer cell cycle. Mechanistically, it has been demonstrated that NETs-associated proteases (e.g. neutrophils elastase (NE), and cathepsin G (CG)) induce degradation of extracellular matrix (ECM) *in vitro* and *in vivo* which results in an increased cancer cells proliferation and invasion [47]. Altogether, these studies propose an unconventional role of NETs in cancer metastasis.

2.3.   Summary

Recently, studies have begun to address the important roles of NETs in malignancy. NETs were shown to be required for pre-metastatic niche formation, and awakening dormant cancer cells. The contribution of platelets to NET generation has been investigated to some degree in mice models of infection, venous thrombosis and inflammation. However, it is not well understood if similar mechanisms work in the context of cancer as their role in CAT is still under debate. Our published works [6,259], one of which is included in this chapter, indicate that platelets are the primary target of NETs in thrombosis. Altogether, accumulating research data suggest that the therapeutic approaches to counteract the effects of NETs in cancer may be based on targeting platelet contribution.
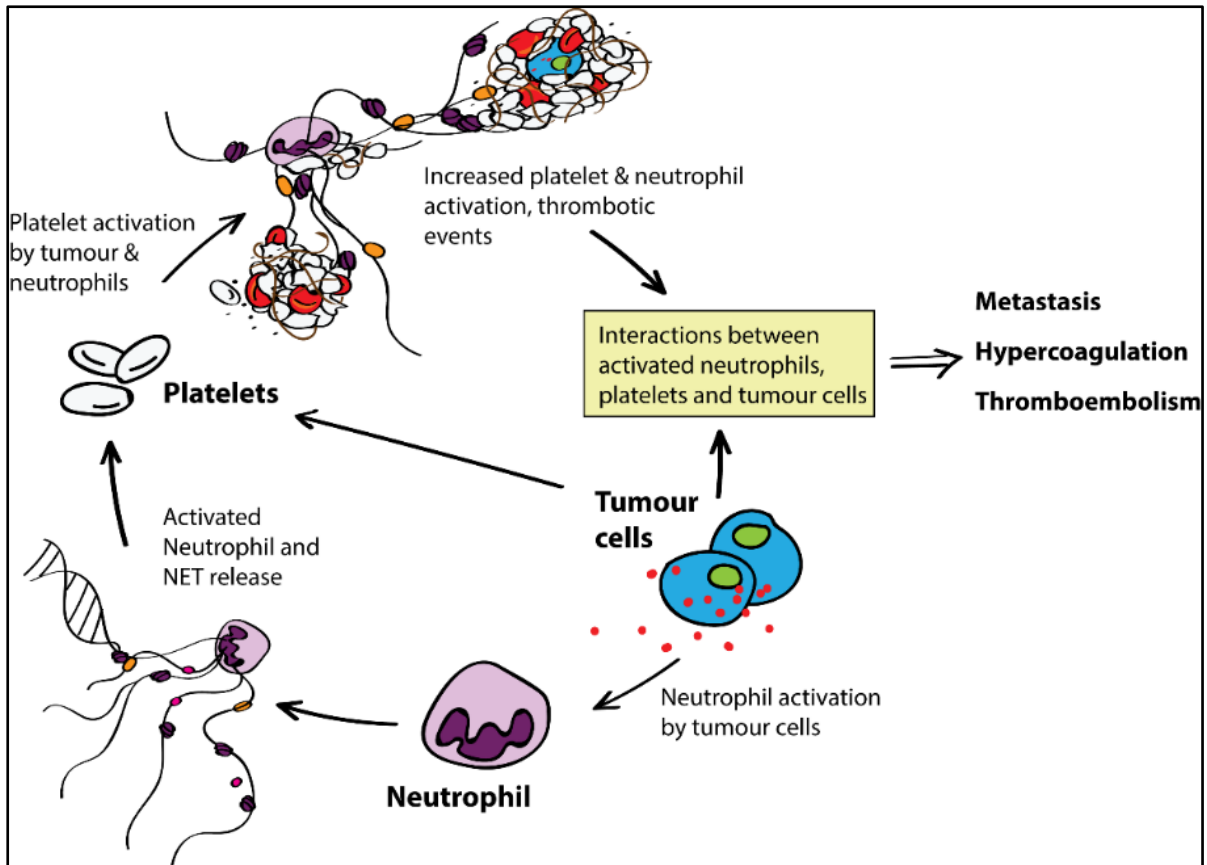
Fig. 1*: **Platelets interaction with cancer cells and neutrophils aid cancer progression**. Cancer cells are capable of activating neutrophils to release NETs. Cancer-activated platelets can attract neutrophils to the tumour microenvironment, as well as activating neutrophils to release NETs. Activated platelets can promote cancer growth, angiogenesis, metastasis, and chemotherapy resistance. NETs, however, can awaken dormant cancer cells, and induce aggressive metastasis.

*Figure from Abdol Razak, **Elaskalani O** et al., [6].

Elaskalani O, Abdol Razak NB, Metharom P. *Neutrophil extracellular traps induce aggregation of washed human platelets independently of extracellular DNA and histones.* Cell Commun Signal. 2018 May 29;16(1):24. doi: 10.1186/s12964-018-0235-0.

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Neutrophil extracellular traps induce aggregation of washed human platelets independently of extracellular DNA and histones

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Abstract

Background: The release of neutrophil extracellular traps (NETs), a mesh of DNA, histones and neutrophil proteases from neutrophils, was first demonstrated as a host defence against pathogens. Recently it became clear that NETs are also released in pathological conditions. NETs released in the blood can activate thrombosis and initiate a cascade of platelet responses. However, it is not well understood if these responses are mediated through direct or indirect interactions. We investigated whether cell-free NETs can induce aggregation of washed human platelets in vitro and the contribution of NET-derived extracellular DNA and histones to platelet activation response.

Methods: Isolated human neutrophils were stimulated with PMA to produce robust and consistent NETs. Cell-free NETs were isolated and characterised by examining DNA-histone complexes and quantification of neutrophil elastase with ELISA. NETs were incubated with washed human platelets to assess several platelet activation responses. Using pharmacological inhibitors, we explored the role of different NET components, as well as main platelet receptors, and downstream signalling pathways involved in NET-induced platelet aggregation.

Results: Cell-free NETs directly induced dose-dependent platelet aggregation, dense granule secretion and procoagulant phosphatidyl serine exposure on platelets. Surprisingly, we found that inhibition of NET-derived DNA and histones did not affect NET-induced platelet aggregation or activation. We further identified the molecular pathways involved in NET-activated platelets. The most potent single modulator of NET-induced platelet responses included NET-bound cathepsin G, platelet Syk kinase, and $P2Y_{12}$ and $\alpha IIb\beta 3$ receptors.

Conclusions: In vitro-generated NETs can directly induce marked aggregation of washed human platelets. Pre-treatment of NETs with DNase or heparin did not reduce NET-induced activation or aggregation of human washed platelets. We further identified the molecular pathways activated in platelets in response to NETs. Taken together, we conclude that targeting certain platelet activation pathways, rather than the NET scaffold, has a more profound reduction on NET-induced platelet aggregation.

Keywords: Neutrophil, Neutrophil extracellular traps, Platelet, Aggregation, DNA, Histones, Cathepsin G

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Background

Neutrophils are well known for their crucial role in innate immunity, providing the first line of defence against pathogens through multiple mechanisms [1]. The discovery of a relatively new antimicrobial mechanism, whereby activated neutrophils expel their DNA and proteins forming an extracellular matrix, termed neutrophil extracellular traps (NETs) [2], has gained much interest recently. NETs possess antimicrobial function either by entrapping and immobilising pathogens or presentation of NET-bound antimicrobial proteins [2, 3]. However, NETs can serve as more than just a host defence mechanism, as studies have implicated the role of NETs in inflammatory and autoimmune diseases and pathological conditions including thrombosis [4]. Notably, as the role of NETs in thrombosis is being investigated extensively in recent times, there is potential for NETs to not only serve as therapeutical target for thrombotic diseases but several other clinical conditions such as diabetes, systemic lupus erythematosus, pre-eclampsia and certain types of cancers, all which are known to be associated with increased risk of thrombosis [5–8].

Increasing number of studies are recognising NETs as a procoagulant surface, which is capable of promoting thrombosis both in vitro and in animal models of deep vein thrombosis and arterial thrombosis [9–13]. The NET structure can serve as a scaffold for platelet adhesion and aggregation [9, 14] thus providing a platform for the subsequent formation of thrombi. Furthermore, NETs have been shown to directly promote the activation of intrinsic coagulation pathway leading to thrombin generation [15]. Besides intact NETs being capable of activating coagulation, several components within the NET structure have been reported to activate platelets and initiate or promote coagulation. Cell-free DNA, which makes up the major backbone of NETs, has previously been shown to activate thrombin generation via the intrinsic pathway of coagulation [16]. The second most abundant constituent and protein found on NETs, extracellular histones, have been studied extensively and known to activate platelets and promote coagulation through multiple mechanisms [9, 17–19]. For example, histones are capable of generating thrombin in the presence of plasma and activating platelet aggregation which has been suggested to be mediated through toll-like receptor (TLR) 2 and TLR 4 [18]. However, the involvement of TLRs in platelet aggregation is not clear as Clark et al. have shown that LPS induced platelet activation but not aggregation [20] Furthermore, neutrophil granular enzymes that are bound to NETs such as neutrophil elastase (NE) and cathepsin G (Cat G), can separately promote coagulation and thrombus growth by facilitating intravascular fibrin formation and degrading tissue factor pathway inhibitor [21], while myeloperoxidase (MPO) can prime platelets [22].

Collectively, NETs are a potentially potent agonist of platelet activation and promoter of coagulation, thereby amplifying and supporting thrombus formation. However, despite many studies reporting NETs as promoters of thrombosis, these studies were conducted in whole blood assays or in the presence of plasma, implicating a role of plasma coagulation factors. Thus the capacity of intact cell-free NETs to directly activate washed platelets is not clearly understood. In this study, we investigated the effect of NETs on platelet function including aggregation, secretion, and surface expression of receptors. We also begin to determine molecular mediators and signalling pathways by examining the effect of antagonists of specific NET components and antiplatelet drugs, on the impact of NETs on platelet activation.

Methods

Materials

Purified anti-human TLR2, TLR4 blocking antibodies and their matching isotype controls were obtained from BioLegend, Inc., USA. Bay 61–3606 and Phorbol 12-myristate 13-acetate (PMA), ticagrelor and Cell Detection ELISA PLUS kit were from Sigma-Aldrich, Australia. ML-171, aspirin, RGDS, cathepsin G inhibitor I, neutrophil elastase inhibitor (1-(3-methylbenzoyl)-1-H-indazole-3-carbonitrile), myeloperoxidase inhibitor 1 (4-Aminobenzoic acid hydrazide), losartan were obtained from Cayman Chemical, USA. Abciximab (ReoPro) and low molecular weight heparin (Clexane enoxaparin sodium) were from Eli Lilly and Sanofi Aventis Australia Pty Ltd., respectively. DNAse I solution was purchased from STEMCELL Technologies Australia Pty Ltd. Collagen and thrombin were from Chrono-log Corporation, USA. Human PMN Elastase ELISA kit was obtained from Abcam Biotechnology, Cambridge, UK.

Preparation of washed human platelets

Blood was drawn from healthy volunteers into a syringe containing acid-citrate-dextrose (ACD; 1:7 (*v*/v) with informed consent in concordance with the Curtin University Human Research Ethics Committee (approval number HR54/2014). Blood donors were medication-free 2 weeks prior to the day of donation. Washed platelets were prepared, with some modifications, as previously described [23, 24]. Briefly, blood was centrifuged at 150 x *g* for 20 min. Platelet-rich plasma (PRP) was collected and centrifuged at 800 x *g* for 10 min in the presence of 1 μM prostaglandin E1 (PGE1; Cayman Chemical). Platelets were then washed three times in CGS buffer (14.7 mM trisodium citrate, 33.33 mM glucose and 123.2 mM NaCl, pH 7), in the

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presence of PGE1 (1 μM). Platelets were adjusted to 1×10^9/mL with calcium-free Tyrode-Hepes buffer (5 mM HEPES, 5.5 mM glucose, 138 mM NaCl, 12 mM NaHCO$_3$, 0.49 mM MgCl$_2$, 2.6 mM KCl, 0.36 mM NaH$_2$PO$_4$, pH 7.4). Platelets were supplemented with 1.8 mM CaCl$_2$ (final concentration) prior to experimentation.

Preparation of neutrophils and cell-free neutrophil extracellular traps (NETs)

Neutrophils were isolated from human blood using PolymorphPrep (Axis-Shield, Norway), with minor changes to the manufacturer's protocol. Briefly, blood anticoagulated with EDTA (2 mM) was layered over Polymorph-Prep and centrifuged at 500 x g for 40 min. The neutrophil fraction was collected and washed twice at 4 °C in Hank's buffered saline solution (without calcium or magnesium) and resuspended in X-VIVO 15 media (Lonza, Switzerland). Neutrophil purity was > 95% as determined with a haematology analyser (Mindray, BC-VET2800). Cell-free NETs were isolated as previously described [25] with minor changes to the protocol. This method of NET isolation does not involve using DNase or EDTA [26], which may confound platelet response to NETs. Briefly, neutrophils (2.5×10^6/mL) were stimulated with 500 nM PMA for 3 h at 37 °C and 5% CO$_2$. The supernatant, containing PMA, was discarded and the NET monolayer was detached with phosphate-buffered saline (PBS). The cell debris was pelleted by centrifugation at 480 x g for 10 min at 4 °C. The supernatant was further centrifuged at 15,000 x g for 20 min at 4 °C to pellet DNA then resuspended in PBS at 100 μl per 1×10^7 of stimulated neutrophils to obtain cell-free NETs. Cell-free NETs were characterised by detecting DNA-histone complex and neutrophil elastase using Cell Detection ELISA PLUS kit (Sigma Aldrich) and Human PMN Elastase ELISA kit (Abcam), respectively. Cell-free NETs were incubated with platelets at 10% of final reaction volume (i.e. 1-volume NET solution to 9-volume platelets).

Platelet aggregation assay

Washed platelets (3×10^8/mL) in Tyrode-HEPES buffer supplemented with 1.8 mM calcium chloride were incubated in the presence of cell-free NETs (10% of final reaction volume) and platelet aggregation was monitored at 37 °C with continuous stirring at 1200 rpm in a light transmission aggregometer (Model 700 Aggregometer, Chrono-log Corporation, USA) for at least 20 min. Tyrode-HEPES buffer was used as a blank. Where inhibitors were used, platelets were pre-incubated for 15 min at 37 °C prior to incubation with NETs. Control samples were incubated with the corresponding volume of buffer.

Platelet-dense granule secretion assay

Platelet secretion was determined by measuring ATP release using luciferin/luciferase reagent (Chrono-Lume, Chrono-log Corporation, USA). Briefly, 90 μl of platelets (1×10^8/mL) in Tyrodes-HEPES buffer (with calcium) were incubated with 10 μl of NETs with gentle shake at 37 °C for 1 and 10 min before adding 5 μl of Chrono-Lume reagent. The luminescence was measured using Enspire Multimode Plate Reader (PerkinElmer, USA). Where anti-platelet drugs were used, platelets were pre-incubated with the drugs for 15 min at 37 °C before incubating with NETs.

P-selectin exposure and αIIbβ3 activation

Platelet activation was measured by detecting P-selectin and active-form αIIbβ3 on the platelet surface using flow cytometry. Where inhibitors were used, platelets were pre-incubated for 15 min at 37 °C before adding NETs. Whenever inhibitors of components of NETs (i.e. DNAse I, cathepsin G, myeloperoxidase, and elastase inhibitors) were used, NETs were pre-incubated for 30 min at 37 °C. The specificity of inhibitors used was also examined for their effect on thrombin (0.1 U/mL) and collagen (5 μg/mL)–induced platelet activation. Inhibitor-, or vehicle-, treated washed human platelets (1×10^8/mL) were treated with NETs (10% of final volume) and stained with phycoerythrin-conjugated mouse anti-human CD62P (P-selectin) and fluorescein isothiocyanate-conjugated mouse anti-human PAC1 (active-form αIIbβ3), or suitable isotype control antibodies for 15 min in the dark. All antibodies were from BD Biosciences. Samples were analysed by flow cytometry (BD LSRFortessa™ cell analyzer).

Phosphatidylserine (PS) exposure

Platelets (3×10^8/mL) were incubated with NETs for 30 min at 37 °C with continuous stirring at 1200 rpm. Whenever inhibitors were used, NETs were pre-incubated for 30 min at 37 °C. Thrombin (0.1 U/mL) was used as positive control. Platelets were then stained with Annexin V-FITC (BioLegend, USA) in binding buffer according to manufacturer's instructions for 15 min in the dark. Samples were then washed in binding buffer and analysed by flow cytometry (BD LSRFortessa™ cell analyzer).

Antibodies and western blot

Rabbit antibodies specific for p-Akt (Ser473), p-Erk1/2 (Thr202/Tyr204), p-Syk (Tyr352), p-Tyr1000 and α-actinin were obtained from Cell Signalling Technology (USA). Platelets (3×10^8/mL) were incubated with NETs (10% of final reaction volume) at 37 °C for 3 min with continuous stirring at 1200 rpm. Platelets were then lysed in Laemmli sample buffer supplemented with

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Protease/Phosphatase Inhibitor Cocktail (Cell Signalling Technology) and β-mercaptoethanol. Forty-five μl of protein sample was loaded per lane and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis then transferred to a polyvinylidene difluoride (PVDF) protein blotting membrane with 0.2 μm pore size (GE Healthcare Life Sciences). The PVDF membrane was blocked in 5% non-fat powdered milk in Tris-buffered saline with 0.1% Tween 20 (or 3% bovine serum albumin (BSA, Bovogen Biologicals Pty Ltd., Australia) in TBS-T for detection of p-Tyr-1000) at room temperature for 1 h. After a brief rinse with TBS-T, the membrane was incubated overnight at 4 °C with primary antibodies at 1:1000 dilution. The primary antibody was detected with secondary horseradish peroxidase-conjugated anti-rabbit antibody (Jackson Immune Research, USA) at 1:40000 dilution. The membrane was developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, USA), and chemiluminescence was detected using ChemiDoc imaging system (Bio-Rad, USA).

Statistical analysis

Data were analysed using GraphPad PRISM 4.0 software. Results are expressed as the mean ± standard error (SEM). One-way ANOVA with posthoc Bonferroni's Multiple Comparison Test were used to examine the statistical significance between means. Differences were considered significant at $P < 0.05$.

Results

NETs induce aggregation, secretion and activation of washed human platelets

We first examined the ability of cell-free NETs to directly induce aggregation of washed human platelets independently of the coagulation pathway. Platelets were washed three times in CGS buffer to remove any contaminating plasma, then resuspended in Tyrode-HEPES buffer (with 1.8 mM calcium chloride). Light transmission aggregometry was used to measure platelet aggregation. Autologous cell-free NETs at different dilutions induced marked platelet aggregation (Fig. 1a). Interestingly, platelet aggregation response was apparent only after 5 mins in the presence of the highest concentration of NETs used (10% of final reaction volume) (Fig. 1b). For all experiments, it was imperative that NETs were used within the same day of isolation as freezing or storing NETs at 4 °C for more than 24 h completely abolished their activity.

As platelet secretion amplifies platelet aggregation, we assessed the ability of NETs to induce platelet dense granule secretion of ATP/ADP using a luminescence assay. NETs triggered significant ATP/ADP secretion from platelet dense granules compared to vehicle control

(Fig. 1c). Platelet secretion was examined after 1 and 10 min incubation with NETs to identify the time required for secretion compared to aggregation. NETs induced significant platelet secretion by 1 min, while aggregation did not occur until 5 min (Fig. 1b-c), indicating that NET-induced platelet secretion precedes aggregation. Furthermore, we also examined if platelet functional response occurred concomitantly with intracellular signalling, particularly the phosphorylation of proteins at tyrosine residues. Indeed, NETs induced tyrosine phosphorylation of several substrates with strong migrating bands at ~ 134, 80 and 60 kDa compared to vehicle control (Fig. 1d).

NETs induce surface expression of receptors and phosphatidyl serine exposure on platelets

We also examined α-granule secretion by measuring the surface expression of P-selectin. Platelets incubated with NETs for 10 to 15 min showed a significant increase of P-selectin surface expression as detected by flow cytometry (Fig. 2a-b). A conformational change in platelet αIIbβ3 receptor is required for platelet aggregation [27]. Therefore we assessed platelet surface expression of active-form αIIbβ3 using PAC1 monoclonal antibody. As shown in Fig. 2a-b, NETs induced a conformational change of αIIbβ3 from resting to an activated state. As PS exposure on platelets can propagate coagulation [28], we assessed the ability of NETs to induce PS exposure on the platelet surface. Platelets were incubated with NETs for 30 min at 37 °C with continuous stirring at 1200 rpm before analysing PS expression. Annexin V-FITC was used to stain PS and was analysed by flow cytometry. NETs induced a marked increase in PS expression on platelet's surface (Fig. 2c-d), suggesting that NET-activated platelets can provide a procoagulant surface.

The role of NET-derived DNA, histones, cat G, and MPO in NET-induced platelet response

As the major components of NETs are DNA and histones, we first investigated whether the effect of NETs on washed platelets is mediated via NET-derived DNA and/or histones. DNA-histone complexes were confirmed in cell-free NETs using Cell Detection ELISA PLUS kit. Based on comparisons between whole neutrophil lysates and NET dilution samples, the concentration of DNA-histone complex in cell-free NETs used in majority of experiments (i.e. 10% final NETs) was equivalent to the amount present in approximately $3.8 \pm 1.2 \times 10^6$/mL of neutrophil lysate (Additional file 1: Figure S1). Additionally, the 10% cell-free NET solution was determined to contain 292 ± 172 pg/mL of elastase, another major protein component of NETs (Additional file 1: Figure S2).

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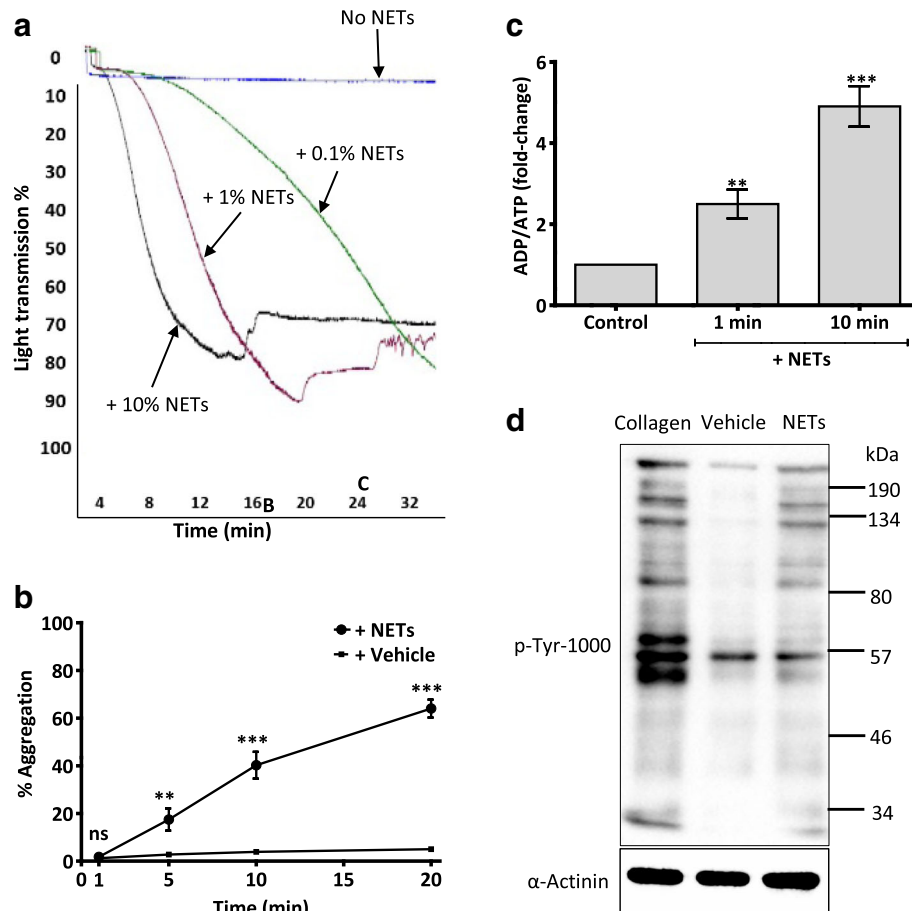
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Fig. 1 NETs directly induce aggregation of washed human platelets. **a** Representative traces showing dose-dependent aggregation of washed human platelets (WP, 3×10^8 /mL) in response to cell free-NETs. 0.1, 1 and 10% NETs refers to NETs constituting 0.1, 1 or 10% of final reaction volume, respectively (e.g. 10% NET is 1-volume NET solution to 9-volume platelets). Platelet aggregation was measured by light transmission aggregometer (Chrono-log). NETs were used at 10% of final reaction volume for following experiments*. **b** Progress time curve displaying % of NET-induced platelet aggregation which steadily increased over 20 min. **c** ATP/ADP release from NET-stimulated platelets. WP (1×10^8/mL) were incubated with NETs with a gentle shake at 37 °C for 1 and 10 min before adding Chrono-Lume reagent. Luminescence was measured using Enspire Multimode Plate Reader (PerkinElmer). NETs induced significant ATP/ADP secretion from platelets at 1 and 10 min. Results represent fold change in luminescence arbitrary absorbance unit relative to vehicle (PBS) control. Data expressed as mean ± SEM; **$P < 0.01$, ***$P < 0.0001$, $N \geq 5$. **d** Platelet signalling in response to NETs was examined by Western blot analysis. WP (3×10^8/mL) were incubated with collagen (5 μg/mL), vehicle (PBS) or NETs for 3 min. Twenty μl of total cell lysate was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted for phospho-Tyrosine (p-Tyr-1000). Equal loading was verified by α-actinin. Western blots indicate tyrosine-phosphorylated proteins in NET-stimulated platelets. *10% NET solution is prepared from 1×10^7/ml of PMA-activated neutrophils, however due to losses during preparation steps, 10% NET is estimated to contain histone/DNA complexes equivalent to $3.8 \pm 1.2 \times 10^6$/mL neutrophils (Additional file 1: Figure S1). The elastase content present in a 10% NET reaction volume is 292 ± 172 pg/mL

Calf thymus histones (CTH) are well-established platelet agonists [29] and were used as a positive control. CTH (1, 5, 20 and 40 μg/mL) induced a dose-dependent aggregation of washed human platelet (Fig. 3a). Heparin can strongly bind to and abate the effect of histones on platelets [30–32]. Indeed, heparin 20 U/mL completely abated CTH but not NET-induced platelet aggregation (Fig. 3 b, d). Collagen (5 μg/mL) was added to the CTH-heparin reaction in order to verify that the platelet functional

response to another agonist remained unaffected (Fig. 3b).

In order to test the impact of DNA on NET-induced platelet aggregation, NETs were pre-treated with DNase (20, 200 U/mL) for 30 min at 37 °C before adding to platelets (where NETs was used at 10% of the final reaction volume). DNase can dismantle NET-DNA (Additional file 1: Figure 3). However, DNase did not reduce the ability of NETs to induce aggregation of washed human platelets (Fig. 3c & d), suggesting neither DNA nor
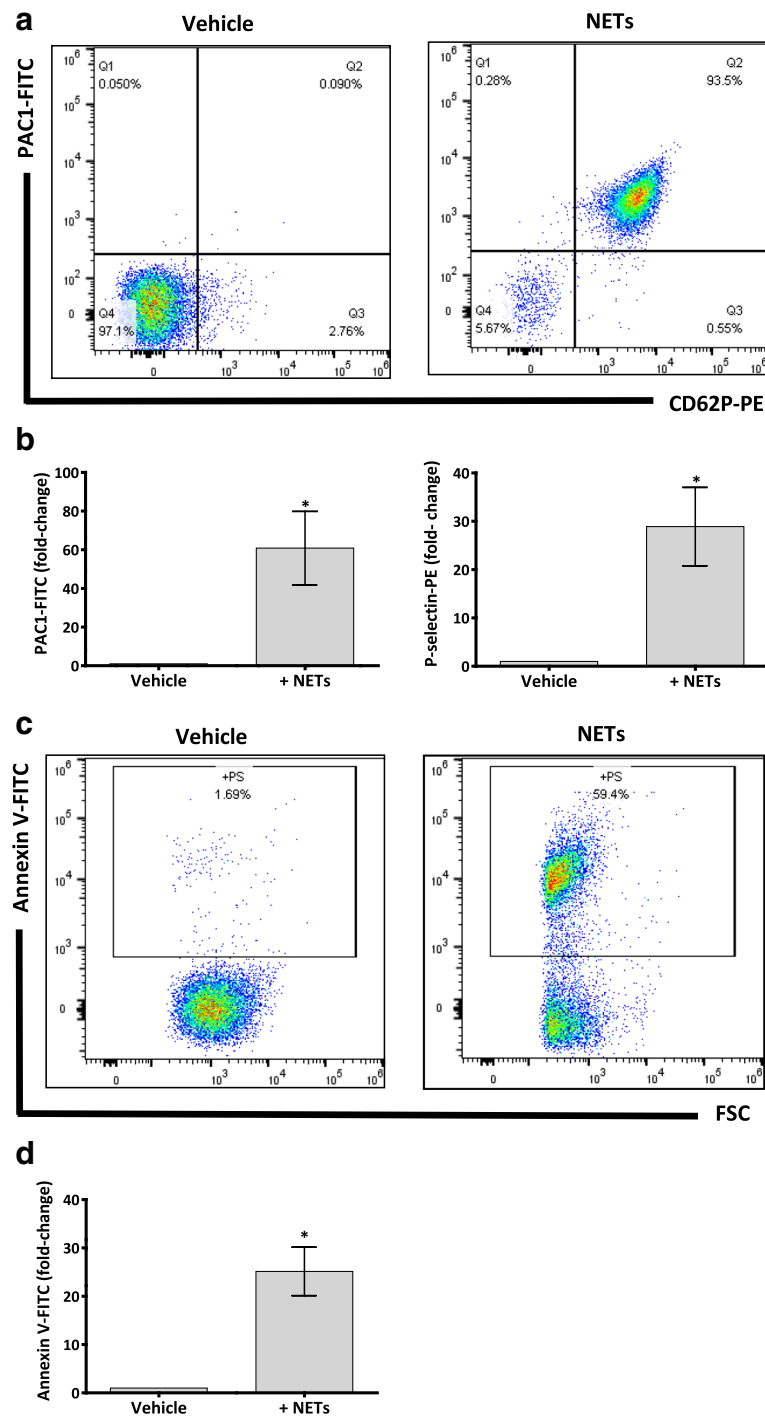
Fig. 2 NETs induce platelet activation and phosphatidyl serine (PS) exposure on washed human platelets. **a** Representative dot plot of CD62P (P-selectin), PAC1 (active-form αIIbβ3) fluorescence. WP (1×10^8/mL) were treated with vehicle (PBS) or NETs then incubated with labelled monoclonal antibodies phycoerythrin (PE)-conjugated CD62P and fluorescein isothiocyanate (FITC)-PAC1 for 10–15 min in the dark. The reaction was stopped by fixing cells in 2% paraformaldehyde before analysing samples with flow cytometry (BD LSRFortessa™ cell analyzer). NETs induced expression of P-selectin and active-form αIIbβ3. **b** Fold change in the geometrical mean fluorescence of P-selectin-PE and PAC1-FITC in NET-activated platelets compared to vehicle-treated platelets. **c** WP (3×10^8/mL) were incubated with vehicle (PBS) or NETs for 30 min at 37 °C with continuous stirring at 1200 rpm. PS was detected by incubating platelets with Annexin V-FITC in binding buffer for 15 min in the dark. Samples were then washed in binding buffer and analysed by flow cytometry (BD LSRFortessa). NETs induced PS exposure compared to vehicle control. **d** Fold change in geometrical mean of fluorescence of Annexin V-FITC in NET-activated platelets compared to vehicle-treated platelets. In all assays, NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. Data are expressed as mean $\pm$ SEM,*$P < 0.05$, $n \geq 4$

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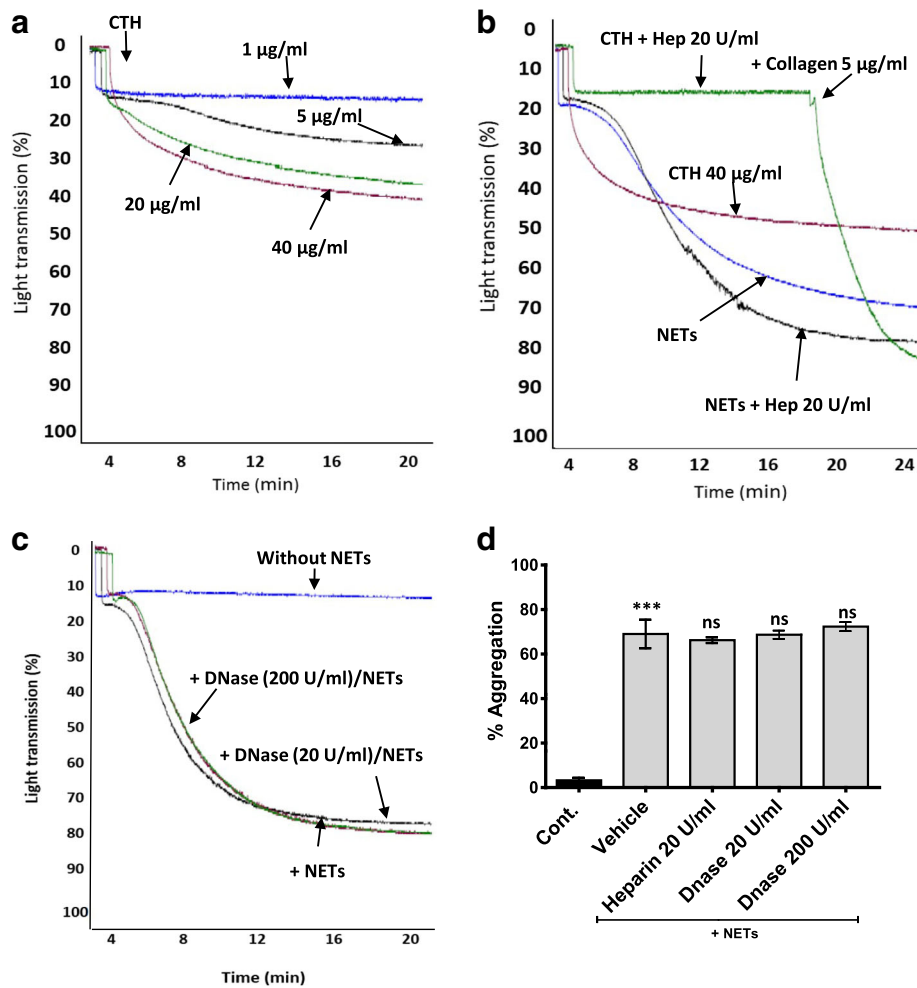
Fig. 3 Heparin and DNase did not reduce NET-induced platelet aggregation. **a** Representative traces showing dose-dependent aggregation of washed human platelets (WP, 3×10^8 /mL) in response to calf thymus histones (CTH; 1, 5, 20 and 40 μg/mL). **b** Representative aggregation traces showing the effect of heparin on NET and CTH-induced platelet aggregation. The reactions were allowed to proceed for 18 min, then collagen (5 μg/mL) was added to the CTH-Heparin reaction to test platelet functional response. **c** Representative aggregation traces showing the effect of DNase on NET-induced platelet aggregation. NETs were pre-treated with DNase (20, 200 U/mL) for 30 min at 37 °C before addition to WP (3×10^8/mL). Traces are representative of 3 independent experiments. **d** Bar graphs depict % of platelet aggregation treated with NET alone or with Heparin and DNase. Percentage platelet aggregation was measured at 20 min. In all assays, NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. Data are expressed as mean $\pm$ SEM,*$P < .05$; ns: non-significant, $n \geq 3$

histones are major contributors to NET-induced washed platelet aggregation. Since histones can activate platelets, which has suggested to be mediated through toll-like receptor (TLR) 2 and TLR4 [18], platelets were pre-incubated with TLR2- and TLR4-blocking antibodies or matching isotype controls (50 μg/mL) for 15 min before incubation with NETs. Similarly, anti-TLR2 and -TLR4 antibodies also did not affect NET-induced platelet activation (Additional file 1: Figure S4). It was recently reported that calf thymus histones can activate platelets through GPVI receptor [29]. However, pre-incubating platelets with a GPVI inhibitor, losartan (30 μM [33–35]) did not affect NET-induced platelet aggregation (Additional file 1: Figure S5). Our data suggest

that neither DNA nor histones contribute to NET-induced washed platelet aggregation or activation.

Extracellular DNA and histones gained considerable interest for their contribution to NET-induced thrombosis, while little attention has been paid to other components of NETs such as neutrophil proteases. Previous studies have reported that Cat G and MPO can modulate platelet response [36, 37]. To determine the effect of NET-derived Cat G and MPO on platelet activation, we used Cat G and MPO inhibitors at concentrations previously described in the literature [36, 37]. Although Cat G inhibition did not significantly affect the maximum aggregation response of platelet to NETs, we observed a trend of increased aggregation lag time (data not

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shown). Interestingly, Cat G inhibitor (Cat G I) but not MPO inhibitor (MPO I) significantly reduced NET-induced platelet expression of P-selectin (% Max response: 100 vs. 85 ± 3.2; $*P < 0.05$, $n = 4$), PAC1 (% Max response: 100 vs. 77.3 ± 3.7; $*P < 0.05$, n = 4) and PS exposure (% Max response: 100 vs. 64.6 ± 12.1; $*P < 0.05$, $n = 4$) (Fig. 4a-c). The concentration of Cat G I used (0.5 μM) did not affect platelet physiological response to thrombin (0.1 U/mL) (Additional file 1: Figure S6), confirming that the inhibitory response is specific to NET-bound Cat G activation of platelets. Additionally, similarly to MPO, neutrophil elastase did not markedly affect NET-induced platelet activation (Additional file 1: Figure S7).

SYK and NOX1 contribute to NET-induced platelet response

We began to delineate the platelet receptors and downstream pathway involved in NET-induced platelet activation. The non-receptor tyrosine kinase Syk mediates signalling from major platelet receptors, including the histone receptors GPVI and TLR2 [29]. Whereas NADPH oxidase (NOX) regulates GPVI-induced reactive oxygen species generation and subsequent thromboxane A2 (TxA2) production [38]. We demonstrate that platelet Syk phosphorylation was augmented upon exposure to NETs, which was accompanied by upregulation of the downstream signalling molecules p-Akt and p-Erk1/2 (Fig.5e). A Syk phosphorylation inhibitor (Bay 61–3606, 5 μM) reduced NET-induced platelet aggregation (% Max Agg: 100 vs. 74.21 ± 8.8; $***P < 0.001$, $n = 7$) (Fig. 5b), dense granule secretion (% Max secretion at 1 min: 100 vs. 52.2 ± 9.2, $***P < 0.001$; at 10 min: 100 vs. 64.4 ± 6.3; $**P < 0.01$, $n = 6$) (Fig. 5e-f), and PAC1 expression (% Max response: 100 vs. 81.7 ± 6.9; $*P < 0.05$, $n = 3$) (Fig. 5c), while P-selectin expression remained unchanged (Fig. 5d). On the other hand, NOX1 inhibitor (ML171, 5 μM) did not alter NET-induced platelet aggregation or activation (P-selectin and active-form αIIbβ3 expression) (Fig. 5a-d), however, it significantly reduced NET-induced platelet dense granule secretion (% Max secretion at 1 min: 100 vs. 62.4 ± 7.1; $**P < 0.01$; at 10 min: 100 vs. 58.6 ± 6.6; $**P < 0.01$, $n = 6$) (Fig. 5d). Collectively, these results highlight the diversity of platelet pathways that are activated by NETs.

P2Y$_{12}$ but not cyclooxygenase pathway is required for NET-induced platelet aggregation

Drugs that target either P2Y$_{12}$ (e.g., ticagrelor) or cyclooxygenase pathway (aspirin) are clinically available and crucial in the management of thrombosis [39]. Therefore, we were interested in investigating their effect on NET-induced platelet response. Ticagrelor markedly reduced NET-induced platelet aggregation (% Max Agg:

100 vs. 56 ± 10.1; $***P < 0.001$, $n = 5$) (Fig. 6b), dense granule secretion (% Max secretion; at 1 min: 100 vs. 69.3 ± 9.8; $P = 0.052$; at 10 min: 100 vs. 62.1 ± 9.4; $*P < 0.05$, $n = 5$) (Fig. 6e-f), and expression of P-selectin (% Max response: 100 vs. 72.9 ± 2.9; $***P < 0.001$, $n = 3$) (Fig. 6d) and PAC1 (% Max response: 100 vs. 40.34 ± 11; $***P < 0.001$, n = 3) (Fig. 6c). Surprisingly, aspirin did not alter NET-induced platelet aggregation or expression of P-selectin and PAC1 (Fig. 6b-d, Additional file 1: Figure S8), however it significantly reduced NET-induced platelet dense granule secretion (% Max secretion; at 1 min: 100 vs. 62.6 ± 7.8; $**P < 0.01$, $n = 5$; at 10 min: 100 vs. 63.5 ± 13.1; $*P < 0.05$, $n = 5$) (Fig. 6e-f). These findings suggest a broader role of ticagrelor, but not aspirin, in reducing NET-induced platelet response.

NET-induced platelet response is dependent on integrin αIIbβ3

Considering the role of NETs in mediating platelet adhesion and spreading [30], we were interested in examining the effect of platelet adhesion receptor αIIbβ3 in NET-induced platelet response. Reopro, a monoclonal antibody that binds to and inhibits the active form of αIIbβ3, dramatically reduced NET-induced platelet aggregation (% Max Agg: 100 vs. 31.2 ± 3.3; $***P < 0.001$, $n = 4$) (Fig. 7b) and dense granule secretion (% Max secretion; at 1 min: 100 vs. 50 ± 12.2; $P = 0.075$; at 10 min: 100 vs. 59.1 ± 10.8; $*P < 0.05$, $n = 5$) (Fig. 7e-f). Moreover, Reopro completely inhibited NET-induced PAC1 expression (Fig. 7c), most likely due to competitive binding to αIIbβ3 which is also the target for the PAC1 antibody. However, Reopro did not reduce NET-induced platelet P-selectin expression (Fig. 7d), suggesting that NETs do not trigger αIIbβ3 outside-in signalling in platelets.

RGDS (100 μM), a peptide that binds to αIIbβ3 and prevents conformational change triggered by inside-out signalling, significantly reduced NET-induced platelet aggregation (% Max Agg: 100 vs. 43.7 ± 5.6; $***P < 0.001$, $n = 3$) (Fig. 7b) and dense granule secretion (% Max secretion; at 1 min: 100 vs. 66 ± 8.7; $*P < 0.05$; at 10 min: 100 vs. 48.2 ± 13.8; $**P < 0.01$, $n = 5$) (Fig. 7e-f). Platelet activation show that RGDS markedly reduced NET-induced platelet P-selectin expression (% Max response: 100 vs. 82.6 ± 3.7; $**P < 0.01$, $n = 3$) (Fig. 7d) and PAC1 expression (% Max response: 100 vs. 38.4 ± 16.2; $**P < 0.01$, $n = 3$) (Fig. 7c). Overall, our results confirm the crucial role of αIIbβ3 in NET-induced platelet response.

Discussion

Our study explored the effect of in vitro-generated NETs on washed human platelets. As described in the methods, cell-free NETs were isolated from PMA-activated human neutrophils. PMA is a known
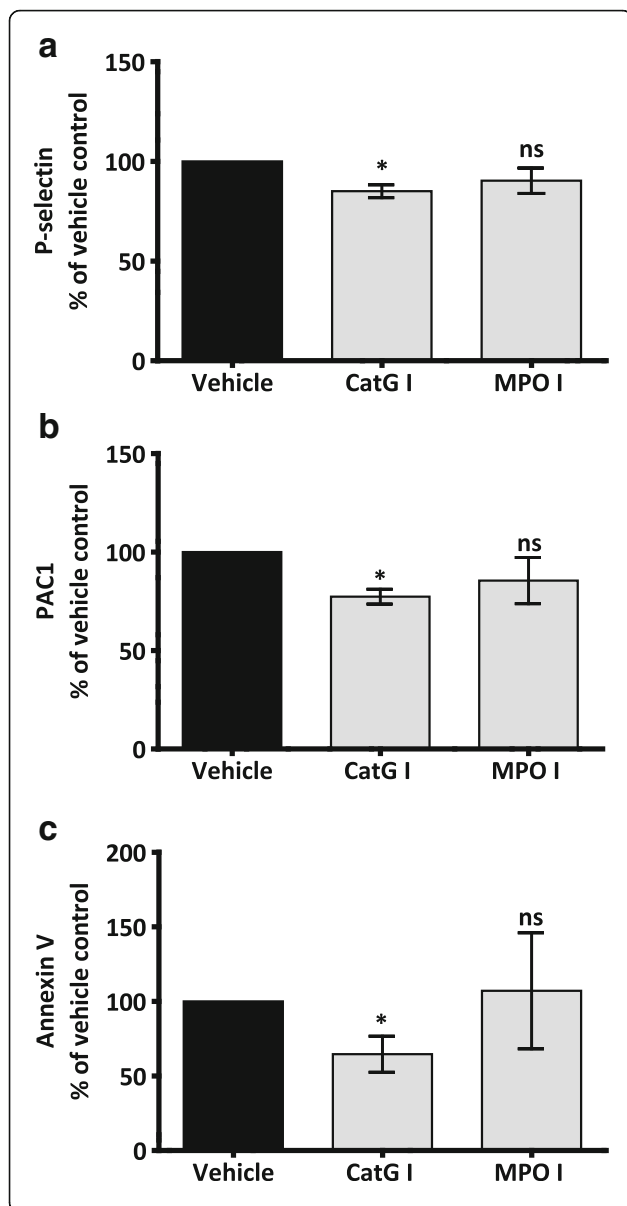
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Fig. 4 Inhibition of Cat G, but not MPO, attenuates NET-induced platelet response. Flow cytometry analysis of P-selectin, PAC1, and PS exposure on platelets. NETs were pre-treated with vehicle or inhibitors for 30 min at 37 °C. **a-b** WP (1×10^8/mL) were incubated with NETs pre-treated with vehicle (0.1% DMSO in PBS), Cat G I (0.5 μM) or MPO I (50 μM) for 10–15 min in the dark with labelled monoclonal antibodies that detect P-selectin (CD62P) and active αIIbβ3 (PAC1). The reaction was stopped by fixing cells in 2% PFA before analysing samples with flow cytometry (BD LSRFortessa). Bar graphs depict the % inhibition in P-selectin, and active αIIbβ3 expression in platelets treated with NETs pre-treated with different inhibitors compared to platelets treated with NETs that were pre-treated with vehicle. Results were normalized for each donor relative to NET-induced platelet response. **c** WP (3×10^8/mL) were incubated with NETs pre-treated with vehicle (0.1% DMSO in PBS), Cat G I (0.5 μM) or MPO I (50 μM) for 30 min at 37 °C with continuous stirring at 1200 rpm. Platelets were then stained with Annexin V-FITC in binding buffer for 15 min in the dark. Samples were then washed in binding buffer and analysed by flow cytometry (BD LSRFortessa). Bar graph depicts the % inhibition in PS expression in platelets treated with NETs pre-treated with different inhibitors compared to platelets treated with NETs pre-treated with vehicle. Results were normalised for each donor relative to NET-induced platelet response. In all assays, NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. Data are expressed as mean ± SEM,*$P < .05$; ns: non-significant, $n = 4$

platelet agonist [40], however PMA was washed out with the culture media after 3 h incubation with neutrophils, thus it is highly unlikely that NET-induced platelet aggregation was confounded by PMA. Unlike the widely used method of cell-free NET preparation by Urban et al., [26], we did not use this method involving DNase/EDTA, as EDTA can hinder platelet functional response [41]. We demonstrate that intact cell-free NETs exhibit the capacity to directly activate several platelet responses, such as aggregation, dense and α-granule secretion (ADP release and P-selectin expression), PS exposure and activation of integrin αIIbβ3, which occurred independently of the presence of coagulation factors or thrombin. NETs triggered a dose-dependent aggregation response in platelets with delayed lag time which correlates with the ability of NETs to first induce rapid platelet dense granule secretion.

In addition to being a procoagulant platform, NETs induced PS exposure on platelet's surface, a characteristic feature of procoagulant platelets. In the presence of small amounts of activated coagulation factors, PS can instigate thrombin generation, which can directly activate platelets and conversion of fibrinogen to fibrin [42]. In washed platelets, strong or multiple agonists can trigger PS-exposing procoagulant platelets [43, 44]. The latter is in line with our data and suggests that NETs are not a single agonist but a platform that presents a number of agonists that can promote platelet activation via multiple pathways.
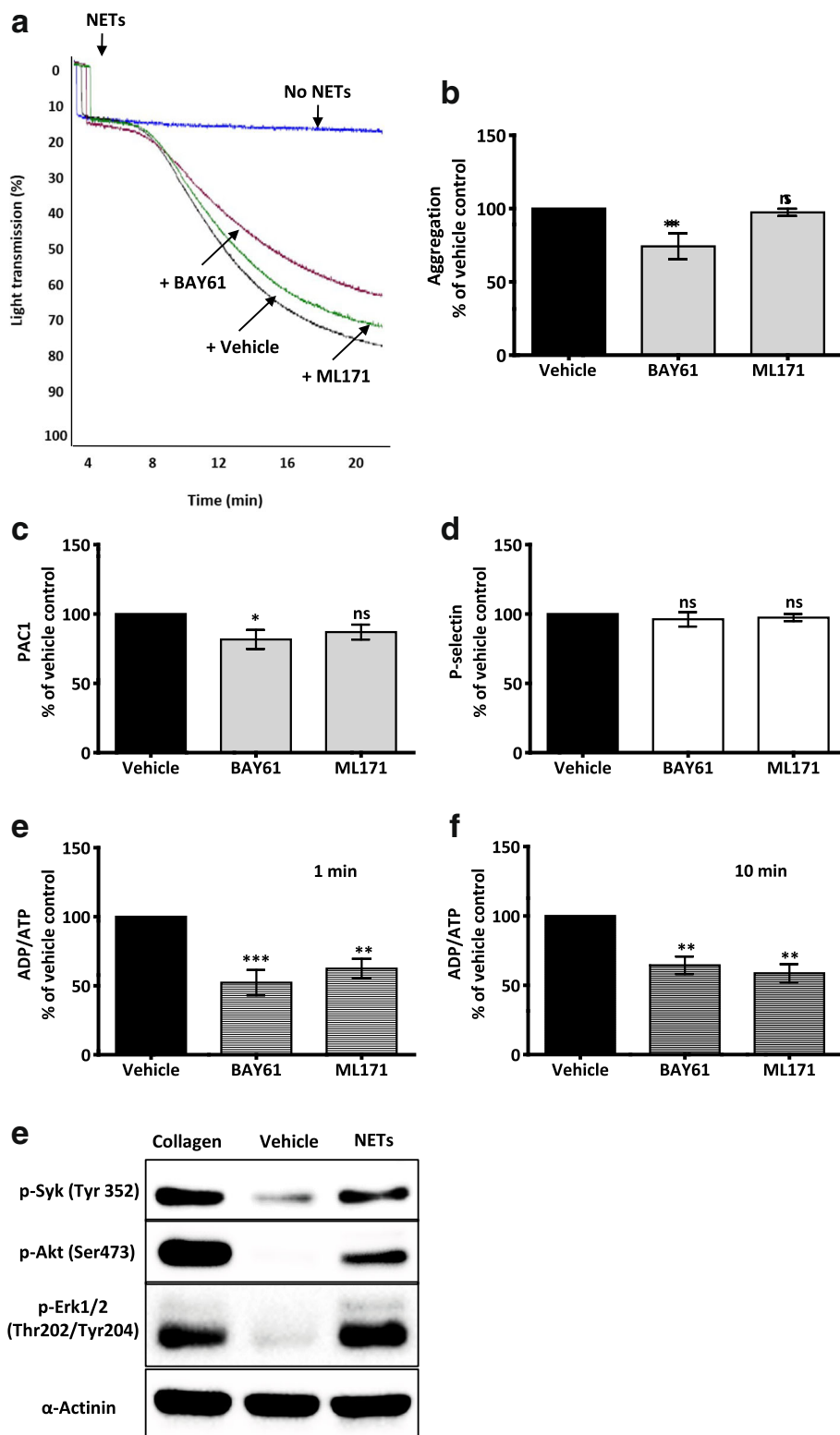
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Fig. 5 (See legend on next page.)

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Fig. 5 Syk, but not NOX1, inhibition attenuated NET-induced platelet aggregation. **a** Representative aggregation traces showing the effect of Syk phosphorylation (BAY61, 5 μM) and NOX1 (ML171, 5 μM) inhibitors on NET-induced platelet aggregation. WP (3×10^8/mL) were pre-incubated with the inhibitors for 15 min at 37 °C before addition of NETs. Platelet aggregation was measured using transmission aggregometer (Chronolog). **b** Bar graph comparing the effect of BAY61 and ML171 on NET-induced platelet aggregation. Platelet aggregation percentage was calculated after 20 min on a light transmission aggregometer. Results were normalized for each donor relative to NET-induced platelet aggregation. Data are expressed as mean ± SEM, ***$P < .001$; ns: non-significant, $n = 7$. (**c-d**) Bar graphs comparing the effect of BAY61 (5 μM) and ML171 (5 μM) on platelet activation (measured by P-selectin and active αIIbβ3 expression, $n = 3$) and (**e-f**) ATP/ADP secretion ($n = 6$) elicited by NETs. Results were normalized for each donor relative to NET-induced platelet response. Data are expressed as mean ± SEM, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: non-significant. **e** Syk phosphorylation is augmented by NETs. WP (3×10^8/mL) were incubated with collagen (5 μg/mL), vehicle (PBS) or NETs for 3 min. Forty-five μl of total cell lysate was then analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted for p-Syk, p-Akt and p-Erk1/2. Equal loading was verified by α-actinin. In all assays, NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. The immunoblots are representative sample of 3 independent experiments

NETs are made up of DNA and proteins (1:1.67 ± 0.26 g, DNA to proteins) [26]. Histones account for 70% of all NET-associated proteins [18, 26, 29, 45]. Previous studies showed the ability of single-strand DNA to bind platelets [46] while double strand DNA can induce platelet aggregation [47, 48]. Degradation of DNA with DNase has been shown to digest NETs and reduce platelet aggregates under flow [30], or platelet adhesion to NETs under static condition [12]. On the other hand, histones are well-established as platelet agonists that can trigger a cascade of platelet responses with defined surface receptors and signalling pathways [18, 29, 45]. Heparin has been reported to bind to histones, thus preventing its binding to platelets [19, 32]. Surprisingly, in this study DNase- and heparin-treated NETs were still capable of aggregating washed platelets and induced expression of P-selectin and active αIIbβ3 to the same extent of untreated NETs. Although DNase and heparin can destabilise the NET structure [9], the presence of freely suspended individual NET components – such as cell free-DNA, histones, and neutrophil proteases – may have greater capacity and exposure to directly bind and activate platelets, as opposed to being restricted on NETs. This presumption is in line with a study that showed DNAse-treatment of NETs resulted in increased coagulation effect [15]. Moreover, nuclear histones have different molecular mass and stoichiometry compared to NET-derived histones [26]. Therefore they may exhibit different biological activity. Indeed, a recent report has found that individual histones and DNA capable of inducing coagulation, but not intact NETs that were released from human neutrophils [49].

Histones are known to induce platelet Syk kinase activation through GPVI, and other tyrosine kinase-linked receptors [29]. We show that inhibition of Syk attenuated NET-induced platelet responses. However, inhibitors of histone receptors on platelets (TLR2, TLR4 and GPVI) did not reduce NET-induced platelet aggregation. Moreover, the sheer magnitude of platelet aggregation response to NETs in washed system precludes a significant contribution of histones which are known to have a

tenfold lower platelet aggregation response in washed platelets compared to PRP [29].

Neutrophil granular proteins are a part of NETs and have separately been shown to activate platelets [37, 50]. Neutrophil serine proteases and histones are negatively charged proteins [51] and would be tightly bound to the positively charged DNA backbone of NETs, thus most likely remain bound after isolation procedures [26]. The pre-treatment of NETs with an MPO inhibitor did not significantly reduce NET-induced upregulation of P-selectin, active αIIbβ3, or PS exposure on platelets, suggesting MPO does not play a major role in NET-induced platelet activation which is consistent with previous studies reporting that MPO is not a robust activator of platelets, but only induces partial activation or priming of platelets [37]. On the other hand, inhibiting Cat G resulted in a significant decrease in platelet surface expression of P-selectin, active αIIbβ3 and PS. This suggests Cat G as a molecular mediator of NET-induced platelet activation, and potentially significant contributor to thrombus formation, as previously described [36]. NE is the second most abundant NET-associated proteins after histones [26] and can potentiate Cat G-induced platelet aggregation [52], however in our hands, NE inhibitor did not affect NET-induced platelet responses (data not shown).

As the NET scaffold contains an array of associated proteins, some of which have been independently associated with platelet activation [26, 36, 37, 50, 52–54], a single inhibitor is highly unlikely to completely abrogate NET-induced platelet responses. However, we were interested mainly in clarifying the major NET components, platelet receptors and downstream signalling molecules that mediate NET-induced platelet secretion and aggregation. Inhibition of the tyrosine kinase Syk activity, P2Y$_{12}$ and αIIbβ3 reduced NET-induced platelet aggregation and secretion. While inhibition of NOX1 and TxA2 reduced NET-induced platelet dense granule secretion, but not aggregation.

The role of NETs in initiating thrombosis in vivo has been established in mice models of different diseases [55]. However, the molecular mechanisms that drive
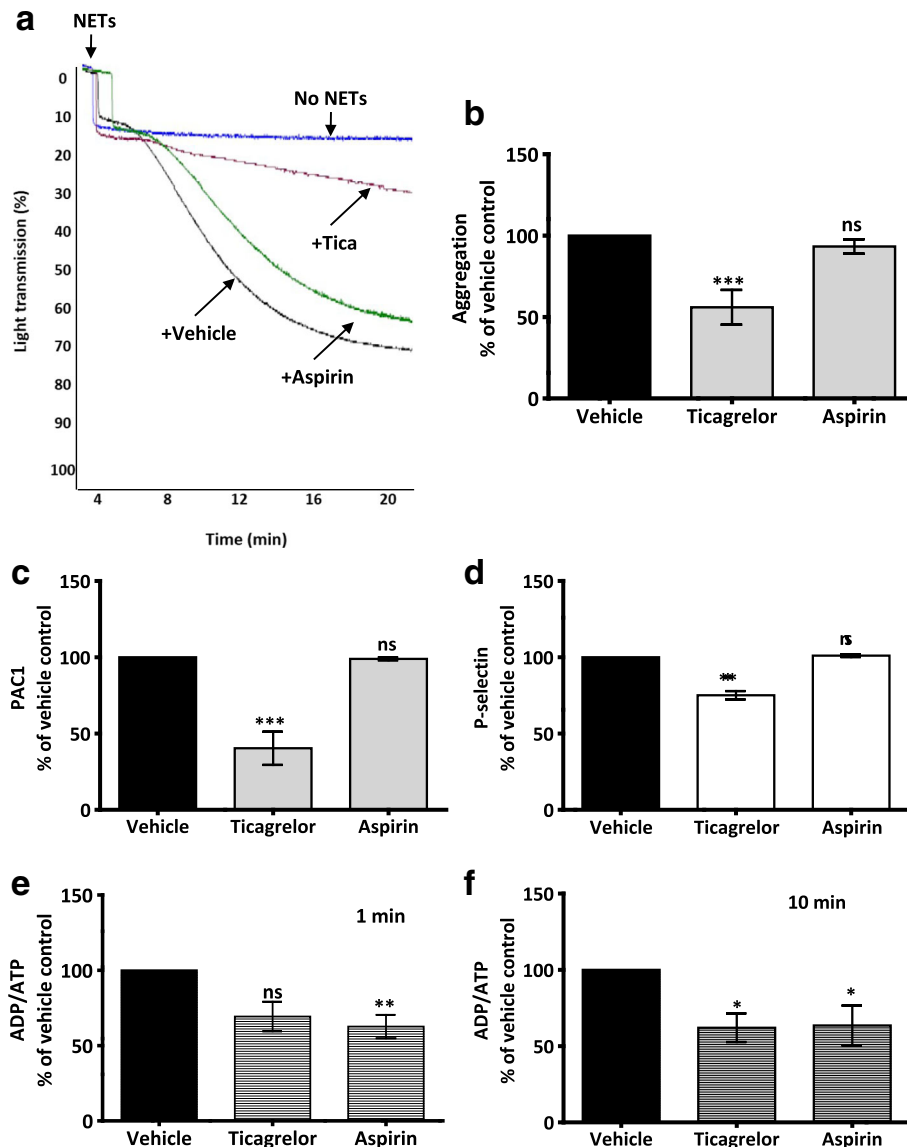
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Fig. 6 Inhibition of cyclooxygenase pathway in platelets attenuated NET-induced platelet secretion but not aggregation, while inhibition of $P2Y_{12}$ affects both. **a** Representative aggregation traces showing the effect of ticagrelor (1 μM) and aspirin (100 μM) on NET-induced platelet aggregation. WP (3×10^8/mL) were pre-incubated with the inhibitors for 15 min at 37 °C before addition of NETs. Platelet aggregation was measured by light transmission aggregometry (Chrono-log). **b** Bar graph comparing the effect of ticagrelor (1 μM) and aspirin (100 μM) on NET-induced platelet aggregation. Platelet aggregation percentage was calculated after 20 min on a light transmission aggregometer. Results were normalized for each donor relative to NET-induced platelet aggregation. Data are expressed as mean ± SEM, ***$P < .001$; ns: non-significant, $n = 3$. (**c-d**) Bar graphs comparing the effect of ticagrelor (1 μM) and aspirin (100 μM) on platelet activation (measured by P-selectin and active αIIbβ3 expression) ($n = 3$) and (**e-f**) ATP/ADP secretion ($n = 5$) elicited by NETs. In all assays, NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. Results were normalized for each donor relative to NET-induced platelet response. Data are expressed as mean ± SEM, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ns: non-significant

NET-induced thrombosis are not well understood. As the recent study by Noubouossie et al., has demonstrated that intact NETs do not directly initiate coagulation [49], we propose that platelets but not coagulation factors are more likely to be the main target of NETs in thrombosis. Our study did not account for the disrupting effect of NETs on endothelium which can also

initiate thrombosis [56, 57]. We propose that platelets adhere mainly to NET-derived DNA, then multiple NET-bound proteins induce platelet aggregation and PS exposure which then can propagate coagulation and thrombin generation. In addition to their pivotal role in thrombosis, platelets can also orchestrate inflammation [58]. Therefore, although dismantling NETs may reduce

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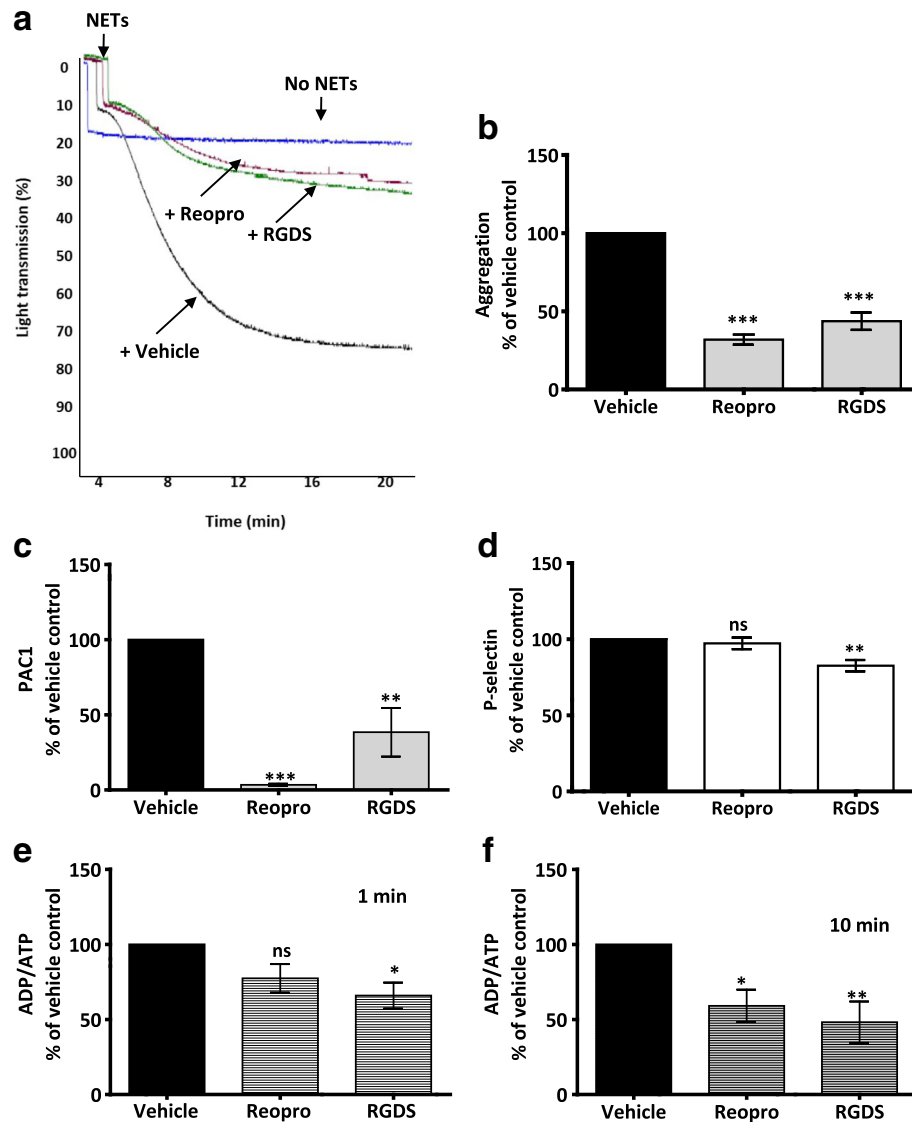
Fig. 7 Inhibition of αIIbβ3 attenuates NET-induced platelet response. **a** Representative aggregation traces showing the effect of Reopro (25 μg/mL) and RGDS (100 μM) on NET-induced platelet aggregation. WP (3×10^8/mL) were pre-incubated with the inhibitors for 15 min at 37 °C before addition of NETs. Both Reopro and RGDS reduced platelet aggregation. Platelet aggregation was measured by light transmission aggregometry (Chrono-log). **b** Bar graph comparing the effect of Reopro (25 μg/mL) and RGDS (100 μM) on NET-induced platelet aggregation. Platelet aggregation percentage was calculated after 20 min on a light transmission aggregometer. Results were normalised for each donor relative to NET-induced platelet aggregation. Data are expressed as mean ± SEM,***$P < .001$. **c-f** Bar graphs comparing the effect of Reopro (25 μg/mL) and RGDS (100 μM) on platelet activation (measured by P-selectin and active αIIbβ3 expression, $n = 3$) and ATP/ADP secretion ($n = 5$) elicited by NETs. In all assays, NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. Results were normalised for each donor relative to NET-induced platelet response. Data are expressed as mean ± SEM, *$P < 0.05$; **$P < 0.01$, ns: non-significant

NET-induced thrombosis [59], inhibition of platelet activity may not only reduce thrombosis but also platelet-mediated inflammation. Indeed, Jansen et al., have recently shown that platelet inhibition with clopidogrel was superior to DNase in reducing granulocyte activation, NET formation and acute kidney injury in a renal reperfusion injury mice model [31]. Apart from NETs inducing thrombosis, Cedervall et al. also showed that the use of DNase to disrupt tumour-induced NETs resulted in decreased neutrophil-platelet complexes in the kidney vasculature, along with improved vascular function in tumour-bearing mice [60]. Thus in these contexts NETs can also be considered as scaffold for platelets that drives inflammatory reactions.

Conclusion

This study showed for the first time that in vitro generated NETs can directly induce marked platelet

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aggregation. We further identified the molecular pathways activated in platelet responses to NETs. It is important to note that aspirin, a widely used antiplatelet, was not as effective at reducing NET-induced platelet aggregation as ticagrelor or Reopro. Finally, pretreatment of NETs with DNase or heparin did not reduce NET-induced activation or aggregation of human washed platelets. Taken together, we conclude that targeting certain platelet activation pathways rather than NET scaffold has a more profound reduction on NET-induced platelet aggregation. Further in vitro studies are needed to compare the effect of different inhibitors on NET-induced platelet responses in a more complex system such as under flow conditions.

Additional file

> **Additional file 1:** Supplementary data and figures. (PPTX 432 kb)

Abbreviations
ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; Cat G: Cathepsin G; CTH: Calf thymus histones; Hep: Heparin; MPO: Myeloperoxidase; NETs: Neutrophil extracellular traps; TLR: Toll-like receptor; Tyr: Tyrosine

Availability of data and materials
All data generated in this study are included in the manuscript.

Authors' contributions
OE and PM contributed to the conception and the design of the study; OE and NA performed the experiments; PM, OE and NA analysed and interpreted the data; OE drafted the article; NA and PM revised the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Venous blood was drawn from healthy volunteers with informed consent in concordance with the Curtin University Human Research Ethics Committee (approval number HR54/2014).

Competing interests
The authors declare that they have no competing interests.

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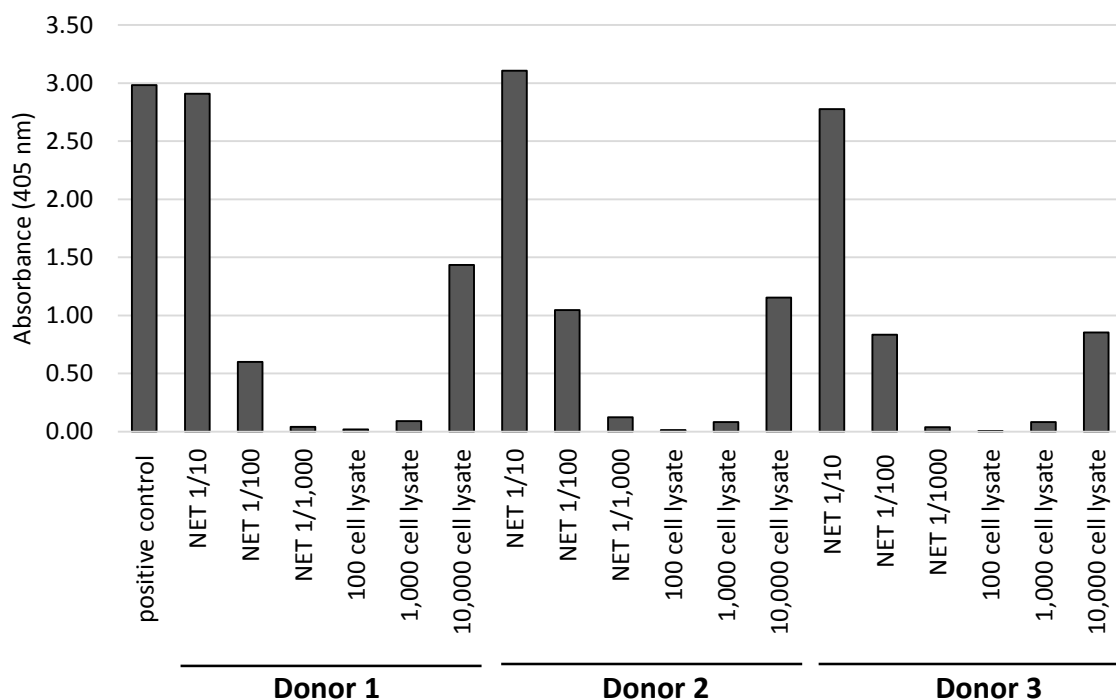
Supplementary Fig 1. Comparing cell-free NET-derived DNA-histone complex to total neutrophil lysates. DNA-histone complexes were confirmed to exist in isolated cell-free NETs from 3 donors using Cell Detection ELISA PLUS kit (Roche #11774425001). Cell-free NETs were isolated as described in materials and methods, and diluted to 1/10, 1/100 and 1/1000 in PBS for analysis in ELISA wells (20 µl per well). Neutrophil cell lysates were also prepared according to manufacturer's protocol and analysed as cell lysates equivalent to 100, 1,000 or 10,000 neutrophils. Thus, the concentration of NET used in majority of experiments (i.e. final 10% NETs) is equivalent to being activated by 3.8 ± 1.2 x 10^6/mL neutrophils. Positive control refers to the DNA-histone complex included in the kit.
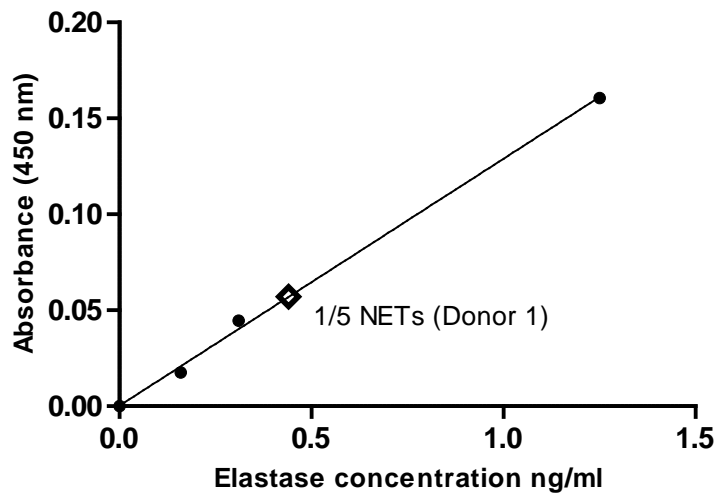
Supplementary Fig 2. Quantification of elastase in cell-free NETs. The elastase content of cell-free neutrophil extracellular traps isolated from healthy donors was quantified using the Human PMN Elastase ELISA kit (Abcam, ab119553) according to the manufacturer's protocol. Cell-free NET were prepared as described in materials and methods, and diluted 1/5 in PBS (i.e. 20% cell-free NET solution) for analysis in ELISA plate wells (100 μl per well). The samples were incubated at room temperature for 1 hr in the elastase antibody pre-coated wells, before addition of HRP-conjugated PMN elastase detection antibody and incubation for another 1 hr. TMB solution was added for 10 minutes and absorbance read at 450 nm. Graph shows standard curve of elastase with 1/5 NET dilution sample from one donor within the standard curve. Results are representative of 3 donors. Calculated elastase concentration of 10% cell-free NETs (i.e. from 1 x 10^7/mL neutrophils preparation) is 292 ± 172 pg/mL.

Supplementary data
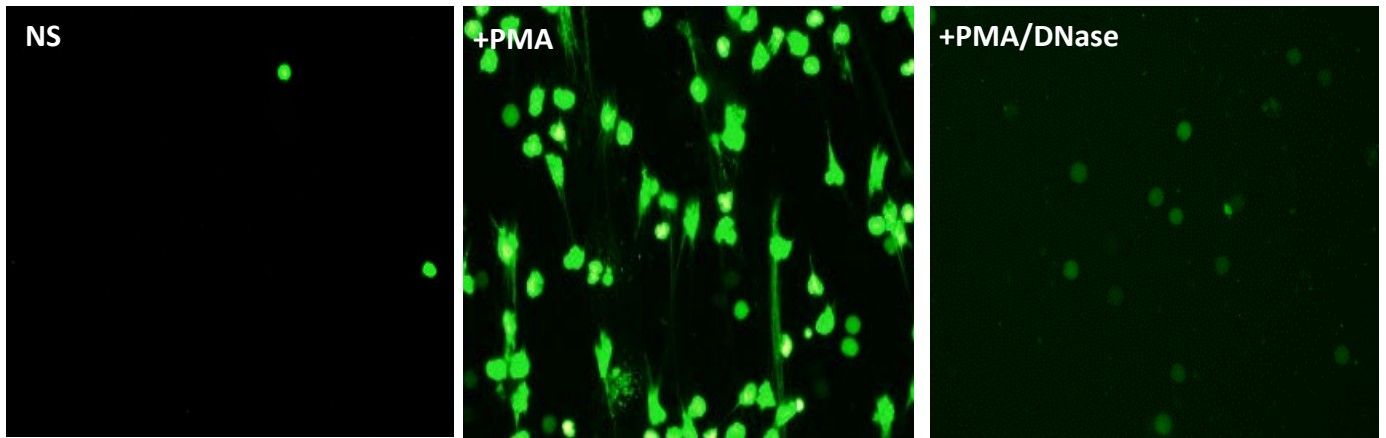
Supplementary Fig 3. Neutrophils stimulated with PMA (25 nM) produced robust NETs, whereas pre-treatment of DNase (200 U/mL) dismantled the extracellular DNA content of NETs. Neutrophils (0.5×10^6/mL) were seeded in a 96-well plate, and left untreated or pre-treated with DNase I (200 U/mL) for 30 min before stimulating with PMA (25 nM) for 3 hr. Extracellular DNA was stained with cell-impermeable SYTOX Green (5 µM) and fluorescence measured on fluorescent plate reader.
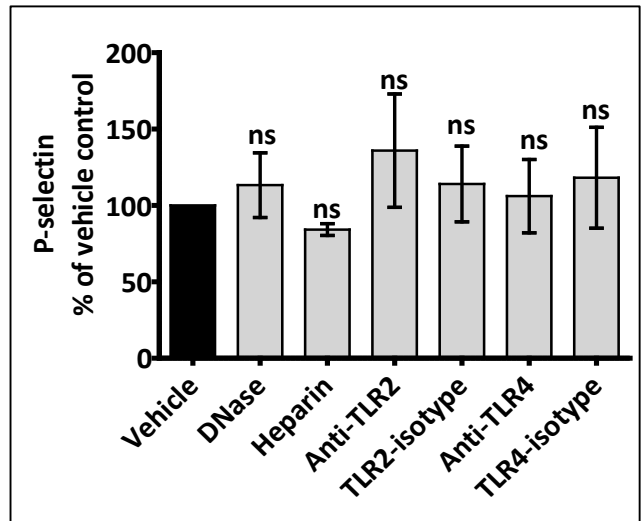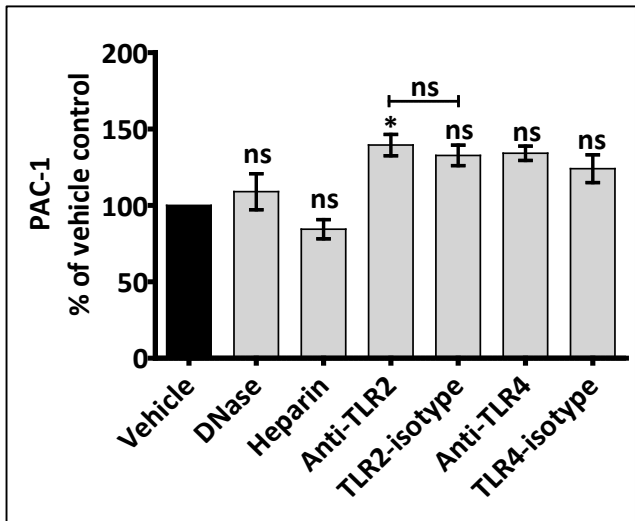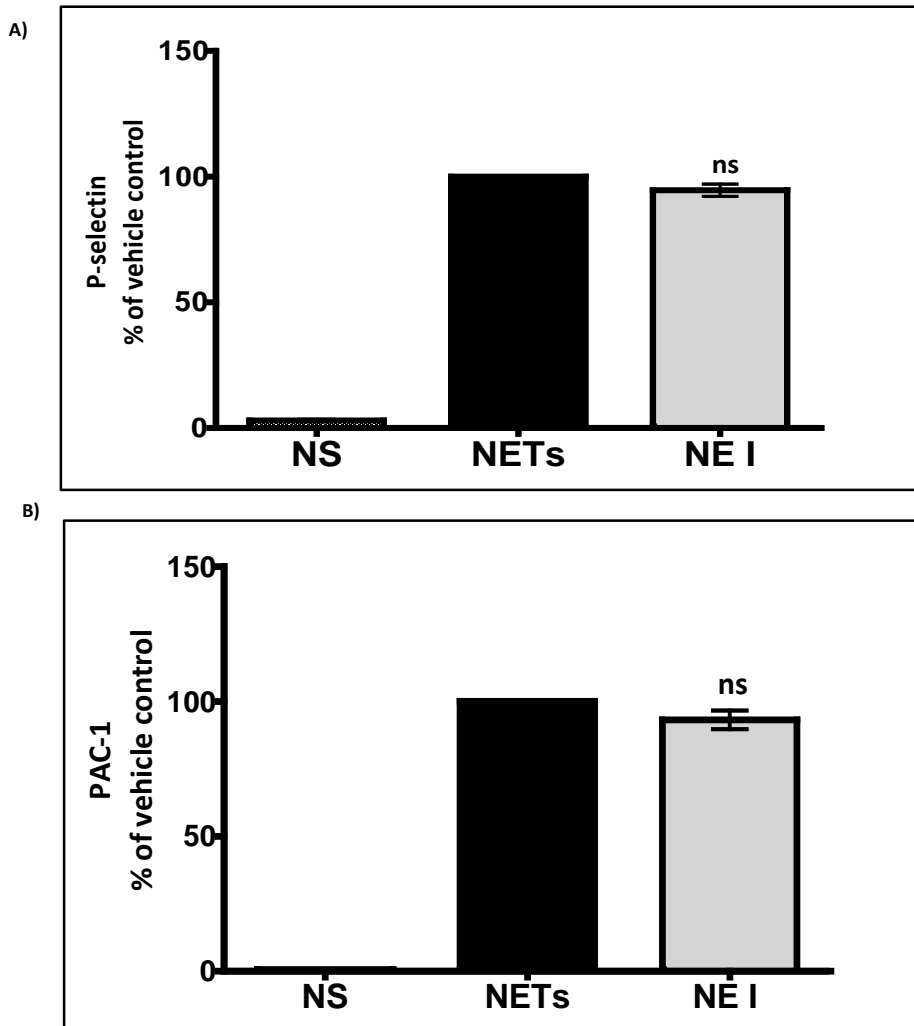
Supplementary Fig 4. Inhibition of platelet toll-like receptors (TLR) 2 and TLR4 did not affect NET-induced platelet aggregation. NETs were pre-treated with vehicle (PBS), DNase 200U/mL, heparin 200 U/mL for 30 min at 37°C before addition to WP (10 % NETs to 1×10^8 platelets/mL). NETs were also added to WP pre-treated with blocking mAbs against human TLR-2 and TLR-4 (50 µg/mL) or isotype controls. NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. WP were then incubated for 10-15 min in the dark with labelled monoclonal antibodies that detect p-selectin (CD62P) and active α2bβ3 (PAC1). The reaction was stopped by fixing cells in 2% PFA before analysing samples with flow cytometry (BD LSRFortessa). Bar graphs depict the % change in P-selectin, and PAC1 expression in platelets treated with the mentioned inhibitors. Data are expressed as mean ± SEM,*P < .05; ns: non-significant, n ≥ 3.
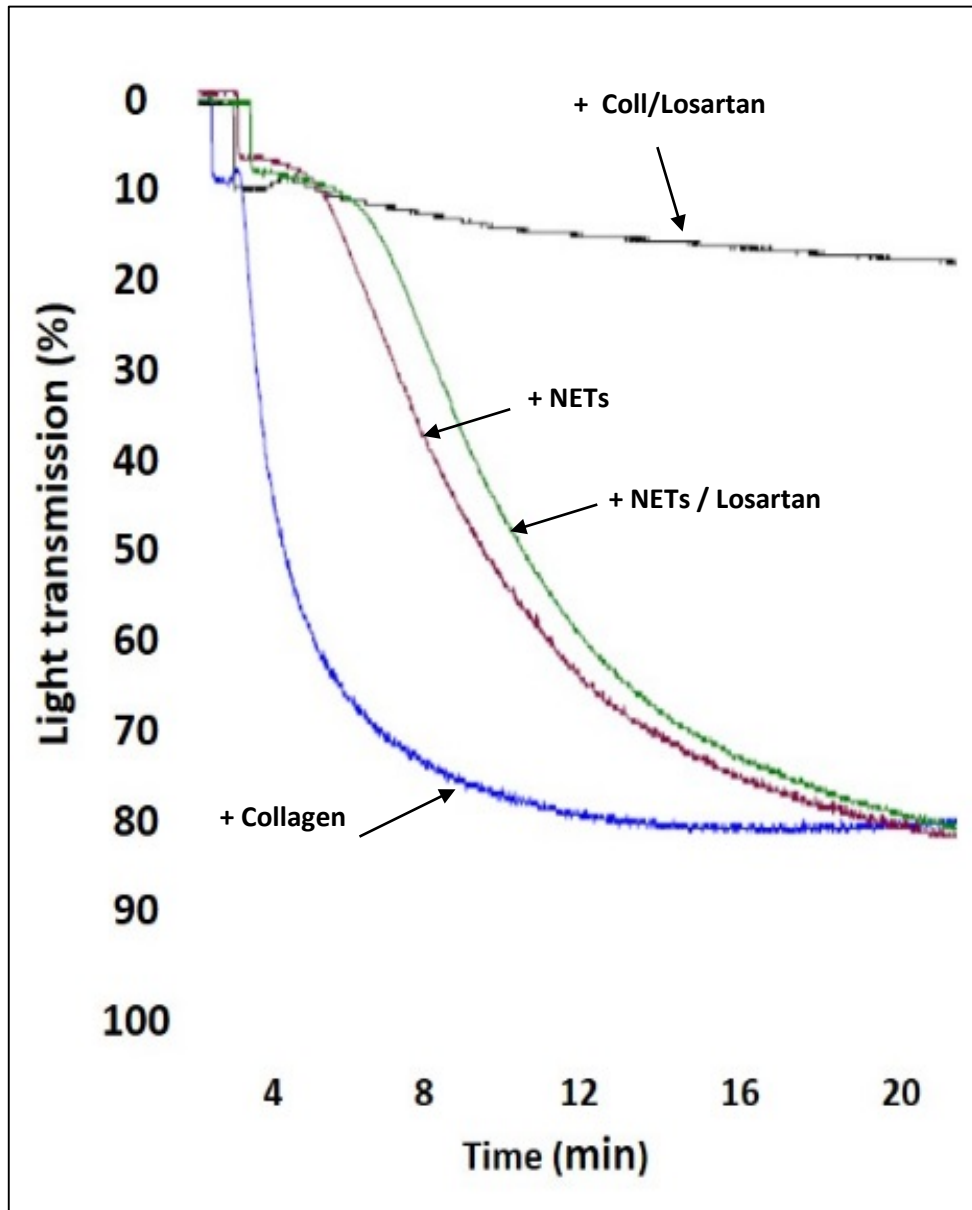
Supplementary Fig 5. Pharmacological inhibition of platelet GPVI did not significantly affect NET-induced platelet aggregation. Representative aggregation traces showing the effect of losartan (GPVI inhibitor) on collagen and NET-induced platelet aggregation. WP (3×10^8/mL) were pre-incubated with losartan (30 µM) for 1 min at 37°C before addition of collagen (5 µg/mL) or NETs. Platelet aggregation was monitored in a light transmission aggregometer (Chrono-log). NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. *Representative sample of two independent experiments.
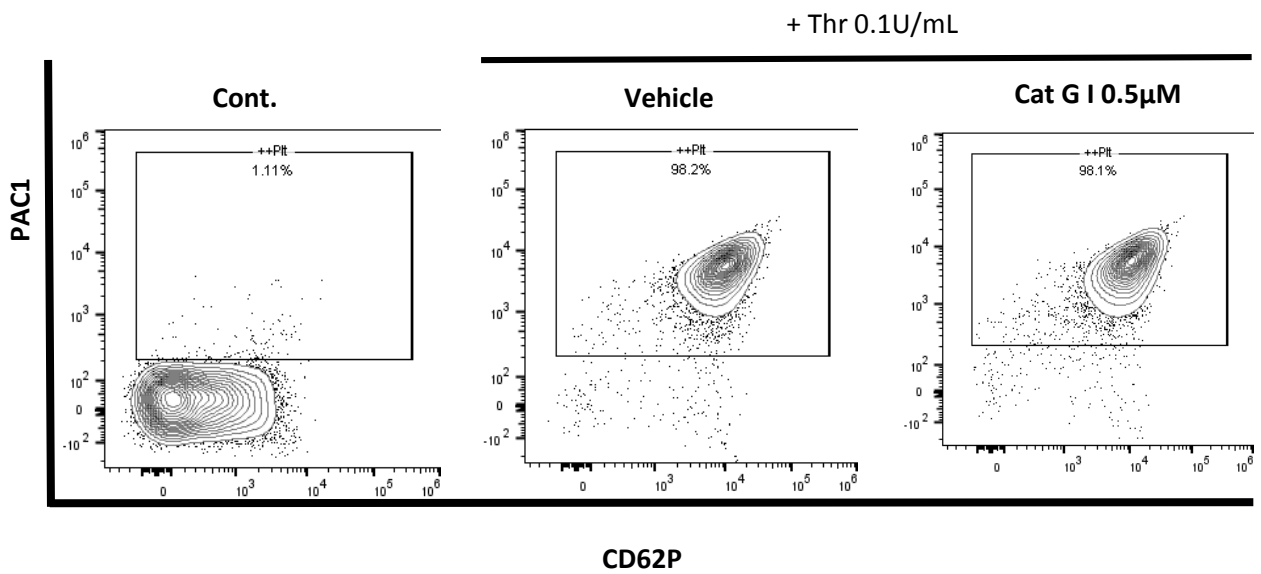
Supplementary Fig 6. Cat G I (0.5 μM) did not affect platelet physiological response to thrombin. (A-C) Platelets were treated with thrombin 0.1 U/mL ± Cat G I (0.5 μM) and labelled with CD62P and PAC1 monoclonal antibodies as explained in Fig. 3. *Representative sample of two independent experiments.

A)

B)

Supplementary Fig 7. Inhibition of NET-derived neutrophil elastase activity did not affect NET-induced platelet activation. Flow cytometry analysis of P-selectin and PAC1 on platelets. NETs were pre-treated with vehicle or neutrophils elastase inhibitor (NE I) for 30 min at 37°C. (A-B) WP $(1 \times 10^8$/ml) were incubated with NETs pre-treated with vehicle (0.1% DMSO in PBS) or NE I (2 μM, final concentration) for 10-15 min in the dark with labelled monoclonal antibodies that detect P-selectin (CD62P) and active αIIbβ3 (PAC1). NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. The reaction was stopped by fixing cells in 2% PFA before analysing samples with flow cytometry (BD LSRFortessa). Bar graphs depict the % inhibition in P-selectin, and active αIIbβ3 expression in platelets treated with NETs pre-treated with inhibitor compared to platelets treated with NETs that were pre-treated with vehicle. Results were normalized for each donor relative to NET-induced platelet response. Data are expressed as mean ± SEM,*P < .05; ns: non-significant, n=3.

A)

Collagen-induced platelet aggregation

+ Collagen

No Collagen

+ Aspirin

+ Vehicle

Light transmission %

Time (min)

B)

NET-induced platelet aggregation

+ NETs

No NETs

+ Vehicle

+ Aspirin

Light transmission %
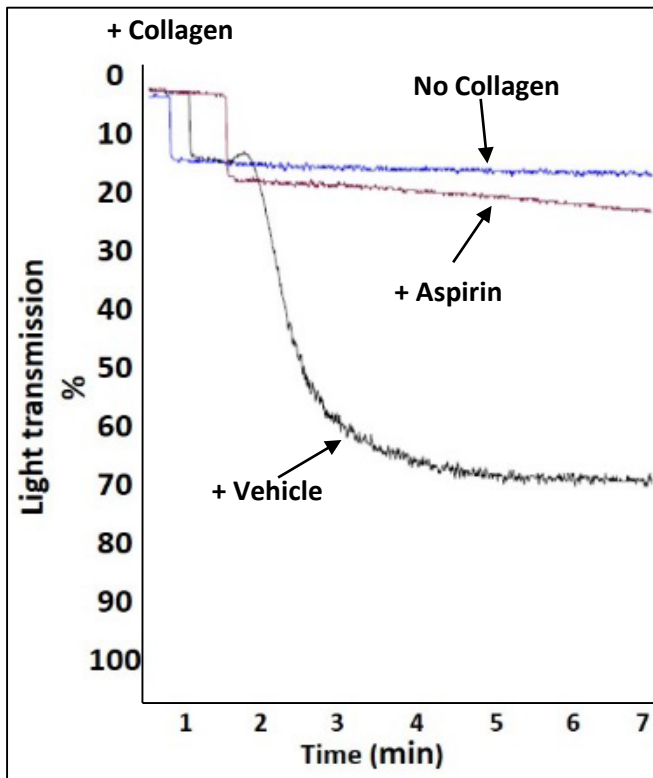
Time (min)
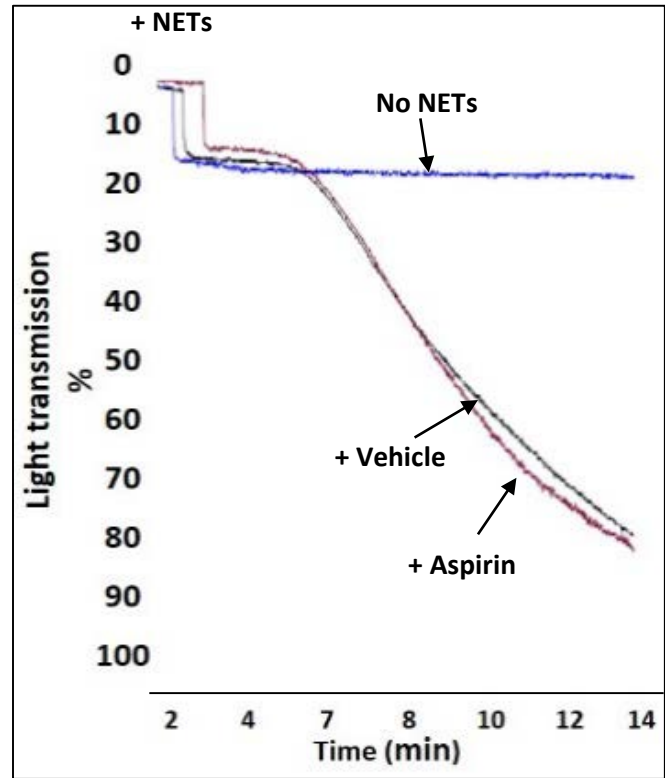
Supplementary Fig 8. Aspirin reduced collagen, but not NET, -induced platelet aggregation. (A-B) Representative aggregation traces showing the effect of aspirin 100 µM on collagen (5 µg/mL) and NET-induced platelet aggregation. WP (3×10^8/mL) were pre-incubated with aspirin for 15 min at 37°C before addition of collagen or NETs. NETs constituted 10% of final reaction volume and contains 292 ± 172 pg/mL of NET-elastase. Platelet aggregation was monitored in a light transmission aggregometer (Chrono-log). Platelets were isolated from the same donor and pre-treated with aspirin before addition of collagen or NETs. *Representative sample of two independent experiments.

Chapter 3:

Platelets activate purinergic signalling in pancreatic cancer; implications in cancer growth and chemotherapy resistance

3.1.   Publications

1. **Elaskalani O**, Abdol Razak NB, Falasca M and Metharom P. *Epithelial-mesenchymal transition as a therapeutic target for overcoming chemoresistance in pancreatic cancer*. World Journal of Gastrointestinal Oncology. 2017 Jan 15;9(1):37-41. doi:10.4251/wjgo.v9.i1.37. Review.

2. **Elaskalani O**, Falasca M, Moran N, Berndt MC, Metharom P.
    The Role of Platelet-Derived ADP and ATP in Promoting Pancreatic Cancer Cell Survival and Gemcitabine Resistance. Cancers (Basel). 2017 Oct 24;9(10). pii: E142. doi:10.3390/cancers9100142.

3.2   Introduction

Among all types of cancers, pancreatic cancer is related with the highest risk of developing thrombotic events, which are associated with poor prognosis [128]. Platelets, the key player in thrombosis, can promote metastasis through activation of epithelial-mesenchymal transition (EMT) in cancer cells. Additionally, the upregulation of EMT in pancreatic cancer has been demonstrated to impart chemotherapy resistance. Gemcitabine is the main chemotherapy drug used in pancreatic cancer; however, the development of acquired resistance hinders its efficacy [32]. This thesis and other published studies indicate that platelet-induced EMT in cancer contributes to chemotherapy resistance [18,53,297]. Several cytokines, primarily tumour growth factor beta 1 (TGF-β1), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) that are enriched in platelets can effectively induce EMT in cancer cells [44,298-300]. The first published article of this chapter will review the role of EMT in chemotherapy resistance in pancreatic cancer [46]. The second article demonstrated

that platelets, through secretion of ADP and ATP, can modulate a network of proteins and transcriptional factors that play a significant role in gemcitabine resistance in pancreatic cancer [53]. Coincidentally, during the course of our study, we discovered that pancreatic cancer cells express P2Y12, a purinergic receptor that is mainly detected on platelets. Therefore, ticagrelor, an ADP-P2Y12 inhibitor and a clinically available antiplatelet medication, was used to investigate the pro-survival effects of platelets on cancer cells. Our results suggest an additional benefit in using P2Y12 inhibitors in the treatment of pancreatic cancer. This thesis is the first to point out the significance of platelet-derived purine nucleotides (ADP and ATP) on cancer cell activity. The following part of the chapter will discuss the most recent advances in regards to the role of purinergic signalling in cancer.

3.2.1 Purinergic signalling in cancer

Recent evidence indicates that extracellular purine nucleotides (e.g. ATP, and ADP) or nucleoside (adenosine) in the tumour microenvironment (TME) promotes cancer growth, angiogenesis and metastasis [102,301]. Tumours are known to release extracellular ATP and ADP under hypoxic condition, which are rapidly degraded to adenosine [102,302]. Activated platelets in the tumour microenvironment are another source of ATP and ADP [63], and they have been shown to rapidly secrete ATP and ADP upon interaction with cancer cells [192]. Due to their role in the interplay between platelets and cancer, the following section will summarise the direct effect of ADP and ATP on cancer cells. ATP and ADP are generated in the TME in response to inflammation, hypoxia, chemotherapy, radiotherapy, and platelet activation [303-307]. Once in the extracellular space, ATP and ADP can bind to a group of receptors known as purinergic receptors. Several families of purinergic receptors were recognised according to selectivity to specific agonist, and intracellular signalling. P2X (P2X1-7) receptors are cationic channels, mainly activated by ATP, whereas P2Y (P2Y1, 2, 4, 6, 11-14) are G-protein coupled receptors that can be activated by ADP or ATP. Adenosine can bind to four different G-protein coupled receptors (A_1, A_{2A}, A_{2B}, and A_3). Reviews on purinergic signalling in several pathological conditions have been published in details elsewhere [102,308,309].

Several pieces of research showed the interaction between ATP and ADP and numerous subtypes of purinergic receptors in cancer cells. For example, Hu et al., have recently demonstrated that extracellular ATP through interaction with the P2Y2 receptor, can promote cancer cell proliferation via the PI3K/AKT signalling pathway. P2Y2 was found to be overexpressed in pancreatic cancer and is associated with poor survival. Moreover, inhibition of the P2Y2 receptor synergised with gemcitabine in a xenograft pancreatic cancer mouse model [310].

In this thesis, we showed that ATP induced upregulation of SLUG, an EMT transcription factor that can impart gemcitabine resistance. SLUG was linked with an increased level of gemcitabine deactivating enzyme, cytidine deaminase (CDD), and downregulation of equiliberative nucleoside transporter 1 (ENT1) which is involved in the drug uptake [32,53]. Although our research suggested that P2Y12 was involved in the process, ATP-induced SLUG via P2Y2 or other purinergic receptors could also contribute.

Extracellular ATP is abundant in the tumour tissues, reaching 100 µM range compared to normal tissues where ATP is barely detectable [309]. In hepatocellular carcinoma, the ATP receptor P2X3 is overexpressed and is associated with poor survival, substantiating the observation that the activation of P2X3 with ATP promoted cell proliferation *in vitro* [311]. In colorectal cancer cells, ATP promoted the expression of multidrug resistance-associated protein 2 (MRP2) through the activation of ERK, a protein kinase that promotes cell survival and inhibits apoptosis, leading to an increase in chemotherapy resistance. ATP effect was indicated to be mediated through P2Y receptors, however, the exact subtype of the receptor was not shown in the study [312].

It has been shown that ATP activation of P2Y6 and P2X7 can lead to interleukin-8 (IL-8/CXCL8) release by cancer cells [313-315]. IL-8 is a chemokine, also known as a neutrophil chemotactic factor, which mediates immune cell recruitment, promotes angiogenesis, cancer cell proliferation and metastasis. Interestingly, a recent study reported that IL-8 is released by breast cancer cells in response to platelets, however, the role of platelet-derived ATP in the induction of IL-8 secretion by cancer cells was not examined [316].

The role of ADP signalling in the TME has attracted much interest recently, especially in the interplay between cancer cells and platelets. Cho et al., recently demonstrated that ovarian cancer cells release more ADP compared to normal cells. The production of cancer-derived ADP induced platelet activation, leading to the release of platelet-derived soluble factors that support cancer growth and metastasis, and the inhibition of the ADP-P2Y12 axis subsequently reduced cancer growth [63]. This thesis demonstrated that ADP, through the P2Y12 receptor, can activate AKT and ERK, and promoted the expression of SLUG and CDD [53]. In chapter 4 of this thesis, we showed that the knockdown of P2Y12 reduced SLUG expression in pancreatic cancer, suggesting P2Y12 has a possible role in mediating chemo-resistance in pancreatic cancer. Intriguingly, in a study by Sarangi S et. al., P2Y12 was found to be equally expressed in normal and malignant cells but inhibition of P2Y12 only potentiated cisplatin cytotoxicity in breast cancer cell lines [64]. In another cancer model, using Glioma C6 cells, ADP was capable of promoting cell proliferation through both P2Y1 and P2Y12 receptors [317].

The relation between ATP, ADP and cancer is complex. Overexpression of a purinergic receptor subtype can change the effect of the agonist (ATP, ADP, and adenosine) on cancer cell activity. Besides, cancer cells can control the availability of ATP and ADP through the expression of ectonucleotidases, which can rapidly degrade ATP and ADP to adenosine. Moreover, several purinergic receptors can signal through trans-activation of an oncogenic receptor. For example, P2Y1, A_{1A}, P2Y2, P2Y12 can trans-activate EGFR (more discussion in chapter 4) [310,318,319]. Several studies have shown that ATP and ADP can have opposing effects on cancer growth, for example, Bz-ATP (a stable ATP derivative) between 5-500 µM promoted cell proliferation in breast cancer and neuroblastoma cells [320,321], whereas a relatively similar concentration range (30-300 µM) activated apoptosis in human cervical epithelial cells and a melanoma cell line [322,323]. These discrepancies may be due to a variety of factors, including the type of the cells, the degree of engagement of certain purinergic receptor subtype, availability of the agonist, and the interplay between a purinergic receptor and other oncogenic receptors [102] (chapter 4).

3.3   Summary

It is well-established that platelets can support cancer growth, angiogenesis and metastasis. Platelets are capable of releasing an array of biological factors in response to cancer cells. However, little attention has been paid to the effects of platelet-derived ADP and ATP on cancer progression. This is surprising since an encounter with cancer cells initiates platelets to promptly release these factors; a rapid reaction that precedes platelet aggregation response. Several lines of evidence have convincingly shown that ATP and ADP can directly modulate cancer cell activity and favour cancer progression. Notably, inhibition of the ATP receptor P2Y2 potentiated gemcitabine activity in pancreatic cancer cells. This chapter showed the ability of ticagrelor, a P2Y12 antagonist, to directly inhibit ADP-mediated survival signals in pancreatic cancer cells. The involvement of ATP/ADP in chemotherapy resistance may allow the integration of purinergic signalling inhibition with chemotherapy as a novel strategy for cancer treatment. More importantly, targeting P2Y12 in pancreatic cancer may offer additional benefits by reducing platelet activation and the risk of patients developing cancer-associated thrombosis.

Elaskalani O, Abdol Razak NB, Falasca M and Metharom P. *Epithelial-mesenchymal transition as a therapeutic target for overcoming chemoresistance in pancreatic cancer*. World Journal of Gastrointestinal Oncology. 2017 Jan 15;9(1):37-41. doi:10.4251/wjgo.v9.i1.37. Review.

MINIREVIEWS

Epithelial-mesenchymal transition as a therapeutic target for overcoming chemoresistance in pancreatic cancer

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Author contributions: Falasca M initiated the manuscript concept; Elaskalani O researched and drafted the review; Elaskalani O and Razak NBA wrote the manuscript; Metharom P revised and contributed to the writing of the manuscript; Metharom P and Falasca M edited the manuscript; Falasca M approved the final version of the manuscript.

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Abstract

Pancreatic cancer has one of the worst prognoses among all cancers due to the late manifestation of identifiable symptoms and high resistance to chemo- and radiation therapies. In recent years, a cancer development phase termed epithelial-mesenchymal transition (EMT) has gained increasing research focus. The process is implicated in tumour metastasis, and emerging evidence suggests EMT also contributes or induces chemoresistance in several cancers. Nevertheless, the applicability of therapeutic targeting of EMT faces many challenges. In this mini-review, we summarise the evidence supporting the role of EMT in pancreatic cancer progression, focusing particularly on its association with chemoresistance.

Key words: Epithelial-mesenchymal transition; Drug resistance; Pancreatic cancer; Chemotherapy

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Core tip: This mini-review examines the role of epithelial-mesenchymal transition in the development of drug resistant pancreatic cancer and a possible use of this process as a drug target.

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a stage of phenotypic alteration in cancer cells instigated by several paracrine and autocrine stimuli, leading to a morphological transformation of epithelial-like cancer cells to an elongated mesenchymal phenotype. The phenotypic change is thought to derive from a shift in the balance between epithelial (*e.g.*, E-cadherin and Claudin-1) and mesenchymal (*e.g.*, N-cadherin, Snail, Zeb-1 and Twist-1) factors. Once described as a key step for successful metastasis in some types of cancers[1], the role of EMT in chemotherapy resistance has attracted much interest recently. Indeed, EMT has been shown to contribute to drug resistance in pancreatic cancer. For instance, in a recent study, the patterns of sensitivity and resistance to three conventional chemotherapeutic agents with divergent mechanisms of action were investigated in several pancreatic cancer cell lines[2]. Interestingly, gene expression profiling revealed that the sensitive and resistant cells formed two distinct groups with resistant cells showing several features consistent with EMT. Additionally, an inverse correlation between E-cadherin and its transcriptional suppressor, Zeb-1, was observed in the gene expression. Moreover, silencing of Zeb-1 restored drug sensitivity in pancreatic cancer cells. The implication of this study is that effectors of EMT, in particular Zeb-1, are potential molecules to target to overcome drug resistance. Recently, a study casts doubt on the role of EMT in metastasis, while a separate study strongly corroborates the key role of EMT in drug resistance. Indeed, both Fischer *et al*[3] and Zheng *et al*[4], demonstrated that not only was EMT able to aid chemotherapy resistance in pancreatic and lung cancer cells but that EMT was not needed for metastasis, as inhibition of EMT did not prevent metastasis of pancreatic and lung cancer cells.

EMT SIGNALLING PATHWAYS IN PANCREATIC CANCER

Several signalling pathways can regulate EMT during tumorigenesis[5-7]. Major pathways which can induce EMT include those activated by growth factors such as transforming growth factor-β (TGFβ), epidermal growth factor and hepatocyte growth factor or the Wnt/beta-catenin and Notch pathway. Notch-2 activation was identified in gemcitabine-resistant (GR) pancreatic cancer cells that have acquired EMT. By using pancreatic cancer cells that have developed gemcitabine resistance through exposure to increasing concentrations of gemcitabine, Wang *et al*[8] were the first to delineate the EMT profile of GR cells. GR but not gemcitabine sensitive (GS) cells had an elongated and irregular shape, increased expression of Zeb-1, vimentin, fibronectin and alpha-SMA, as well as translocation of E-cadherin from the plasma membrane to the nucleus. In concordance with previous reports regarding the role of Notch signalling in EMT, Wang *et al*[8] further characterised the Notch activation status in GR cells. Notch-2 and its ligands, Jagged-1 and Notch-4, were upregulated in GR cells. The Notch signalling also upregulates NF-κB, a critical mediator of the EMT process. Indeed, NF-κB was upregulated in GR cells as well as its downstream target, matrix metallopeptidase 9 (MMP-9). The activity of MMP-9 is governed by urinary plasminogen activator (uPA), also upregulated in GR cells, and both proteins are known for their role in cancer invasion and metastasis. Using Notch-2 and Jagged-1 siRNA, the interrelation between Notch, EMT and NF-κB were further delineated. Notch-2 and Jagged-1 knock-down in GR cells led to mesenchymal-epithelial transition (MET) morphological changes which include upregulation of E-cadherin, and reduction of vimentin, slug, snail, Zeb-1, and NF-κB (p65 subunit). As expected, Notch-2 and Jagged-1 downregulation by siRNA also led to a reduction in GR cells invasion and migration[8]. The study characterised for the first time the Notch-driven EMT pathway in GR pancreatic cancer cells, however, the authors did not address whether silencing or inhibition of Notch could reverse GR.

EMT AND GEMCITABINE RESISTANCE

The development of gemcitabine resistance and its association with EMT phenotype has drawn attention to a possible role of gemcitabine in inducing EMT. Güngör *et al*[9] examined the role of Midkine (MK) in orchestrating the interplay between Notch, EMT and gemcitabine resistance. MK is a heparin-binding growth factor overexpressed in some types of cancers[10,11]. High MK mRNA expression was detected in pancreatic cancer cell lines that developed gemcitabine resistance following repeated exposure to gemcitabine, and in PDAC tumour samples isolated from patients who underwent total pancreaticoduodenectomy. Gemcitabine treatment in GR cells led to a dose-dependent increase in MK mRNA expression and protein secretion, an effect not observed in GS cells. Knockdown of MK with siRNA restored gemcitabine sensitivity in GR cells, while the addition of recombinant human MK (rh-MK) to MK-knockdown or GS cells, restored or induced resistance, respectively. GR cells overexpressing MK displayed EMT characteristics while MK-depleted GR cells displayed MET characteristics as evidenced by downregulation of vimentin and NF-κB, and upregulation of E-cadherin. As expected, there was a reduction in migration and invasion in MK-depleted cells compared to control. As Notch-2 and EMT are associated with gemcitabine resistance, Güngör *et al*[9] further examined the impact of MK on Notch-2 activation. Treatment of GR cells with rh-MK resulted in enhanced cleavage of Notch[ICD] and expression of Hes-1 (Notch-2 target). Expectedly, silencing of Notch-2 improved gemcitabine efficacy in GR cells. Güngör *et al*[9] were the first to pinpoint the role of MK in gemcitabine resistance and its impact on

Notch-2 activation and EMT phenotype.

The role of Notch-2 activation in EMT, metastasis and chemotherapy resistance has attracted attention to target Notch-2 in pancreatic cancer. Palagani *et al*[12] showed the effect of the γ-secretase inhibitor (GSI IX) in preventing Notch-2 activation, EMT, and cancer cell proliferation and migration *in vitro* and pancreatic tumour-initiating CD44+/EpCAM+ xenograft growth and metastasis *in vivo*. Future studies are needed to examine the effect of GSI IX in reducing gemcitabine resistance in pancreatic cancer.

A few microRNAs are implicated in EMT and chemoresistance. Li *et al*[13] were the first to introduce a novel way of reducing gemcitabine resistance in pancreatic cancer (PaCa) through modulation of microRNAs. GR PaCa cells showed downregulation of miR-200 in addition to the typical EMT signature discussed earlier. Upregulation of miR-200 either through the reintroduction of miR-200 or the treatment with the natural compound, isoflavone, resulted in MET as demonstrated by decreased levels of mesenchymal markers (Zeb1, Vimentin and Slug) and induction of epithelial-associated morphological changes, and thus reducing gemcitabine resistance[13]. Using a similar methodology, Ma *et al*[14] recently showed a positive influence of miR-233 in EMT, invasion, migration and gemcitabine resistance in PaCa cells.

The conversion in cancer cells from epithelial to mesenchymal phenotype and its requirement for cancer invasion and migration were clearly demonstrated through *in vitro* studies. However, the consequence of EMT on cancer metastasis had not been investigated *in vivo* until recently. Using the well-established mouse model that mirrors human pancreatic cancer (KPC mice), Zheng *et al*[4], produced KPC mice absent in Snail or Twist protein. Although accumulating evidence suggests the requirement of EMT process for cancer migration, the authors showed the ability of pancreatic cancer to metastasize despite deleted EMT-inducing factors, Snail and Twist. Deletion of either one of these proteins did not affect local invasion, metastasis or overall survival compared to control KPC mice. It also resulted in a reduction in expression of other mesenchymal markers (*e.g.*, Slug, Zeb1 and alpha-SMA) while enhancing the expression of the epithelial factor, E-cadherin. Although the apoptosis of cancer cells was not affected by deletion of Snail or Twist, the proliferation rate of cancer cells significantly increased, while blood dissemination remained unchanged compared to control KPC mice. Examination of EMT profile of the metastatic pancreatic cancer cells at secondary sites (liver, lung and spleen) showed positive for E-cadherin, and negative for Snail or Twist. Moreover, the ability of cancer cells, isolated from either control KPC mice or KPC with deleted Snail or Twist, to form tumour spheres were comparable. In the study, EMT program did not appear essential for primary cancer growth, local invasion, blood dissemination and metastasis. Interestingly, gemcitabine sensitivity was improved in KPC mice with deleted Snail or Twist compared to KPC control mice which could be explained by a significant upregulation of equilibrative nucleoside transporter 1 and concentrating nucleoside transporter 3 (receptors that mediate uptake of nucleosides) in cancer cells lacking Snail or Twist.

GENERAL CONSIDERATIONS ON PHARMACOLOGICAL APPROACHES TO TARGET EMT

Several strategies have been proposed for the design of EMT-based therapies as recently and extensively described and reviewed by Davis *et al*[1]. While major challenges and questions remain regarding the possibility of targeting EMT to counteract metastasis specifically, stronger evidence is accumulating on the use of anti-EMT agents in cancer chemoresistance settings. However, targeting a single receptor, enzyme or transporter that is associated with EMT faces many limitations since several redundant pathways are involved in this process. Strategies focused on targeting micro-RNAs regulating EMT such as miR-200, or transcription factors might represent a more effective approach since they influence the process more broadly. In addition, key components of the tumour microenvironment are also attractive targets for therapeutic intervention. Indeed, recent evidence has revealed that local tumour microenvironment represents a main driving force for EMT, chemotherapy resistance and cancer progression. Inflammatory cells such as neutrophils and macrophages are contained in the tumour microenvironment, which offers multidirectional interactions leading in some cases to increased chemotherapy resistance and metastasis[15-18]. Neutrophils have been shown to induce EMT in pancreatic cancer while macrophages induce gemcitabine resistance *via* promoting cytidine deaminase mediated drug inactivation[15,19,20]. Similarly, platelets were recently shown to be capable of inducing EMT in cancer[21,22]. Pancreatic cancer is associated with a high risk of venous thromboembolism (VTE) caused by tumour-derived or tumour-elicited tissue factor which can indirectly induce platelets aggregation[23,24]. Whether targeting platelets can offer an indirect way to reduce EMT and chemoresistance as well as the risk of VTE in pancreatic cancer is yet to be demonstrated.

Therefore, a systematic testing of different methods for targeting EMT in combination with existing chemo-therapeutic agents is required for each model of therapy relapse. The excitement elicited by the new reinforcement of the link between EMT and chemore-sistance will surely result in a surge of studies in this field, and consequently, further in-depth investigations are warranted, especially in pancreatic cancer.

CONCLUSION

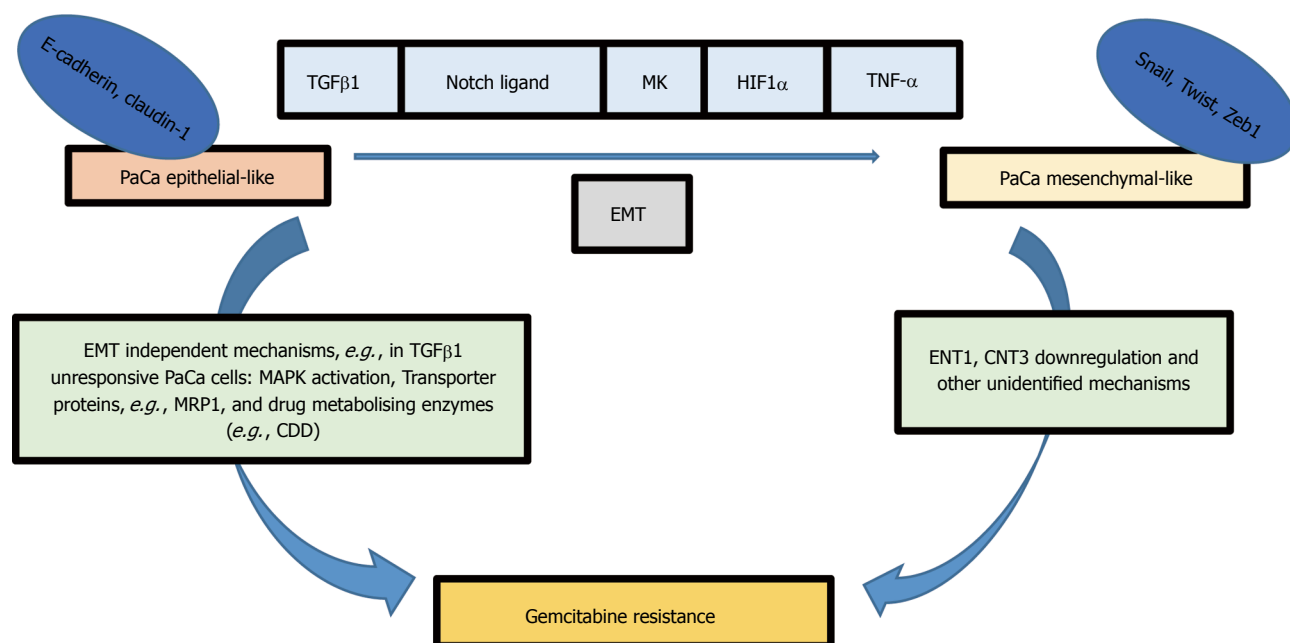In summary, resistance to treatments such as Gemci-tabine in pancreatic cancer can be mediated by several

Figure 1 Several signalling pathways can induce epithelial-mesenchymal transition in pancreatic cancer cells for example Notch, transforming growth factor-β, Midkine, hypoxia-inducible factor-1α and tumor necrosis factor-α. EMT phenotype is associated with gemcitabine resistance; however, the signalling pathway relating EMT factors (*e.g.*, Snail, Twist and Zeb1) to gemcitabine resistance is not clearly identified, although ENT1 and CNT3 upregulation was observed in KPC mice models with deleted Snail or Twist. EMT independent pathways can also lead to gemcitabine resistance for example MAPK activation, transporter proteins, and gemcitabine metabolising enzymes (*e.g.*, CDD). MRP1: Multidrug resistance protein1; CDD: Cytidine deaminase; TGFβ: Transforming growth factor-β; MK: Midkine; HIF1α: Hypoxia-inducible factor-1α; TNF-α: Tumor necrosis factor-α; EMT: Epithelial-mesenchymal transition; ENT1: Equilibrative nucleoside transporter 1; CNT3: Concentrative nucleotide transporter.

EMT-dependent or independent pathways (Figure 1)[15,25-29], making the EMT process an attractive target for reducing chemotherapy resistance in pancreatic cancer. EMT can be regulated by blocking extracellular signalling molecules such as TGFβ1 (a cytokine mediator of EMT in many types of cancer) and EMT signal transduction pathways[1]. Loss of E-cadherin-mediated cell adhesion is a hallmark of EMT and subsequent invasion and metastasis. Since Snail and Zeb family of transcriptional factors mediate E-cadherin translocation, loss of function or downregulation, they can potentially be targeted to avert EMT at its initial steps. Several studies have reported disruption in TGFβ signalling in pancreatic cancer[30-32]. Therefore, the mesenchymal transcriptional factors may be better druggable targets compared to TGF-β receptors to reduce EMT-derived chemoresistance in pancreatic cancer. Despite a significant number of emerging studies examining the role of EMT in cancer, the interplay between different signalling pathways that drive EMT is more complex than we initially thought. The fact that the phenotypic alteration is transient and triggered by several dynamics encountered by tumour cells during their development or metastasis emphasises the challenge of utilising EMT as a lone druggable target. The diversity of EMT-inducing transcriptional factors may enable cancer cells to adapt and survive a single targeted molecular therapy. The association between EMT and chemotherapy resistance is well established in the literature, but it is not well understood how EMT can affect cancer cell survival pathways, drug transporters and drug metabolising enzymes. Delineation of these interactions may uncover novel approaches to inhibit chemoresistance in pancreatic cancer. Nevertheless, at present, there may be potential benefits in tempering cancer EMT in a combined immunotherapy and molecular-targeted drug strategies to treat pancreatic cancer.

ACKNOWLEDGMENTS

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The Role of Platelet-Derived ADP and ATP in Promoting Pancreatic Cancer Cell Survival and Gemcitabine Resistance

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Abstract: Platelets have been demonstrated to be vital in cancer epithelial-mesenchymal transition (EMT), an important step in metastasis. Markers of EMT are associated with chemotherapy resistance. However, the association between the development of chemoresistance, EMT, and the contribution of platelets to the process, is still unclear. Here we report that platelets regulate the expression of (1) human equilibrative nucleoside transporter 1 (hENT1) and (2) cytidine deaminase (CDD), markers of gemcitabine resistance in pancreatic cancer. Human ENT1 (hENT1) is known to enable cellular uptake of gemcitabine while CDD deactivates gemcitabine. Knockdown experiments demonstrate that Slug, a mesenchymal transcriptional factor known to be upregulated during EMT, regulates the expression of hENT1 and CDD. Furthermore, we demonstrate that platelet-derived ADP and ATP regulate Slug and CDD expression in pancreatic cancer cells. Finally, we demonstrate that pancreatic cancer cells express the purinergic receptor $P2Y_{12}$, an ADP receptor found mainly on platelets. Thus ticagrelor, a $P2Y_{12}$ inhibitor, was used to examine the potential therapeutic effect of an ADP receptor antagonist on cancer cells. Our data indicate that ticagrelor negated the survival signals initiated in cancer cells by platelet-derived ADP and ATP. In conclusion, our results demonstrate a novel role of platelets in modulating chemoresistance in pancreatic cancer. Moreover, we propose ADP/ATP receptors as additional potential drug targets for treatment of pancreatic cancer.

Keywords: platelets; ADP; ATP; pancreatic cancer; gemcitabine

1. Introduction

Despite remarkable advancements in our understanding of cancer development and progression, pancreatic ductal adenocarcinoma (PDAC) continues to be one of the most aggressive types of cancer, with a mortality rate that has not changed for the last 50 years. In addition to relatively late stage symptoms and high metastasis, chemotherapy resistance contributes significantly to increased mortality in PDAC [1]. Hypercoagulable disorders often characterise people living with PDAC, marked particularly with an increased risk of venous thromboembolism (VTE) [2]. The association of PDAC with thrombotic events thus suggests a close interplay between cancer and platelets, the key player in haemostasis and thrombosis.

Within the blood compartment, tumour cells can form aggregates with platelets to avoid natural killer cell-mediated cytotoxicity [3–5]. PDAC cells can induce platelet activation and aggregation [6]. Once activated, platelets release growth factors and angiogenic factors which contribute to cancer progression [7–9]. Platelets through direct contact or release of Transforming growth factor beta 1 (TGFβ1) can activate epithelial-mesenchymal transition (EMT) in tumour cells, an important step in cancer metastasis [10,11]. EMT is a phenotypic alteration in cancer cells towards a more metastatic phenotype characterised by elongated shape and advanced ability to invade and migrate to distant organs, associated with a change in the level of several adhesion proteins and transcriptional factors. For example, Slug, Snail, Twist and Zinc finger E-box-binding homeobox 1 (Zeb-1) are described as mesenchymal transcriptional factors. Upregulation of some or all these factors may predispose cells to a mesenchymal phenotype [12]. Recently it has been shown that EMT may play a role in cancer progression other than by promoting metastasis. For example, Snail and Slug have been associated with chemotherapy resistance [13,14]. Moreover, gemcitabine-resistant PDAC cells display an EMT signature [15], while deletion of the EMT program in a pancreatic cancer mouse model enhanced gemcitabine sensitivity [16].

Gemcitabine is a prodrug cytidine analogue that is clinically used to manage PDAC. Like most cytidine analogues, the activity of gemcitabine is dependent on its cellular uptake, drug activation and deactivation rates. Gemcitabine is a hydrophilic drug. Therefore penetration through the lipid bilayer of the plasma membrane is a crucial step in gemcitabine-mediated cytotoxicity. The human nucleoside transporters mediate cellular uptake of gemcitabine. Deoxycytidine kinase mediates the phosphorylation-dependent activation of gemcitabine, while cytidine deaminase (CDD) catalyses gemcitabine deactivation [17–19]. Indeed, overexpression of hENT1 was associated with enhanced gemcitabine response while downregulation of CDD reduced gemcitabine resistance [20,21]. CDD expression is relatively high in some organs like liver [22], where most drugs passage on their transit to the target organ.

In this study, we have explored if platelets can drive gemcitabine resistance in PDAC cells. To investigate this, we exposed cancer cells to platelets or the releasate from aggregated platelets and examined the level of several proteins that are known to impart gemcitabine resistance in PDAC (Slug, CDD, hENT1) and the phosphorylated level of the survival signalling molecules such as Protein kinase B (also known as Akt) and Extracellular signal-regulated kinase (Erk). We also found that the platelet-derived nucleotides, ADP and ATP, are the main soluble mediators that drive gemcitabine resistance, which is completely blocked by ticagrelor, an ADP P_2Y_{12} receptor antagonist, and that the P_2Y_{12} receptor antagonist is expressed not only on platelets but also on PDAC cells.

2. Results

2.1. Platelet Releasate Promotes Proliferation and Survival Signals in PDAC Cells Challenged with Gemcitabine

High platelet count is associated with chemotherapy resistance and poor prognosis [23]. It is known that activated platelets release a variety of growth factors that support cancer cell proliferation and survival [24–26]. In order to address if platelets also enable PDAC cells to survive the antiproliferative effect of gemcitabine, platelet releasate isolated from collagen-related peptide (CRP)-aggregated platelets was incubated with cancer cells at increasing concentrations of gemcitabine (0–100 μM) for 72 h. As shown in Figure 1A,B, platelet releasate induced a significant increase in proliferation in PDAC cell lines, AsPC-1 and BxPC-3, despite gemcitabine challenge ($n \geq 4$; AsPC-1, $p < 0.05$ and BxPC-3, $p < 0.05$ at 10 μM and 1 μM gemcitabine, respectively).

Next, we examined if platelet releasate can initiate survival signals in cancer cells challenged with gemcitabine. AsPC-1 cells are considered gemcitabine-resistant, while BxPC-3 cells are gemcitabine sensitive. Therefore, two different concentrations of gemcitabine were used with the two cell lines. AsPC-1 and BxPC-3 cells were incubated with platelet releasate ± gemcitabine (25 μM and 10 μM, respectively) for 2 h in Roswell Park Memorial Institute (RPMI) serum-free medium.

As shown in Figure 1C,D, platelet releasate triggered a significant upregulation of phosphorylation of the survival signalling molecules, p-Erk and p-Akt, in both AsPC-1 and BxPC-3 cells, which was unaffected by the presence of gemcitabine.
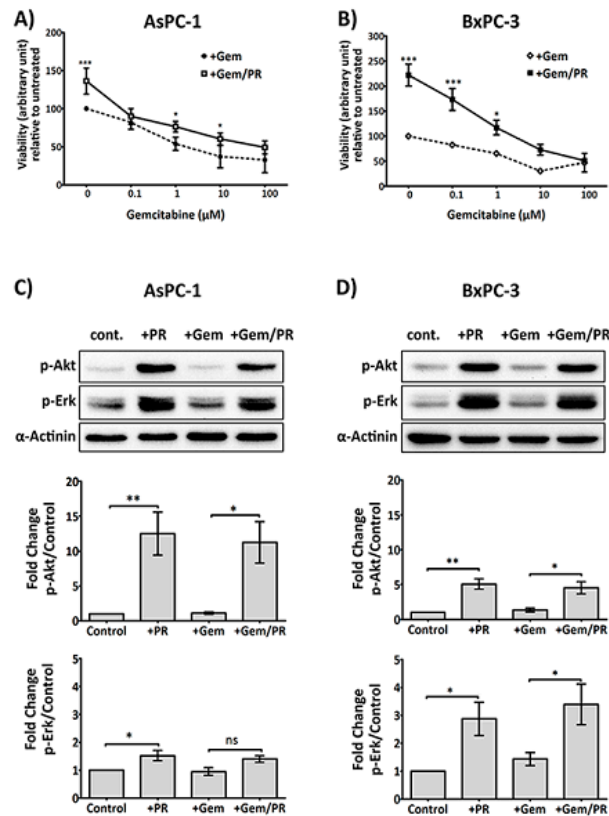

Figure 1. Platelet releasate (PR) promotes pancreatic ductal adenocarcinoma (PDAC) cell survival in the presence of gemcitabine. PDAC cell lines, AsPC-1 (**A**) or BxPC-3 (**B**), were seeded at 5000 cells/well, in a 96-well plate for 24 h, then treated with platelet releasate (PR) $\pm$ gemcitabine (0 to 100 μM) for 72 h. PR was prepared from xl-CRP (1 μg/mL)-aggregated platelets (5×10^9 platelets/mL) and used in cell culture at 1:10 dilution, resulting in the final concentration equivalent to the amount of releasate from 5×10^8 platelets/mL. Cell proliferation was quantified by Alamar blue reagent, a non-toxic cell health indicator dye that is converted to fluorescent red colour in living cells. The fluorescence intensity (ex/em 570 nm/610 nm) was quantified by a multi-mode plate reader (PerkinElmer). Statistics were calculated using two-way ANOVA, with $n = 5$ for (**A**) and $n = 4$ for (**B**), *** $p < 0.0001$, * $p < 0.05$. Changes in the phosphorylation status of Protein kinase B (Akt) and Extracellular signal-regulated kinase (Erk) in cancer cells at 2 h were determined following high-dose gemcitabine treatment (25 μM for AsPC-1 and 10 μM for BxPC-3). Briefly, cancer cells were seeded at 3×10^5 cells/well in a 6-well plate for 24 h, serum starved for 6 h, then treated with PR $\pm$ gemcitabine for 2 h. Cell lysates were separated by SDS-PAGE and immunoblotted using phospho-specific antibodies for Akt (upper panel) and Erk1/2 (middle panel). Alpha (α)-actinin (lower panel) was used as loading controls for each protein. The expression level of the protein of interest was quantified and normalised to the loading control with automated software Image Lab (version 5.1, BioRad, CA, USA) and GraphPad Prism 5 (GraphPad Software, Inc, CA, USA). The columns represent fold changes in protein expression level compared to vehicle control-treated cells. Data are presented as mean $\pm$ SEM. One-way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean, with $n \geq 4$ for (**C**) and $n \geq 3$ for (**D**), ** $p < 0.001$, * $p < 0.05$. Abbreviations: Gem—gemcitabine, xl-CRP—cross-linked Collagen-related peptide, PDAC—pancreatic ductal adenocarcinoma, PR—platelet releasate, Akt—Protein kinase B, Erk—Extracellular signal-regulated kinase, SDS-PAGE—sodium dodecyl sulfate polyacrylamide gel electrophoresis, ANOVA—Analysis of variance.

2.2. Platelet Releasate Induces A Rapid Upregulation of the EMT Transcription Factor, Slug, Independent of the TGFβ/Smad Pathway

Platelets can affect a change in cancer cells by upregulation of relevant transcription factors involved in the EMT process, including Snail, Slug, and Twist [10,27,28]. One such marker, Slug, has recently been implicated in supporting chemotherapy resistance activity in tumour cells [13,14,29]. The main platelet-derived soluble factor that has been suggested to regulate Slug expression is TGFβ1, through the Smad effector pathway [10,30]. However, TGFβ/Smad signalling defects and non-Smad signalling pathways have been observed in several conditions and cell types [31–33]. Therefore, we investigated the expression of Slug and TGFβ/Smad signalling in AsPC-1 and BxPC-3 cells stimulated with platelet releasate.

The releasate used in our study was determined to contain approximately 10 ng/mL of TGF-β1 by ELISA (Supplementary Figure S1), which is in line with the previous reported literature [34]. As shown in Figure 2A,B, platelet releasate induced a rapid upregulation of Slug expression after 2 h in both AsPC-1 and BxPC-3. Interestingly, the increase in Slug by platelet releasate was not entirely due to TGFβ1, as Slug level remained upregulated in the presence of a TGFβ1 receptor inhibitor (SB431542 (Tocris Bioscience, Bristol, UK), Figure 2C,D. And the increase of Slug expression by TGFβ1 is much more elevated in BxPC-3 compared to AsPC-1, suggesting the cell lines may have distinct responses to TGFβ1 (Supplementary Figure S2). Additionally, SB431542 reduced the phosphorylation of Smad2/3 in both platelet releasate-stimulated AsPC-1 and BxPC-3 cells. Moreover, platelet releasate was able to sustain Slug upregulation in cancer cells challenged with gemcitabine (Figure 2E,F).
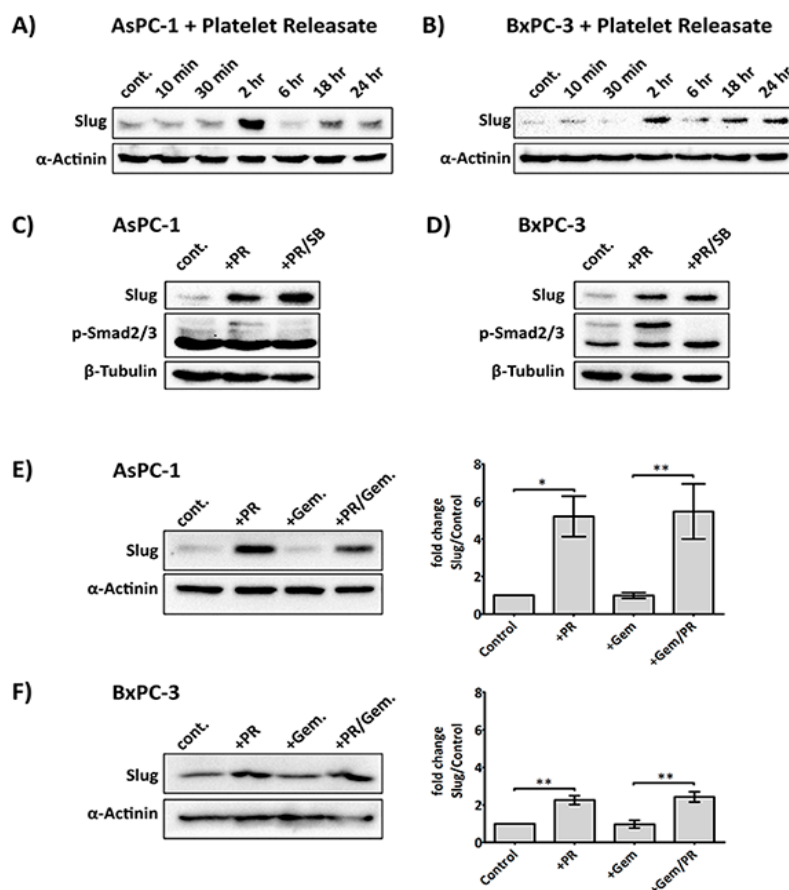
Figure 2. PR induces a rapid upregulation of Slug, an EMT and chemotherapy resistance marker, independent of the TGFβ1/Smad pathway. Representative immunoblots (**A**) and (**B**) show Slug expression in AsPC-1 and BxPC-3 cells after time course treatment with platelet releasate (PR). 2×10^5 cancer cells per well were seeded in a 12-well plate for 24 h, serum starved for 6 h, then PR was added to the culture media (final concentration of PR was equivalent to releasate from 5×10^8 platelets/mL) for 10 min, 30 min, 2 h, 6 h, 18 h and 24 h. Representative immune blots (**C**) and (**D**) show Slug and pSmad2/3 expression in AsPC-1 and BxPC-3 cells after treatment with PR $\pm$ 10 μM SB431542 (TGFβ1 receptor inhibitor) or 0.1%DMSO (vehicle control, Sigma-Aldrich, St. Louis, MO, USA) for 2 h. Cell lysates were separated by SDS-PAGE and immunoblotted using specific antibodies for Slug (upper panel), p-Smad2/3 (middle panel) and loading control protein α-actinin or β-tubulin (lower panel). Each of the immunoblots (**A**)–(**D**) is representative of two independent experiments with similar results. Representative immune blots (**E**) and (**F**) and the associated bar graphs show Slug expression in AsPC-1 and BxPC-3 cells after 2 h PR $\pm$ gemcitabine treatment. The expression level of the protein of interest was quantified relative to the loading control. The graph columns represent fold changes in protein expression level compared to vehicle-treated cells (*n* = 4 for (**E**) and *n* > 3 for (**F**)). One way ANOVA with post.-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean (** $p < 0.001$, * $p < 0.05$).

2.3. Platelets Modulate hENT1 and CDD in PDAC Cells

As platelet releasate could promote cancer cell proliferation and activation of key kinases involved in supporting cell survival, Erk and Akt, despite the presence of gemcitabine, we hypothesised that platelet-derived factors could modulate the cellular metabolism and uptake of gemcitabine to counteract its cytotoxic effects. The influx of gemcitabine depends primarily on the nucleoside transporters [35]. Once inside the cell, gemcitabine can be deactivated by CDD, an enzyme whose increased expression has been implicated in chemoresistance [36,37]. As shown in Figure 3, platelet

releasate (equivalent to 1×10^8 platelets/mL) significantly augmented the expression of CDD in both AsPC-1 and BxPC-3 cell lines. The level of hENT1 was decreased by platelet releasate in the two cell lines, with statistically significant reduction observed in AsPC-1. Unstimulated or degranulated platelets, however, were less effective at modulating the expression levels of hENT1 and CDD.
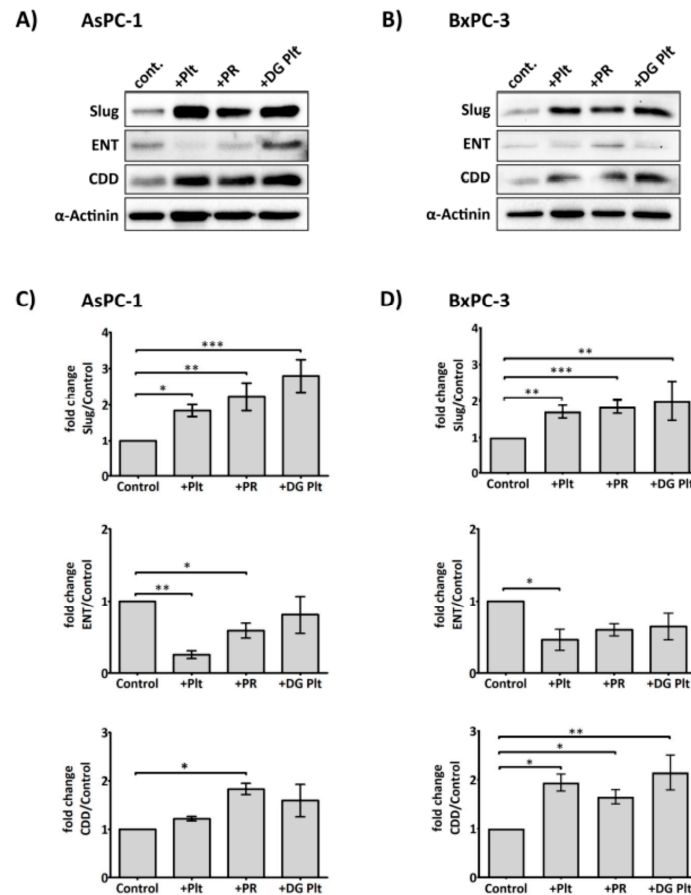

Figure 3. Platelets modulate the expression of Slug, human equilibrative nucleoside transporter 1 (hENT1) and cytidine deaminase (CDD) in PDAC cells. Representative immunoblots (**A**) and (**B**) show the expression of Slug, hENT1 and CDD in AsPC-1 and BxPC-3 after treatment with platelets (Plt), platelet releasate (PR) or degranulated platelets (DG Plt) for 24 h. PR and degranulated platelets (DG Plt) were isolated from activated platelets. Briefly, platelets (1×10^9/mL) were aggregated by incubating with 1 µg/mL CRP for 30 min then the supernatant (i.e., PR) and the pellet (i.e., DG Plt) were separated by centrifugation (5000 g for 10 min). The pellet was resuspended in Tyrode's buffer, using the initial volume. The cancer cells were seeded in a 6-well plate at 3×10^5 per well for 24 h, then incubated with Plt, PR or DG Plt to the final concentration equivalent to 1×10^8 platelets/mL for 24 h in serum-free media. Cell lysates were separated by SDS-PAGE and immunoblotted using specific antibodies for Slug, hENT1, CDD and loading control protein α-actinin. Bar graphs (**C**) and (**D**) show the changes in the expression of Slug, hENT1 and CDD in AsPC-1 and BxPC-3 after different treatments. The expression level of the protein of interest was quantified relative to the loading control and normalised to the negative control group ($n \geq 3$). Data are presented as mean $\pm$ SEM. One way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean. *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$.

2.4. Slug Modulates Expression of CDD and hENT1 in PDAC Cells

As platelet releasate was demonstrated to significantly increase Slug and alter the levels of CDD and hENT1 in PDAC cells, we next examined whether Slug is necessary for the modulation of CDD

and hENT1 expression. A *SLUG* messenger RNA (mRNA) knockdown assay, using two different short interfering RNA (siRNA) sequences specific for *SLUG* mRNA and an irrelevant siRNA negative control, was performed to assess the changes in the expression of CDD and hENT1 in PDAC cells. Figure 3 clearly shows that the expression of CDD was significantly suppressed and, conversely, hENT1 was significantly increased in the absence of Slug in AsPC-1 cells. The protein expression change trends were also observed in the BxPC-3 cells, but the modulation was less pronounced and not statistically significant.
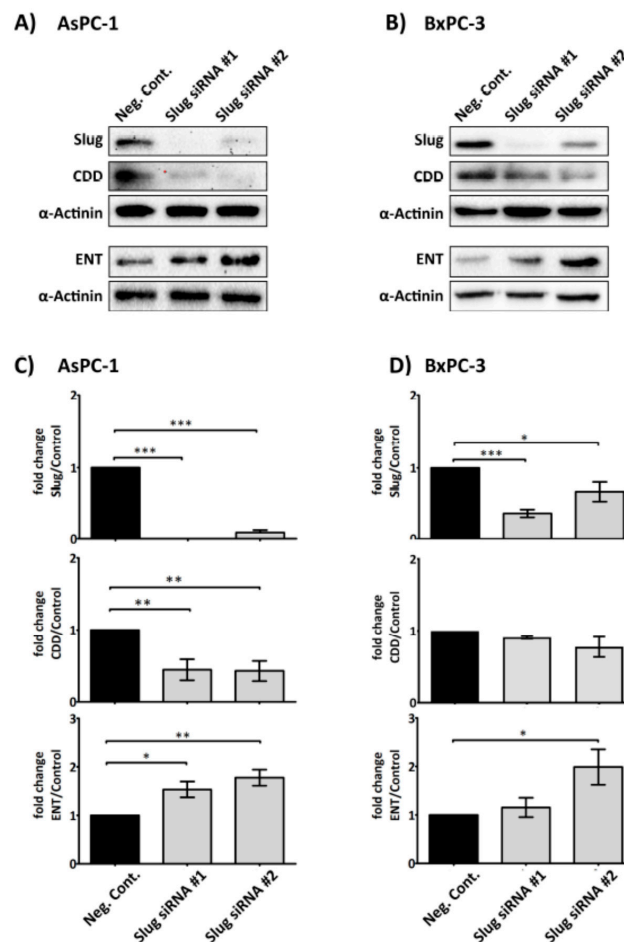
Figure 3. Slug mediates the expression level of CDD and hENT1 in PDAC. Representative immunoblots (**A**) and (**B**) show the expression of Slug, hENT1 and CDD in AsPC-1 and BxPC-3 after Slug-specific siRNA treatment. 3×10^5 cancer cells were seeded in 6-well plate for 24 h. Slug siRNAs (the number #1 and #2 designate two different Slug siRNA sequences) or a negative control siRNA (75 nM) in serum/antibiotic-free media were added to the adherent cells and incubated for 24 h. The media was then replaced with fresh media (+10% fetal bovine serum (FBS)) and cells incubated for a further 24 h before cells were lysed and examined for the expression of the proteins of interest. Bar graphs (**C**) and (**D**) show the expression of Slug, hENT1, CDD and loading control α-actinin in AsPC-1 and BxPC-3 post siRNA treatment. The expression levels were quantified relative to the loading control. The columns represent the fold change of protein levels relative to the negative control siRNA treated cells ($n \geq 3$). Data are presented as mean ± SEM. One way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$. Neg. Cont.: negative control.

2.5. Platelet-Derived ADP and ATP Promote Slug and CDD Expression Levels in PDAC Cells

Several purinergic receptors are reported to be expressed on cancer cells, including PDAC cells, ((e.g., $P2Y_1$ and $P2X_7$, which are adenosine diphosphate (ADP) and adenosine triphosphate (ATP) receptor, respectively)) [38,39]. More interestingly, recent studies suggest that purinergic signalling can promote invasiveness and EMT in prostate cancer cells [40,41]. Since ADP and ATP are secreted from activated platelets, and our above data indicated that platelet-derived soluble factors were responsible for modulating Slug and CDD expression, we next investigated whether the enzyme apyrase—which catalyses the hydrolysis of ATP and ADP—could ameliorate the effect of platelet releasate on Slug and CDD expression in PDAC cells. Our results show that apyrase (1 U/mL) significantly negated PR-induced upregulation of Slug and CDD in cancer cells (Figure 4A,B) but apyrase did not significantly modulate hENT1 expression level (Supplementary Figure S3). Exogenous ADP and ATP (100 μM) also increased the expression levels of Slug and CDD in PDAC cells (Figure 4C,D).
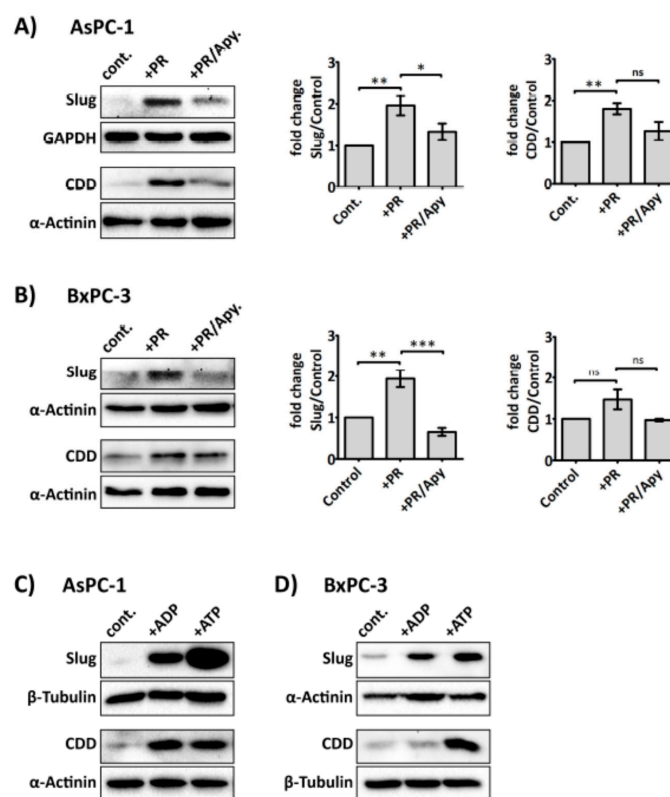

Figure 4. Platelet-derived ADP and ATP mediate Slug and CDD expression in PDAC cells. Representative immunoblots and bar graphs (**A**) and (**B**) show Slug and CDD expression in AsPC-1 and BxPC-3 cells after incubation with PR or PR pre-treated with apyrase (1 U/mL, for 30 min at 37 °C). 3×10^5 cells were seeded in a 6-well plate for 24 h, then PR or apyrase pre-treated PR were added to the cancer cells for a further 24 h in serum-free media. The final concentration of PR used was equivalent to releasate from 5×10^8 platelets/mL. Cell lysates were prepared and used in SDS-PAGE and immunoblotting as previously described. The expression levels of the proteins of interest were quantified relative to the loading control. The columns represent fold-change in protein expression level compared to control, vehicle-treated cells. Data are presented as mean ± SEM. One way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean. $n \geq 4$. *** $P < 0.0001$, ** $P < 0.001$, ** $P < 0.05$. Representative immunoblots (**C**) and (**D**), from two independent experiments, show Slug and CDD expression in AsPC-1 and BxPC-3 after exogenous ADP or ATP (100 μM) treatment for 24 h. cont.: control.

2.6. The Antiplatelet Drug, Ticagrelor, Reduces PR-Induced Akt, Erk Activation and Slug Upregulation in Cancer Cells

In view of the fact that platelet-derived ADP/ATP could significantly promote upregulation of Slug and CDD, two important markers of gemcitabine resistance, we, therefore, assessed whether AsPC-1 and BxPC-3 expressed the ADP receptor, $P2Y_{12}$ by western blot analysis (Supplementary Figure S4), which confirmed its expression in both cell lines. We therefore assessed whether the clinically available $P2Y_{12}$ receptor antagonist (ticagrelor) reduced PR-initiated signals in cancer cells. Cancer cells adherent in 6-well plates were treated with PR $\pm$ ticagrelor (10 µM) and incubated for 2 h in serum-free media. As shown in Figure 5, ticagrelor negated the PR effect on Akt, Erk activation and Slug upregulation in AsPC-1 and BxPC-3. Exogenous ADP and ATP (100 µM) were also able to induce Akt, Erk activation and Slug upregulation in cancer cells and these effects were completely blocked by ticagrelor (10 µM) (Supplementary Figure S5).
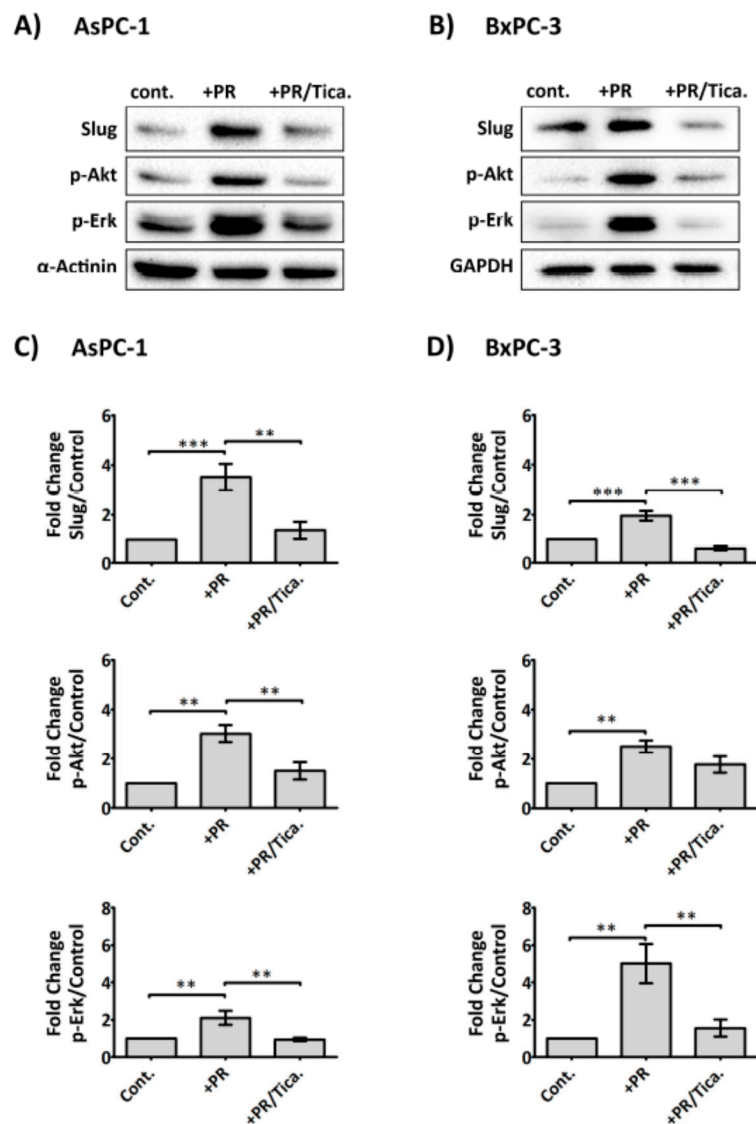
Figure 5. Anti-platelet drug ticagrelor, an antagonist of the purinergic receptor P2Y$_{12}$ receptor, reduces the effects of PR on cancer cells. Representative immunoblots show Slug, p-Akt and p-Erk expression in AsPC-1 and BxPC-3 cells after incubation with PR $\pm$ ticagrelor (Tica, 10 μM) for 2 h. Sample preparations and immunoblotting were performed as previously described. The final concentration of PR used was equivalent to releasate from 5×10^8 platelets/mL. Bar graphs (**C**) and (**D**) show the expression levels of the proteins of interest relative to the loading control. Columns represent fold change in protein expression level compared to control non-treated cells. $n \geq 5$. *** $p < 0.0001$, ** $p < 0.001$, ** $p < 0.05$. Data are presented as mean $\pm$ SEM. One way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean. cont.: control.

3. Discussion

Various types of cancer cells, including PDAC cells, can activate platelets, which in turn, can support tumour cell growth and metastasis [10,42–44]. Recent clinical studies have indicated an association between platelets and poor chemotherapeutic responses in cancer patients [45,46]. However, the mechanisms by which platelets confer chemoresistance to cancer cells have remained unclear. In accordance with several investigative studies on the impact of platelets on tumour growth [9,47,48], our data showed that platelet-derived factors could promote proliferation of AsPC-1 and BxPC-3 cancer cells and protected them from the cytotoxic effects of gemcitabine (Figure 1A,B). Previous

studies indicated that cellular Akt or Erk activity was associated with gemcitabine resistance [49–52]. Here, after exposure to platelet releasate, a significant increase of Akt and Erk phosphorylation was detected in both cell lines, and the activation of these survival signalling molecules remained in the presence of gemcitabine (Figure 1C,D). Our results suggest that platelet releasate contained an active component (or components) capable of regulating Akt and Erk phosphorylation and promoting cell growth under chemotherapy.

Platelet-derived TGF-β1 has been implicated as the major signalling regulator of cancer progression towards an invasive phase, through an induction of EMT [10,53,54]. Critical downstream effectors of TGF-β1 include receptor-regulated Smad2/3 [55–57] and Slug [58,59], a transcriptional repressor and regulator of EMT and chemoresistance [29,60]. In our study, the expression of Slug in both PDAC cell lines was significantly, and rapidly, augmented by the addition of platelet releasate (Figure 2A,B). However, the level of Slug did not lessen in the presence SB431542, a TGF-β1 receptor blocker. Furthermore, the phosphorylation of Smad2/3 by platelet releasate was inhibited by SB431542 in both cell lines, suggesting that the increase in Slug expression by PR in AsPC-1 and BxPC-3 was independent of TGF-β1R signalling (Figure 2C,D). The exposure to exogenous TGFβ1 showed that the cells may have distinct responses to the cytokine, as the upregulation of Slug expression was much more elevated in BxPC-3. Therefore, our data suggest that inhibition of platelet-derived TGF-β1 signalling was dispensable under these conditions and other factors apart from TGF-β1 could promote Slug expression. More importantly, when the cancer cells were grown in the presence of platelet-derived soluble factors and challenged with gemcitabine, Slug expression stayed elevated (Figure 2E,F). The signalling cascade through the complex Smad2/3/4 may be very important in some type of pancreatic cancer cells as Smad4 is a critical mediator of TGFβ signalling. However, Smad4 is also known to be inactivated in more than 50% of PDAC patients, and several in vitro studies have indicated that both AsPC1 and BxPC3 lack Smad4 protein expression [61–65], therefore its contribution to signal transduction is negligible in regards to these particular cancer cell lines.

The effectiveness of gemcitabine relies on the function of several proteins, including the influx protein, ENT1 [35], deoxycytidine kinase (dCK) (phosphorylates gemcitabine into an active form) [66], and CDD (deactivates gemcitabine) [36,37]. In our hands, we observed a statistically significant upregulation of CDD expression in cancer cells cultured with platelet releasate (Figure 3), suggesting that soluble factors from platelets could increase the capacity of cancer cells to deactivate gemcitabine. Inversely, the expression of hENT1 was decreased, at a statistically significant level in AsPC-1 and a trend reduction in BxPC-3. Reduced hENT1 implies that a lesser amount of gemcitabine could be transported inside the cells, thus increasing cell survival. Interestingly, degranulated platelets were also capable of instigating a significant upregulation of Slug in the cancer cells, suggesting a physical interaction between the surface molecules of platelets and cancer cells could induce intracellular signalling down the EMT pathway. Similarly, Labelle et al. have previously observed that a direct contact between cancer cells and platelets can activate a nuclear factor kappa-light-chain-enhancer of activated B cells, Nf-κB, pathway and EMT-like transformation in cancer cells independent of platelet-released factors [10]. Although studies indicate a common pathway for Nf-κB and Slug in regulating EMT [67,68], currently it is still unclear which receptors are involved in this cascade, and more studies are required to understand this interaction better.

The results of Slug knockdown indicated that the expression levels of CDD and hENT1 were dependent on Slug (Figure 3). Absent or reduction of Slug, especially in AsPC-1 cells, significantly reduced CDD and increased hENT1 expression, respectively. BxPC-3 Slug knockdown showed similar trends but were not statistically significant. This may be due in part to BxPC-3 being relatively more resistant to siRNA transfection. Our data highlight the novel role of Slug in the gemcitabine metabolism pathway and further validate its role in acquired-chemoresistance.

Since the increase of Slug expression by platelet releasate was not significantly dependent of platelet-derived TGF-β1, we hypothesised that Slug expression could be regulated by platelet-derived nucleotides, ADP and ATP. Platelets release ADP and ATP from their dense granules after interacting

with stimuli such as cancer cells. Several studies have suggested nucleotides to be important in promoting tumour metastasis by increasing the permeability of the transendothelial barrier [43], maintaining tumour cell survival under duress [69,70], and eliciting EMT-related genes in cancer cells [40]. Here, we show that degradation of ATP and ADP by apyrase prevented the platelet releasate-induced increase of Slug and CDD expression in PDAC cells (Figure 4A,B). Furthermore, exogenously supplied ADP and ATP to AsPC-1 and BxPC-3 increased both the Slug and CDD expression level, suggesting a regulatory role for ADP and ATP in EMT and gemcitabine metabolism in cancer cells.

Activation of purinergic receptors, including $P2Y_1$ [71] and $P2X_7$ [39,72], in PDAC cells have been shown to promote cancer cell growth. Here, we report a low-level expression of the ADP receptor $P2Y_{12}$ in PDAC cell lines AsPC-1 and BxPC-3 (Supplementary Figure S4). This corroborated with the Human Protein Atlas data (https://www.proteinatlas.org/ENSG00000169313-P2RY12/pathology) which indicated elevated $P2Y_{12}$ expression in pancreatic cancer tissue and negligible detection level in normal tissue [73,74]. $P2Y_{12}$, considered the predominant receptor that mediates ADP-induced platelet activation [75] and detected primarily in platelets and brain tissues [76,77] has also been detected in other cell types including smooth muscle cells [78,79] and pancreatic islets [80]. Here, our data show that ticagrelor, an ADP receptor $P2Y_{12}$ antagonist and clinically available antiplatelet drug, was effective at reducing platelet releasate-induced cancer cell activation (Figure 5). This was a direct effect of ticagrelor on the cancer cells as the cells were exposed only to platelet-derived soluble factors, not whole platelets. As ATP is an unstable molecule and can be rapidly hydrolysed to ADP and phosphate, the effects observed with exogenously administered ATP on the cancer cells may also be the results of the action of ADP. This is corroborated by the inhibitory effect of ticagrelor on cancer cells supplied with extracellular ATP (Supplementary Figure S5). It must be noted that the action of ticagrelor on the cell lines may also be due to its ability to weakly inhibit the uptake of adenosine by hENT1 [81,82], therefore preventing the pro-survival effect provided by adenosine uptake [83]. Furthermore, a drug review information (NDA number 22-433) from the U.S. Food and Drug Administration indicated that ticagrelor, at lower μM concentrations, can inhibit adenosine A3 receptor, and phosphodiesterase 5 (PDE5) [84]. Studies have shown that a blockage of A3 receptor reduced viability and chemotherapy resistance in glioblastoma stem-like cancer cells [85], whereas, inhibition of PDE5 potentiated gemcitabine in pancreatic cancer cells [86].

In summary, we show here for the first time that factors released from activated platelets can support PDAC cells to better survive under gemcitabine challenge by modulating CDD and hENT1 expression, both of which are controlled by Slug. The chemoresistance was mediated largely through platelet-derived nucleotides ADP and ATP. As tumours are exposed to high levels of extracellular nucleotides and nucleosides within the microenvironment [87–89], a supplementary strategy to target platelet activation and purinergic signalling in cancer treatment may be beneficial. Additionally, we showed a direct anti-cancer effect of ticagrelor, warranting a further assessment of the drug in the context of cancer therapy.

4. Materials and Methods

4.1. Reagents and Cell Lines

Apyrase, ADP, ATP, gemcitabine and ticagrelor were obtained from Sigma-Aldrich, (St. Louis, MO, USA). Inhibitor of the transforming growth factor-β (TGF-β) type I receptor, SB431542 was from Tocris Bioscience, Bristol, UK. AsPC-1 and BxPC-3 cell lines were obtained from ATCC and tested negative for mycoplasma (tissue culture facility routine testing). Cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM Sodium Pyruvate, 1 mM non-essential amino acids (all from Gibco® Life Technologies Australia Pty Ltd., Mulgrave, Australia), and 10% Foetal Bovine Serum (FBS, from Bovogen Biologicals, Keilor East, Australia).

4.2. Preparation of Human Washed Platelets

Blood from healthy volunteers was drawn into ACD (acid-citrate-dextrose—15% *v/v*) with informed consent in concordance with the Curtin University Human Research Ethics Committee (approval number HR54/2014). Washed platelets were prepared as previously described [90]. Platelet count was adjusted to 5×10^9/mL and suspended in HEPES-Tyrode's buffer (5 mM HEPES, 5.5 mM glucose, 138 mM NaCl, 12 mM NaHCO$_3$, 0.49 mM MgCl$_2$, 2.6 mM KCL, 0.36 mM NaH$_2$PO$_4$, 1.8 mM CaCl$_2$, pH 7.4). To prepare degranulated platelets (DG Plt) and platelet releasate (PR), washed platelets were activated with cross-linked CRP (collagen-related peptide—1 µg/mL, Auspep, Tullamarine, Australia) at 37 °C for 30 min. The DG Plt pellet was separated from the PR supernatant by centrifugation at 5000 *g* for 10 min.

4.3. Cell Viability

Cancer cells were seeded at 5000 cells/well in a 96-well plate. After 24 h, the medium was replaced with serum-free RPMI 1640 medium supplemented with platelet releasate and gemcitabine (0.1 to 100 µM) and incubated for a further 72 h. Cell viability was measured by detecting the metabolic activity of live cells using Alamar blue (Resazurin sodium salt, Sigma-Aldrich, St. Louis, MO, USA) as previously described [91]. Briefly, $10 \times$ Alamar blue was added as 10% of the sample volume; then the plate was incubated for 1 h (AsPC-1) or 2 h (BxPC-3) at 37 °C. Alamar blue reagent is a non–toxic cell health indicator that is converted to fluorescent red colour in living cells, and the fluorescence (ex/em 570 nm/610 nm) was quantified by a plate reader (EnSpire Multimode, PerkinElmer®, Waltham, MA, USA).

4.4. Western Blot:

The level of protein expression was examined by Western blot. Specific antibodies against Slug, p-Akt (Ser473), p-Smad2 (Ser465/467)/Smad3 (Ser423/425), p-Erk1/2(Thr202/Tyr204), β-tubulin, α-Actinin and Glyceraldehyde 3-phosphate dehydrogenase (GADPH) were obtained from Cell Signaling Technology® (Danvers, MA, USA). Rabbit anti-CDD and hENT1 antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit P$_2$Y$_{12}$ [EPR18611] monoclonal antibody was obtained from Abcam Biotechnology (Cambridge, UK). Tumour cells were seeded in a 6-well plate. After 24 h, cells were serum starved for 6 h, then subjected to different treatments, and the plate was incubated for the specified time. Cells were then washed 3 times with ice-cold Tris Buffered Saline (TBS, 137 mM, sodium chloride, 20 mM Tris, pH 7.6) and lysed in ice-cold cell extraction RIPA buffer (ThermoFisher Scientific Inc., Waltham, MA, USA) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology®, Danvers, MA, USA). Forty-five µg of cell lysate was then analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted for the relevant protein. Equal loading was verified by immunoblotting for the house-keeping proteins (β-tubulin, α-actinin or GADPH).

4.5. Slug Knockdown

Cancer cells were seeded at 3×10^5 cells/well in a 6-well plate and maintained for 24 h. Slug siRNAs (two different Slug-specific siRNA sequences) or a negative control siRNA (75 nM), prepared using DharmaFECT1 transfection agent (GE Dharmacon, Lafayette, CO, USA) in serum/antibiotic-free media, were added to the adherent cells and incubated for 24 h. The two Slug-specific siRNAs GAAUGUCUCUCCUGCACAA (#1) and UCUCUCCUCUUUCCGGAUA (#2) were purchased from GE Dharmacon, Lafayette, CO, USA. The Silencer® negative control (sequence: proprietary, designed to have no significant similarity to human sequences, catalogue #AM4635) was from Ambion®, (ThermoFisher Scientific Inc., Waltham, MA, USA). The media (containing the transfection mixture) was then replaced with fresh media (RPMI + 10% FBS), and the plate was further incubated for 24 h. After washing cells with TBS, the cells were then lysed with RIPA buffer, and western blots used to

examine protein levels of expression. It must be noted that the BxPC-3 cell line was relatively difficult to transfect, likely due to the expression of extracellular DNA on the cell surface [91], and to achieve efficient transfer of siRNAs, the cells were treated with DNase (60 U/mL, STEMCELL Technologies, Vancouver, BC, Canada) for 3 h, then washed 3 times with EDTA (50 mM), before addition of the transfection mixture as described above.

4.6. Statistical Analysis

Data were analysed using GraphPad PRISM 5.0 software (GraphPad Software, Inc, CA, USA). Results are expressed as the mean $\pm$ standard error (SEM). One-way ANOVA with posthoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean. Differences were considered significant at p-value less than 0.05.

5. Conclusions

Our results report for the first time that platelets can regulate the expression of markers of gemcitabine resistance in pancreatic cancer cells, hENT1 and CDD. Slug, a mesenchymal transcription factor and mediator of EMT processes, was shown to regulate the expression of both hENT1 and CDD. Furthermore, we showed that platelet-derived ADP and ATP could modulate the level of Slug and CDD, and activated survival signals in cancer cells. Finally, ticagrelor, a P2Y$_{12}$ inhibitor, could act directly on the cancer cells and abrogated the survival signals initiated in cancer cells by platelet-derived ADP and ATP. Future studies are required to examine whether targeting platelet activation and purinergic signalling in cancer cell could reduce chemotherapy resistance and cancer metastasis.

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Supplementary Materials: The Role of Platelet-Derived ADP and ATP in Promoting Pancreatic Cancer Cell Survival and Gemcitabine Resistance

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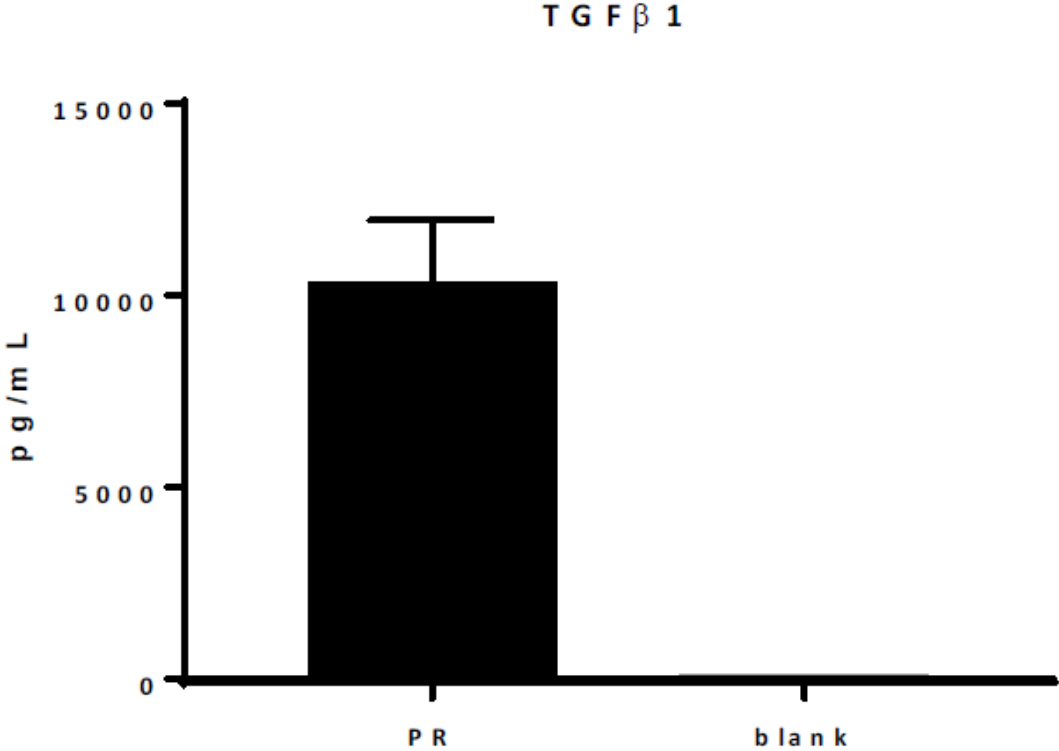
Figure S1. TGF-β1 in platelet releasate.PR from four different donors were prepared at 5 × 10^8platelets/ml and were analysed for the presence of human TGF-β1with an ELISA kit (Biosensis, Pty Ltd, CA, USA). n = 4, mean = 10360 ± 3195 pg/ml.
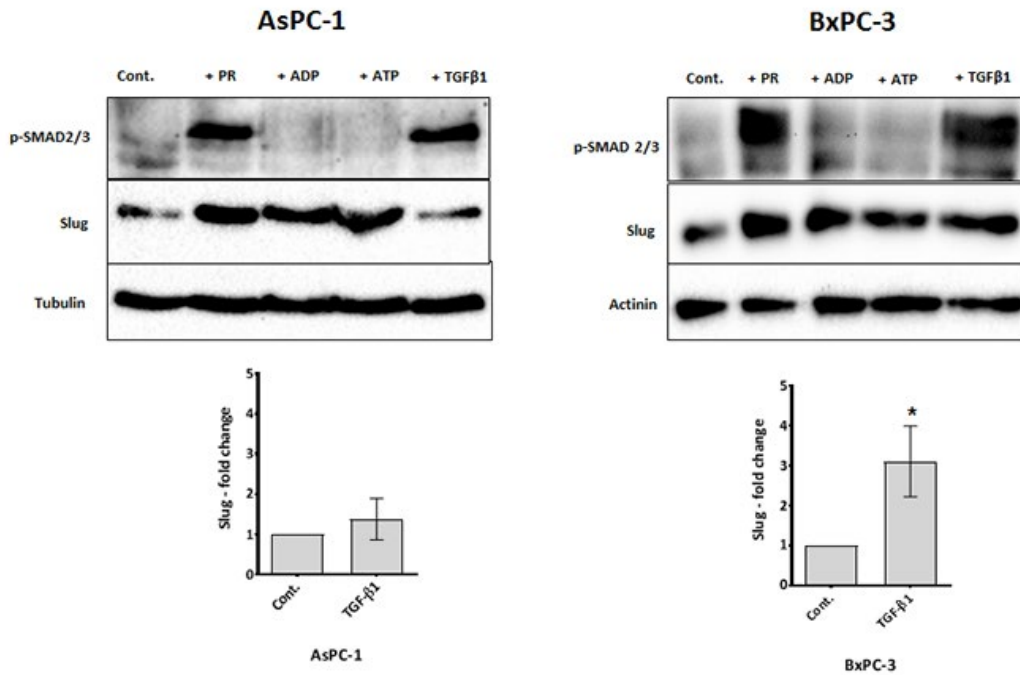
Figure S2. TGF-β1 upregulates Slug in BxPC-3 but not in AsPC-1. Cancer cells were treated with platelet releasate (PR), ADP (100 μM), ATP (100 μM), and TGF-β1 (10 ng/mL) for 2 h. PR, ADP and ATP induced Slug upregulation in both cell lines. TGFβ1 activated SMAD2/3 in both cell lines, however, highly upregulated Slug in BxPC-3, but not AsPC-1. * Each blot is a representative sample of 3 independent experiments with similar results.
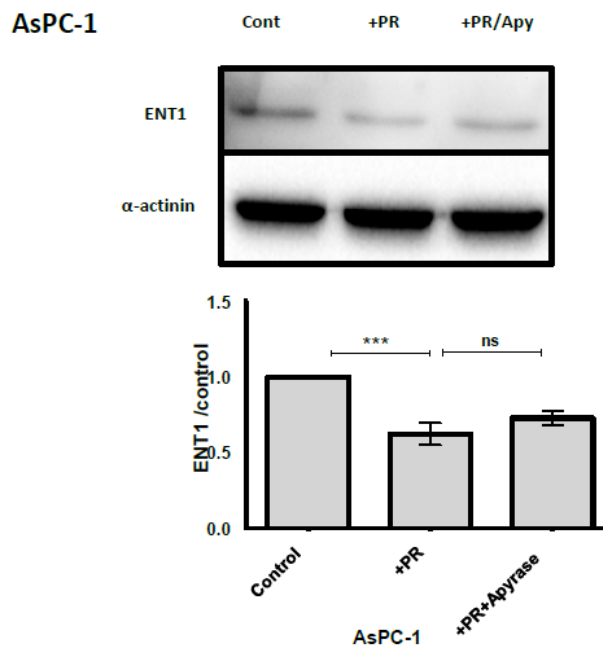

Figure S3. Apyrase did not significantly alter hENT1 expression in PR-treated cancer cells. Representative immunoblots and bar graphs show hENT1 expression in AsPC-1 cells after incubation with PR or PR pre-treated with apyrase (1 U/mL, for 30 min at 37 °C). 3×10^5 cells were seeded in a 6-well plate for 24 h, then PR or apyrase pre-treated PR were added to the cancer cells for a further 24 h in serum-free media. The final concentration of PR used was equivalent to releasate from 5×10^8 platelets/mL. Cell lysates were prepared and used in SDS-PAGE and immunoblotting as previously described. The expression levels of the hENT1 were quantified relative to actinin. One way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean. $n = 4$. *** $p < 0.0001$.
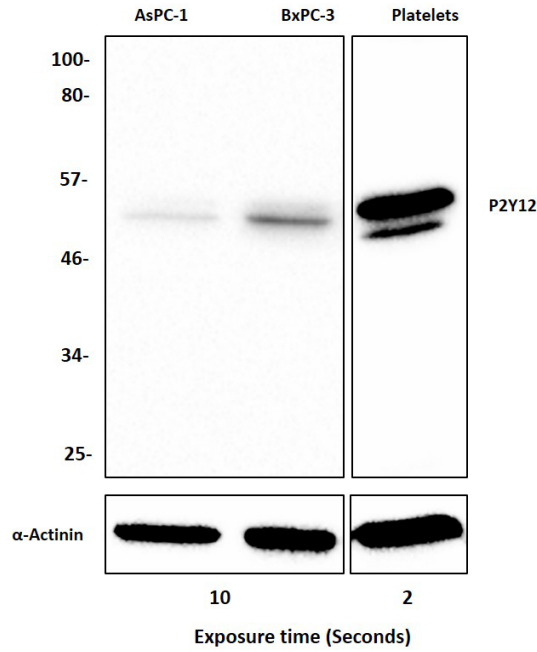
Figure S4. Expression of P2Y12 in PDAC cell lines AsPC-1 and BxPC-3. Cell lysates from AsPC-1, BxPC-1 and platelets were prepared using RIPA lysis buffer supplemented with protease inhibitor cocktail (Cell Signaling Technology). 20 μg of cell lysate with Laemmli sample buffer was loaded per lane, and immunoblotting was performed as mentioned in methods and materials. Rabbit anti-P2Y12 [EPR18611] monoclonal antibody, from Abcam Biotechnology Company was used to detect P2Y12 and, after membrane stripping, equal loading was verified using the house keeping protein α-Actinin (Cell Signaling Technology). P2Y12 is highly expressed in platelets, therefore the development of the membrane was performed using signal accumulation mode (ChemiDoc, Biorad Imaging system), and the bands in PaCa cells and platelets are shown at different exposure time.
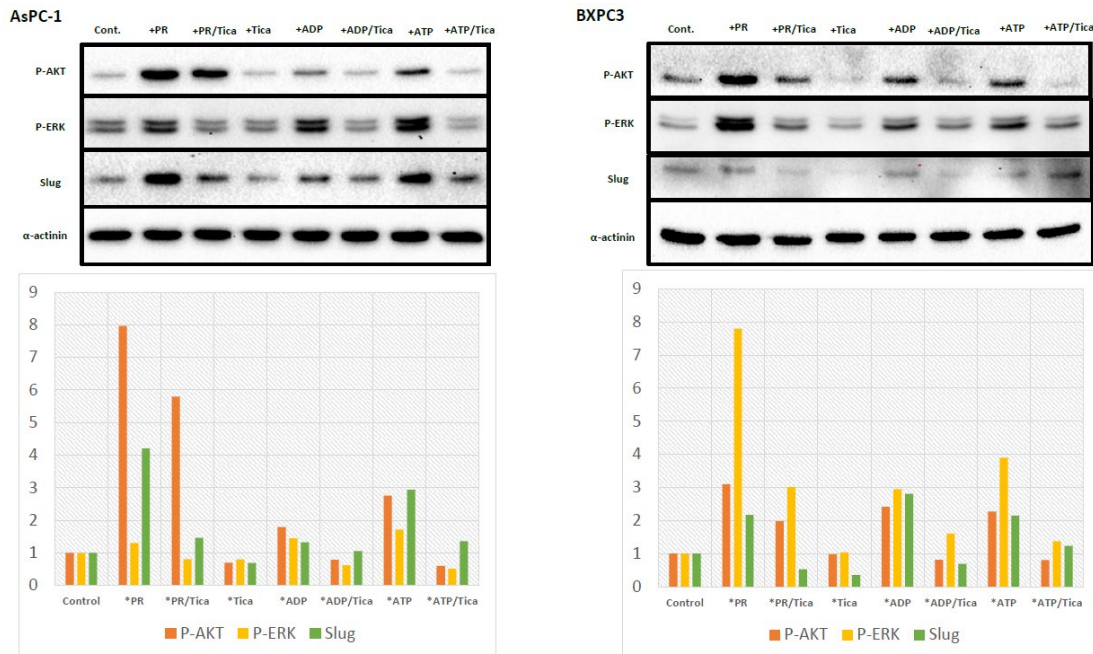

Figure S5. Ticagrelor inhibits the effects of ADP and ATP in the cancer cells. Cancer cells were treated with platelet releasate (PR), ADP (100μM) and ATP (100 μM) ± ticagrelor (10 μM) for 2 h. ticagrelor (10 μM) reduced PR, ADP and ATP-induced p-Akt, p-Erk and Slug upregulation, possibly by the blocking purinergic receptor P2Y12 that is found to be expressed on both AsPC-1 and BxPC-3. ATP is an unstable molecule, and its effects can also include the effects of its hydrolysed product, ADP. * Each blot is a representative sample of 2 independent experiments with similar results.

Chapter 4:

Ticagrelor - an antiplatelet agent for cancer treatment

4.1.   Publications

1. Adamska A, **Elaskalani O**, Emmanouilidi A, Kim M, Abdol Razak NB, Metharom P, Falasca M. *Molecular and cellular mechanisms of chemoresistance in pancreatic cancer*. Review. Advances in Biological Regulation. 2017 Nov 22. pii: S2212-4926(17)30176-8. doi: 10.1016/j.jbior.2017.11.007.

2. **Under preparation for submission**
   Elaskalani O, Abdol Razak NB, Domenichini A, Falasca M, Metharom P. *Antiplatelet agent ticagrelor potentiates the antitumour effect of the chemotherapy drug gemcitabine in pancreatic cancer*

4.2.   Introduction

The tumour microenvironment is increasingly considered as a significant contributor to chemotherapy resistance in pancreatic cancer. Publication 1 of this chapter provides a literature review on different mechanisms of chemotherapy resistance in pancreatic cancer [32]. The author (Elaskalani O) was responsible for sections (4, and 8-13, and figure 3) and contributed to the overall design of the paper. The second part of the chapter is a study that is currently being prepared for submission. In this study, we investigated the antitumour activity of ticagrelor, a P2Y12 inhibitor and a clinically available antiplatelet *in vitro and in vivo*.

Adamska A, **Elaskalani O**, Emmanouilidi A, Kim M, Abdol Razak NB, Metharom P, Falasca M. *Molecular and cellular mechanisms of chemoresistance in pancreatic cancer*. Review. Advances in Biological Regulation. 2017 Nov 22. pii: S2212-4926(17)30176-8. doi: 10.1016/j.jbior.2017.11.007

Molecular and cellular mechanisms of chemoresistance in pancreatic cancer

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ARTICLE INFO

ABSTRACT

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the most chemoresistant cancers, and current therapies targeting cancer-associated molecular pathways have not given satisfactory results, owing in part to rapid upregulation of alternative compensatory pathways. Most of the available treatments are palliative, focussing on improving the quality of life. At present, available options are surgery, embolization, radiation, chemotherapy, immunotherapy and use of other more targeted drugs. In this review, we describe the cellular and molecular effects of current chemotherapy drugs such as gemcitabine, FOLFIRINOX (5-fluorouracil [5-FU], oxaliplatin, irinotecan, and leucovorin) and ABRAXANE (nab-Paclitaxel), which have shown a survival benefit, although modest, for pancreatic cancer patients. Nevertheless, gemcitabine remains the standard first-line option for advanced-stage pancreatic cancer patients and, as resistance to the drug has attracted an increasing scientific interest, we deliberate on the main intracellular processes and proteins vital in acquired chemoresistance to gemcitabine. Lastly, our review examines various microenvironmental factors capable of instigating PDAC to develop resistance to chemotherapeutic drugs.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most chemoresistant cancers due to the broad heterogeneity of genetic mutations and the dense stromal environment. Thus far, therapies targeting cancer-associated molecular pathways have not given satisfactory results (Adamska et al., 2017). Although tumour resection is the most realistic option for PDAC patients, less than 20% of them can undergo surgery at the time of diagnosis (Sohn et al., 2000) due to the fact that metastasis has already occurred. In case the tumour has only spread to the local blood vessels, then it is characterised as borderline resectable, and the patients are subjected to neoadjuvant therapy. The prognosis for metastasised cancer cases is poor, and the only treatment option is chemotherapy.

Since 1997, for nearly 15 years, gemcitabine has been a reference first-line therapy drug for patients with a good performance status (PS) (Burris et al., 1997). Therefore, gemcitabine in combination with different cytotoxic and biological agents is the predominant therapeutic option for patients. However, it only manages to diminish the symptoms of the disease and increase survival rate, and its toxicity limits the application to patients with good PS. Therefore, with the PS as a point of reference, patients are

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subjected to either single-agent treatment or combination therapy. A combination that exhibited a significant response is that of gemcitabine with albumin-bound paclitaxel, also known as ABRAXANE (Von Hoff et al., 2013), which increased the capacity of gemcitabine to penetrate the tumour (Frese et al., 2012). Due to the increased survival rate of the patients who responded to this combination, the FDA established it as a first-line therapy option, along with another multidrug combination called FOLFIRINOX (Conroy et al., 2011). However, their advantage over single-agent gemcitabine was quite moderate, with mostly palliative effects. Factors such as cell plasticity, heterogeneity of the tumour, composition of the tumour stroma, epithelial-to-mesenchymal transition (EMT), altered metabolism and cancer cells-derived vesicles, can highly impact treatment outcomes (Elaskalani et al., 2017c; Falasca et al., 2016). The development of drug resistance is the main cause of the lack of efficacy of current treatments for pancreatic cancer, and in this review, we will summarise the main mechanisms of chemoresistance in pancreatic cancer with emphasis on the emerging role played by the tumour microenvironment.

2. Chemotherapy in pancreatic cancer: mechanism of action and cellular metabolism

In this section, the mechanism of action of drugs such as gemcitabine, FOLFIRINOX (5-fluorouracil [5-FU], oxaliplatin, irinotecan, and leucovorin) and ABRAXANE (nab-Paclitaxel), which have shown a survival benefit for pancreatic cancer patients, will be described.

3. 5-FU (5-fluorouracil)

5-Fluorouracil (5-FU) has been widely used in cancer therapies for approximately 50 years and it still remains one of the main drugs for PDAC therapies (Manji et al., 2017). 5-FU is an S-phase-specific uracil analogue also known as a pyrimidine analogue by incorporating into DNA, RNA or both, leading to accumulation of 5-FU in cells which results in increased cytotoxicity, eventually causing cell death (Longley et al., 2003). 5-FU is converted intracellularly to fluorodeoxyuridine monophosphate (FdUMP), which then forms a complex with thymidylate synthase (TS), inhibiting the production of deoxythymidine monophosphate (dTMP), which is essential for DNA replication. 5-FU can be also converted to 5-fluorouridine 5′-triphosphate (FUTP) which is then incorporated into RNA transcribed by RNA polymerase, mainly causing interference of mRNA synthesis. The incorporation of 5-FU into RNA not only affects mRNA synthesis but also inhibits ribosomal RNA maturation, post-transcriptional modification of transfer RNAs, and pre-messenger RNA splicing. In addition, it has been reported that 5-FU induces apoptosis through activation of caspase-6, upregulation of phospho-Bcl-2, and generation of mitochondrial reactive oxygen species (ROS) (Chan et al., 2008; Hwang et al., 2001). However, 5-FU has marginal efficacy in PDAC therapies due to low stability inside the cells (Heggie et al., 1987). The majority of administered 5-FU tends to be easily broken down to *dihydrofluorouracil* (*DHFU*) by dihydrophyrimidine dehydrogenase (DPD) in the liver. Thus, DPD is a vital element for the activity of 5-FU, impacting on the sensitivity and resistance of the drug. DPD can be also an important molecule in terms of the toxicity of this drug. It has been reported that deficiency of DPD causes fluorouracil-related severe toxicity (Milano et al., 1999).

4. Gemcitabine

Research has focused on improving patient survival and, towards this direction, gemcitabine has proven to be a good candidate, showing a significantly higher level of efficacy, compared to 5-FU. More specifically, the comparative phase III studies of single agent gemcitabine and 5-FU revealed that 5-fold more patients experienced clinical benefit from gemcitabine over 5-FU, and the 1-year survival rate was 9-fold higher (Burris et al., 1997). Gemcitabine (2′, 2′-difluorodeoxycytidine, dFdC) is a nucleoside cytidine (pyrimidine) analogue that can be incorporated into replicating DNA, thereby inhibiting DNA synthesis, which results in premature chain termination (Fig. 1) (Hertel et al., 1990). Gemcitabine is a prodrug, and its cellular uptake is mediated by nucleoside transporters, molecules pivotal for cell sensitivity to drugs (Baldwin et al., 1999; Plunkett et al., 1995). Once inside, deoxycytidine kinase (dCK) mediates phospho-activation of gemcitabine into gemcitabine di- (dFdCDP) and triphosphate (dFdCTP), which is an essential step in gemcitabine-mediated cytotoxicity. These active drug metabolites have multiple inhibitory actions on DNA synthesis. As an inhibitor of ribonucleotide reductase, dFdCDP prevents the *de novo* synthesis of deoxyribonucleotides, while dFdCTP is perceived by the cell as a cystidine and is incorporated into the DNA chain during replication (Huang et al., 1991). Incorporation of the active drug forms into nucleic acids terminates DNA polymerase action, eventually leading to cell death (Huang and Plunkett, 1995). Moreover, gemcitabine metabolites can inhibit a plethora of other enzymes including cytidine triphosphate synthetase (CTP synthetase) (Heinemann et al., 1995) or deoxycytidine monophosphate deaminase (dCMP-deaminase) (Heinemann et al., 1992), which creates a positive feedback loop extending gemcitabine activity and efficacy by reducing dCTP pools and increasing the incorporation of dFdCTP into DNA.

5. Cisplatin & oxaliplatin

Cisplatin is a platinum complex surrounded by ammonia and chlorine atoms, incorporated into cells by the copper transporter (Ctr1) (Ishida et al., 2002). Intracellularly, chloride ions in cisplatin are displaced by water molecules converting it into a charged electrophilic compound (active form). This active form has the ability to bind to nucleophilic groups such as oxygen, nitrogen and sulphur atoms, which are present in amino acid side chains and purine bases of DNA or RNA, to form DNA adducts, such as intrastrand cross-linked d(GpG) and d(ApG). This leads to the bending of the DNA helix, followed by binding of the high-mobility-
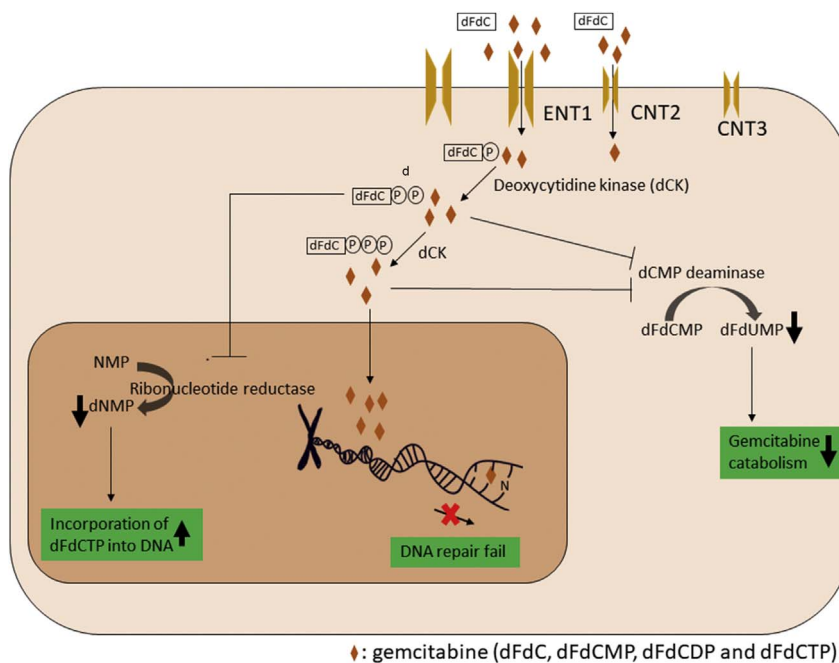
♦: gemcitabine (dFdC, dFdCMP, dFdCDP and dFdCTP)

Fig. 1. Mechanistic representation of gemcitabine metabolism. Gemcitabine (dFdC) is transported by multiple active nucleoside transporters (e.g. ENT1, CNT2 and CNT3). The transported dFdC is consecutively phosphorylated and is converted to gemcitabine diphosphate and tri-phosphate (dFdCDP and dFdCTP). These two activated metabolites (dFdCDP and dFdCTP) disturb DNA synthesis. dFdCDP inhibits ribonucleotide reductase which catalyses the *de novo* synthesis of deoxyribonucleotides (dNMPs). Decreased dNMPs help dFdCTP to bind into DNA by reducing competing natural deoxyribonucleotide pools. The incorporation of gemcitabine to DNA by DNA polymerase terminates DNA chain elongation. Interestingly, exonuclease enzymes are not able to detect and repair this gemcitabine-incorporated DNA because DNA polymerase permits one more nucleotide to pair after binding gemcitabine to DNA chain. dFdCDP and dFdCTP also inhibit dCMP deaminase which converts dFdCMP to dFdUMP. This activity allows a decrease of gemcitabine catabolism and perpetuates gemcitabine's own activity and survival.

group (HMG) proteins to this changed structure. The binding of HMG proteins to cisplatin-DNA adducts is structurally similar to their natural binding formation and contributes to the cells' inability to fix the DNA lesions. A charged electrophilic cisplatin also induces cellular oxidative stress that can damage mitochondria (Saad et al., 2004). On the other hand, binding of cisplatin to other nucleophiles, e.g. glutathione or metallothionein, can contribute to the gain of cisplatin resistance.

Oxaliplatin is a platinum analogue and a component of the combination therapy, FOLFIRINOX. Unlike cisplatin, the platinum atom of oxaliplatin is surrounded by an oxalate group and 1,2-diaminocyclohexane (DACH) (Raymond et al., 1998). Oxaliplatin forms inter- and intra-strand DNA adducts similarly to cisplatin, but it remains active in cisplatin-resistant tumour by means of a mechanism that is not fully understood.

The application of gemcitabine and of the described platinum analogues combination therapy has given contradictory results since in some trials pancreatic cancer patients did not respond, whereas in others, the median overall survival (OS) was increased from 6 months to 7.5 (Heinemann et al., 2006; Louvet et al., 2005).

6. Taxanes

Taxanes, including paclitaxel and docetaxel, are anti-microtubule agents which suppress microtubule dynamics, eventually leading to the arrest of the cell cycle. Their mechanism of action includes prevention of microtubule rearrangement by binding to α-tubulin and formation of highly stable tubulin polymers that inhibit the function of the spindle during cell division, resulting in cell arrest (G2/M phase) and, ultimately, cell death (Fig. 2) (Abal et al., 2003). Until recently, delivery of these drugs had limitations associated with a poor solubility. Solvents such as Cremophor EL (CrEL) and polysorbate 80 (Tween 80) were utilised but exhibited drawbacks, due to a change of the disposition of intravenously administered drugs, hypersensitivity reactions and peripheral neurotoxicity (Gelderblom et al., 2001). Recently, a solvent-free nanoparticle albumin-bound (Nab) paclitaxel combined with gemcitabine (ABRAXANE) showed a significant increase in efficacy in the treatment of advanced PDAC patients (Hennenfent and Govindan, 2006; Von Hoff, Ervin, 2013).

In a phase III trial (n = 861), the ABRAXANE-gemcitabine group exhibited a median OS of 8.5 months compared to the 6.7 months of the gemcitabine group, and higher survival rates at all time points (Von Hoff et al., 2013). The superiority of this combination treatment to the single-agent therapy led to its approval by the FDA and its establishment as a first-line therapy for PDAC patients with dismal prognosis.
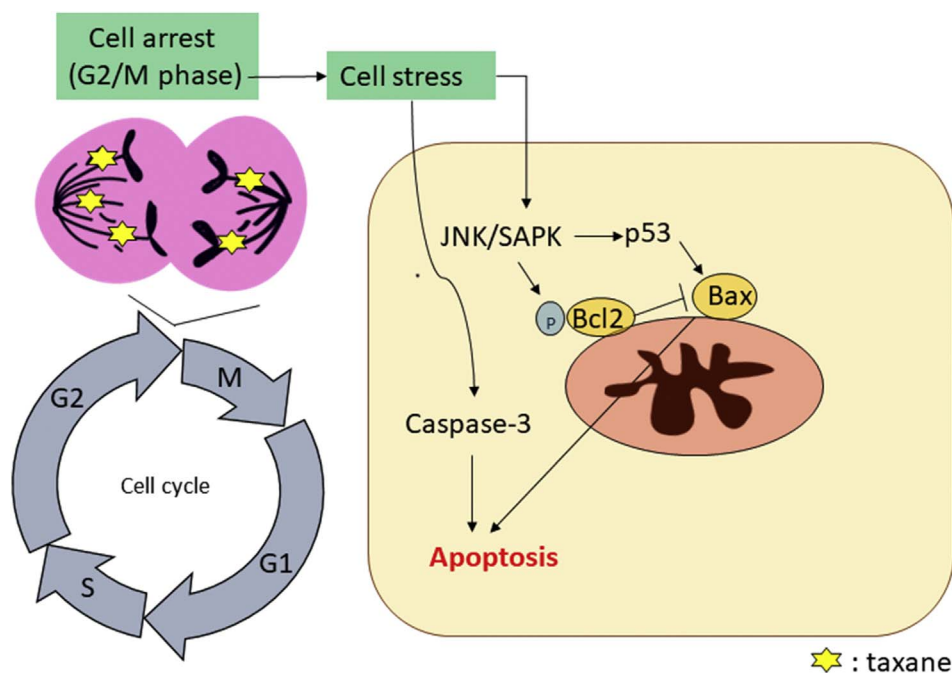
Fig. 2. Mechanistic representation of Taxane action. Taxane binds to α-tubulin and forms stable tubulin polymers during cell division. This activity results in inhibiting the function of the spindle and arresting G_2/M phase of cell cycle. The cell stress caused by the cell arrest allows JNK/SAPK phosphorates Bcl-2 which is an anti-apoptotic protein. The phosphorylated Bcl-2 is unlikely to bind to pro-apoptotic Bax protein, and then free Bax proteins can easily activate apoptosis. Moreover, cell stress induces high expression of p53 and caspase-3 which are related to apoptosis.

7. FOLFIRINOX

Recently, FOLFIRINOX has been used as an effective first-line therapy, especially for patients experiencing metastasis (Conroy et al., 2011). FOLFIRINOX is a combination of drugs that have proven their efficacy both individually and synergistically (Azrak et al., 2004; Ducreux et al., 2004; Ueno et al., 2007). It comprises of 5-Fluorouracil (a pyrimidine antagonist acting in a similar way with gemcitabine), Leucovorin (a folinic acid which acts to reduce its side effects), Oxaliplatin (a DNA repair inhibitor), and Irinotecan (a topoisomerase inhibitor which blocks DNA duplication).

In all three phases of clinical trials, FOLFIRINOX surpassed gemcitabine in all efficacy parameters, increasing OS of 1.6-fold, and the progression-free survival (PFS) and 1-year survival rate of almost 2-fold (Conroy et al., 2011; Ychou et al., 2003). However, due to the unfavourable safety profile of FOLFIRINOX (Gourgou-Bourgade et al., 2012), its use is constrained to patients under 75 years of age, and with a good PS; nevertheless, it remains a first-line option for advanced-stage patients.

8. Mechanism of acquired resistance to gemcitabine

High resistance to chemotherapy and delayed diagnosis make pancreatic cancer one of the most aggressive and malignant tumours. Mechanisms of acquired resistance can be attributed to genetic mutations arising from treatment, adaptive responses and natural selection of a drug-resistant tumour subpopulation. Gemcitabine is one of the most clinically utilised options for pancreatic cancer, and the acquired gemcitabine resistance has attracted recent interest in literature. An increased understanding of the molecular mechanisms involved in adaptive responses in pancreatic cancer during chemotherapy may facilitate the development of novel treatment strategies. In the sections below, we detail key intracellular processes and proteins involved in acquired chemoresistance to gemcitabine (Fig. 3).

9. Nucleoside transporters

Gemcitabine is a hydrophilic molecule, and its molecular targets are intracellular. Therefore, cellular uptake of gemcitabine requires specialized transporters to pass through the lipid bilayer of the plasma membrane.

Human equilibrative and concentrative nucleoside transporters (ENT, CNT) are two structurally unrelated families of proteins that mediate nucleoside transport through the plasma membrane (Griffiths et al., 1997a, 1997b). Several studies have examined the validity of using ENT1 as a biomarker to anticipate gemcitabine efficacy; however, the results are contradictory. Deficiency in hENT1 is associated with significant gemcitabine resistance in *vitro* (Mackey et al., 1998). *In vivo,* there have been reports indicating an association of better overall survival with the expression of hENT1 (Morinaga et al., 2012; Spratlin et al., 2004). On the contrary, in a
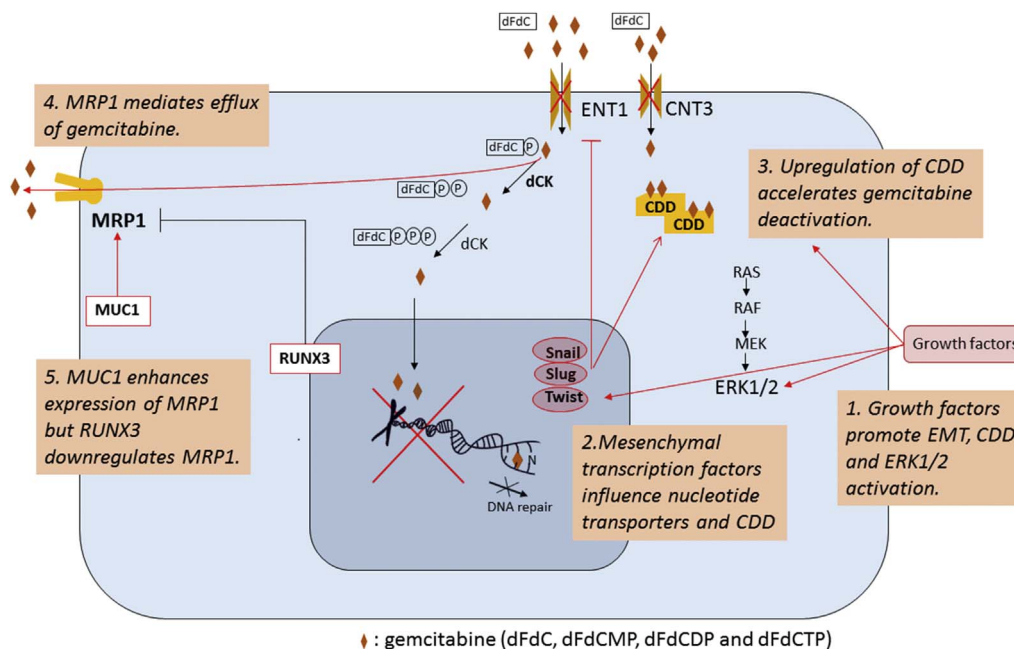
Fig. 3. Mechanistic representation of gemcitabine resistance pathway. Stroma or autocrine derived growth factors promote EMT, CDD upregulation and ERK1/2 activation. The mesenchymal transcription factors (e.g. snail, slug and twist) could influence resistance by targeting nucleotide transporters (e.g. ENT1 and CNT3) and CDD. CDD upregulation accelerates deactivation of gemcitabine. MRP1 mediates efflux of gemcitabine. MUC1 enhances gemcitabine resistance by bolstering expression of MRP1, while RUNX3 reduces resistance by downregulating MRP1 expression.

group of pancreatic cancer patients receiving neoadjuvant therapy including gemcitabine, a desirable prognosis appeared to be associated with hENT1 negative patients (Kawada et al., 2012). More recently, the results of the randomised clinical trial (CONKO-001) showed that high expression of ENT1 in the gemcitabine group was not associated with disease-free survival (Sinn et al., 2015).

10. Expression of ATP-binding cassette transporters

One of the principal mechanisms of chemoresistance in pancreatic cancer is the regulation of drug efflux. For instance, multidrug resistance-associated proteins (MRP)s are ATP-binding cassette (ABC) pumps that mediate the outflow of a range of chemotherapeutic agents, gemcitabine among others, thereby decreasing the intracellular drug concentration and contributing significantly to resistance. The normal function of these proteins is to mediate transport of amphiphilic anions which are overexpressed in some types of cancer (reviewed in (Borst et al., 2000; Deeley and Cole, 1997)), including pancreatic cancer (Miller et al., 1996). The discovery of MRP1-dependent chemotherapy resistance has resulted in the development of MRP1 as a drug target. Studies indicated inhibitors of MRP1 could significantly elevate the intracellular accumulation of chemotherapeutic drugs (Zhang et al., 2015).

The control of MRP1 expression in cancer has been examined in several basic studies with promising results. For instance, expression of runt-related transcription factor 3 (RUNX3), a tumour suppressor gene, in a group of pancreatic cancer cell lines resulted in enhanced gemcitabine sensitivity by reducing MRP1 expression (Horiguchi et al., 2013). Undoubtedly, further examination of the influx/efflux protein regulation pathway is required in order to better utilise them as anti-cancer targets.

11. Nucleoside enzymes

Different enzymes participating in the regulation of nucleosides metabolism are directly involved in gemcitabine resistance. For instance, deoxycytidine kinase (dCK) phosphorylates and activates gemcitabine once it reaches the cytoplasm, and low levels of this enzyme correlate with significantly decreased OS (Marechal et al., 2010). The levels of cytidine deaminase (CDD), the enzyme that catalyses the metabolic inactivation of gemcitabine, were also found to correlate with clinical outcome (ref PMID: 21652582). In addition, the enzyme ribonucleotide reductase (RRM1 and RRM2), that catalyses the conversion of ribonucleotides to deoxynucleosides and therefore has a key role in DNA synthesis, is also associated with gemcitabine resistance and its high levels correlate with worse prognosis (Aye et al., 2015).

12. Gemcitabine resistance mediated by epithelial-mesenchymal transition (EMT)

EMT is a stage of phenotypic alterations in cancer cells that favours a more mesenchymal phenotype with enhanced invasive properties. The process is characterised by a morphological transformation in cancer cells as well as by changes at the genomic and

protein level (Huber et al., 2005; Zavadil et al., 2008). However, a possible role of the mesenchymal transcriptional factors (e.g., Snail, Slug, and Zinc finger E-box-binding homeobox 1 - Zeb1) in chemotherapy resistance has recently attracted more attention. Inactivation of *Zeb1* results in cellular plasticity and stemness abrogation, reduction of the number of genes related to TGF-β signalling and metabolic changes which affect the capacity of cancer cells to colonize distant organs (Krebs et al., 2017). The knockdown of slug resulted in an enhanced sensitivity of CD133^{+} pancreatic cancer cells to gemcitabine (Tsukasa et al., 2015). Interestingly, EMT has been found to impart gemcitabine resistance in pancreatic cancer mouse models (Zheng et al., 2015). Moreover, tumour microenvironment is enriched with mediators of EMT, including cytokines (e.g. TGF-β1), growth factors (e.g. EGF kinase receptor) and hypoxia (Halder et al., 2005; Jiang et al., 2011; Lo et al., 2007). Nevertheless, the molecular signalling that links mesenchymal transcriptional factors (e.g., snail and slug) to gemcitabine resistance in pancreatic cancer has yet to be fully identified.

13. Gemcitabine resistance mediated by ERK

Extracellular regulated kinase (Erk) is a critical effector of mitogen-activated protein kinases (MAPK) that transduces several cellular signalling stimuli. Tumour microenvironment contains a plethora of mediators that strengthen chemotherapy resistance by activating Erk-dependent pro-survival pathways in cancer cells. Therefore, Erk1/2 hyperactivity can contribute to gemcitabine resistance in pancreatic cancer. Activation of Erk1/2 triggers upregulation of the pro-survival Bcl2 proteins and downregulation of the pro-apoptotic Bax proteins, leading to resistance of gemcitabine-mediated apoptosis (Wang et al., 2015). The anti-proliferative activity of gemcitabine on a panel of pancreatic cancer cell lines was enhanced by the addition of Erk1/2 activation inhibitor (U0126) (Zheng et al., 2013). Moreover, gefitinib, a tyrosine kinase inhibitor, restored gemcitabine sensitivity by inhibiting Erk activation and reversed the gemcitabine enhanced expression of multidrug resistance proteins (e.g., MRP1) in BxPC3 pancreatic cancer cells (Xiao et al., 2012). On the contrary, CXCL-12, a chemokine released by pancreatic cancer stromal cells, has been shown to potentiate gemcitabine resistance via activation of several survival pathways including Erk (Singh et al., 2010).

14. Microenvironmental factors affecting chemoresistance

The tumour microenvironment also contributes significantly to the chemotherapy resistance process and cancer relapse. Desmoplasia is the main characteristic in PDAC, and its main components are cancer-associated fibroblasts (CAFs) which originate from pancreatic stellate cells (PSCs), as well as endothelial and inflammatory cells (Nielsen et al., 2016). Interestingly, the role of the desmoplastic stroma in PDAC is controversial. On the one hand, it prevents the chemotherapeutic drugs from being effective by reducing tumour perfusion, but on the other hand, it possibly averts PDAC cells from penetrating the surrounding tissue and thus metastasizing (Özdemir Berna et al., 2014).

14.1. Pancreatic stellate cells

Pancreatic stellate cells (PSCs) are an important stromal component in PDAC (Jaster, 2004), as well as a representative key player in chronic pancreatitis (Sönke et al., 2006). They are activated in the region surrounding defective cells, as a result of innate defence of the stromal cells to encapsulate the damage and restrain its spread. In turn, activated PSCs start expressing extracellular matrix (ECM) proteins which initiate hypoxia and fibrosis within the stroma (Fig. 4). The barrier that is gradually being created can affect the genetic stability of the neighbouring normal cells and, subsequently, aggressive and chemoresistant cells are positively selected (Erkan, 2013). Cancer initiation or tissue injury can activate quiescent PSCs through secreted factors such as the tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukins 1, 2, 10 (IL1, IL2, IL10), and the platelet-derived growth factor (PDGF). After activation, PSCs can develop autocrine signalling by producing PDGF and TGFβ themselves (Nielsen et al., 2016). Apart from conferring resistance, this cell type can also promote pre-metastatic niche formation, since it was found to co-metastasize with cancer cells. Interestingly, PSCs seem to play a role in immune surveillance escape by secreting galectin1, which can block T cell activation and induce their death (Tang et al., 2012). Moreover, higher deposition of gemcitabine within the tumour can be achieved by the Hedgehog pathway inhibition in PSCs (Olive et al., 2009).

14.2. Tumour-initiating cells

Within a heterogeneous tumour, the highly proliferative cell populations that can survive intense chemotherapy or radiation and give rise to the tumour from which they were derived are called tumour-initiating cells (TICs); a term sometimes used interchangeably with cancer stem cells (CSCs) (Amp et al., 2009). Pancreatic TICs show all the properties of normal stem cells and are also implicated in many aspects of pancreatic cancer, including drug resistance (Fitzgerald et al., 2015; Fitzgerald and McCubrey, 2014). The actual mechanism through which tumour-initiating cells support chemoresistance in PDAC has not been fully elucidated, however the existence of hypoxic regions enriched in CD133^{+} cells expressing increased levels of ABC transporters and anti-apoptotic genes such as Bcl-2 has been recently revealed (Banerjee et al., 2014).

14.3. Fibroblasts

Cancer-related fibroblasts (CAFs) and PDAC are inextricably linked in aspects of differentiation and proliferation. Conditioned media from PDAC cells has been shown to trigger extracellular matrix (ECM proteins) production from PSCs, and conditioned media
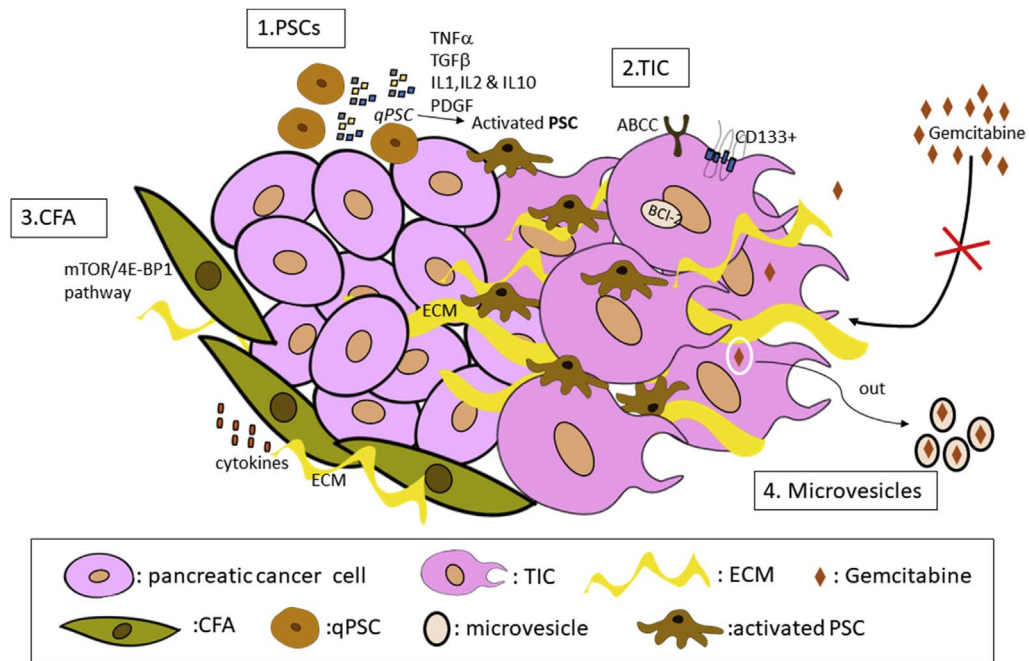
Fig. 4. Major factors contributing to the development of chemoresistance in pancreatic cancer. Pancreatic stellate cells (PSCs) are an important stromal component in PDAC with a possible dual role in neoplastic lesions. PSCs are initially activated in the region surrounding any defective cells in order to encapsulate them and restrain the damage from spreading. However, later on they initiate hypoxia and fibrosis within the stroma and they gradually create a barrier (desmoplasia), which at the end plays a key role in chemoresistance. When quiescent, they store vitamin A and produce matrix metalloproteinases (MMPs), and upon activation resulting from cancer initiation or tissue injury, they start expressing α-SMA, gain a myofibroblast-like phenotype (cancer-associated fibroblasts- CAFs) and lose the capacity of storing vitamin A. Factors contributing to qPSCs activation include tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukins 1, 6, 10 (IL1, IL2, IL10), and platelet-derived growth factor (PDGF). Moreover, a subpopulation of CD133 + cells within the tumour itself, with increased expression of ABC transporters and anti-apoptotic genes such as Bcl-2, have been shown to confer chemoresistance in PDAC. Last but not least, pancreatic cancer cells -derived microvesicles (MVs) are able to eliminate drugs such as gemcitabine (GEM) both from the intracellular space as well as the microenvironment. Based on their protein composition, MVs can either trap the drug or allow it to return to the microenvironment, depending on whether the cells of origin are GEM-resistant or GEM-sensitive, respectively.

from CAFs can promote PDAC cell proliferation, migration and chemo- and radiation-resistance (Bachem et al., 2005; Hwang et al., 2008). The alpha-smooth muscle actin (αSMA)-positive CAFs harbour a highly active mTOR/4E-BP1 pathway which, once inhibited, can reduce chemoresistance. The SOM230 analogue Pasireotide targets the sst1 somatostatin receptor expressed by CAFs and abrogates the mTOR pathway, while at the same time it has translational effects such as cessation of IL-6 protein synthesis. Co-administration of SOM230 and gemcitabine in mouse xenografts resulted in reduced tumour growth and chemoresistance, decreased fibrosis and increased gemcitabine-induced cell death (Duluc et al., 2015). This knowledge is of great importance since very little is known about translation and translational regulation in CAFs. The initiation and rate-limiting steps are known to be linked to elF2α, and PI3K/mTORC1 pathways (Baer et al., 2015; Martineau et al., 2012) and more than 50% of PDAC cases are non-responsive to mTOR inhibitors due to loss of 4E-BP1 expression, which negatively regulates translation (Martineau et al., 2013). IL-6 is identified as an important CAF-derived component as it is considered to be a chemoresistance mediator in pancreatic cancer cells (Lesina et al., 2014). In fact, its concentration in serum from patients is used as a marker for gemcitabine treatment efficacy (Mitsunaga et al., 2013). Another study revealed that when pancreatic ductal epithelial cells (H6c7) and chemosensitive adenocarcinoma cells (T3M4) were co-cultured with PDAC stroma-derived myofibroblasts, they acquired a chemoresistant phenotype and underwent a series of intracellular changes, including decreased levels of caspase expression and the transcription factor STAT1 (Müerköster et al., 2008). Following treatment with 5-azadeoxycytidine – a DNA methylation inhibitor, chemosensitivity was restored, and caspase and STAT1 levels were increased, pointing out a link between epigenetic modification, caspase apoptotic pathway and chemoresistance. Recently, an interesting study has given a different perspective in the field of CAFs research, with its novelty being CAF heterogeneity within the stroma. More specifically, two topologically distinct CAF subtypes, which exclude each other and are reversible, were identified and termed myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs). The first group is located in a site distant to the cancer cells, is triggered in a paracrine signalling manner and expresses low αSMA and high IL6 levels, whereas the second group is found juxtacrine to the cancer cells and contrary to the iCAFs, it expresses high αSMA and low IL6 levels. The detection of these populations might be the answer to conflicting studies on CAFs, and is certainly going to draw attention to the development of therapies that will take into account both the anti- and pro-tumorigenic capabilities of CAFs (Ohlund et al., 2017).

14.4. Microvesicles

There is an increasing amount of interest in regard to extracellular vesicles (EVs) and their role in tumour growth and metastasis. EVs are microparticles with a lipid bilayer membrane, secreted from all cell types in physiological as well as pathological conditions. It is known that microvesicles (MVs) promote chemoresistance by mediating the cross-talk between drug-resistant and drug-sensitive cancer cells, enabling the exchange of drug transporter proteins between them (Bebawy et al., 2009). Lately, it has been reported that pancreatic cancer cells use MVs to efflux gemcitabine and eliminate it from the microenvironment. More specifically, experiments carried out in a panel of human pancreatic cancer cell lines with different levels of gemcitabine revealed that the degree of resistance was proportionate to the amount of MV released, and retention of MV release in resistant cells resulted in them being re-sensitised both *in vitro* and *in vivo*. Interestingly, MVs were able to eliminate drugs both from the intracellular space as well as the microenvironment, and it was gemcitabine that regulated the influx (e.g. ENT1) and efflux (e.g. MRP5, P-gp) of the protein amount present in the vesicles. Based on their protein composition, the MVs could either trap the drug or allow it to return to the microenvironment, depending on whether the cells of origin were drug-resistant or sensitive, respectively (Muralidharan-Chari et al., 2016). According to the latest studies, CAFs that underwent chemotherapy released a high amount of exosomes containing Snail mRNA as well as miR-146a, which once transferred in the recipient cells, increased their chemoresistance as well as proliferation rate. Pharmacological inhibition of exosome release from CAFs reversed this phenomenon, underscoring the significance of these MVs in pancreatic cancer chemoresistance (Richards et al., 2017).

Another research group pointed out the significance of exosomal microRNAs in gemcitabine resistance in PDAC. Prolonged gemcitabine treatment was found to cause an increase in the intracellular levels of microRNA-155, which affected both the levels of exosomes shed by cancer cells as well as their anti-apoptotic activity. Exosomes would, in turn, deliver the specific microRNA in other cells, starting a new cycle. Nevertheless, chemosensitivity was restored by either attenuation of the exosome secretion or the targeting of microRNA-155 (Mikamori et al., 2017).

15. Immune cells and the microenvironment

The innate immune cell population is another significant component of the tumour microenvironment and there have been cases where rather than protecting the host with anti-tumour responses, innate cells promote tumour progression, metastasis and chemoresistance (Grivennikov et al., 2010). Infiltrating immune cells in the tumour microenvironment include macrophages, neutrophils, lymphocytes, and myeloid-derived suppressor cells and platelets. These immune cells may contribute to the various potential mechanisms of drug resistance such as induction of EMT (Zheng et al., 2015), deregulation of key pathways (e.g. apoptotic pathways) and the establishment of cancer stem cells (Long et al., 2011).

15.1. Tumour-associated macrophages

The most abundant immune cells in the tumour microenvironment of many solid tumours, including pancreatic tumours, are the differentiated tumour-associated macrophages (TAMs), which have acquired a tumour-promoting phenotype, augmenting tumour growth, angiogenesis, remodelling of the matrix and suppressing immune responses (Mantovani et al., 2006). In pancreatic cancer, macrophages are implicated in resistance to gemcitabine through the upregulation of cytidine deaminase, an enzyme that metabolises gemcitabine to an inactive form (Weizman et al., 2014). In addition, TAMs were found to suppress anti-tumour immune responses. During the administration of gemcitabine, increased infiltration of anti-tumour CD8^{+} T cells was achieved by blocking the recruitment of TAMs to the tumour site by targeting CCR2 or CSF1R (Mitchem et al., 2013). Furthermore, TAMs also enhanced STAT3 activation, a mediator of immune suppression, which resulted in increased tumour-initiating capacity of pancreatic tumour cells (Mitchem et al., 2013). More recent studies have revealed that TAMs together with activated myofibroblasts are responsible for the production of most of the IGF in the pancreatic cancer microenvironment *in vivo*, whereas almost 25% of pancreatic cancer patients are found to have an activated insulin/IGF1R pathway. Inhibition of this pathway would promote chemosensitivity to gemcitabine in this type of cancer and in fact, specific IGF-blocking antibodies (BI 836845, MEDI-573) being tested in phase II clinical trials, have shown to successfully block proliferation via the insulin and IGF1 receptors. Nevertheless, it is important to mention that TAMs can polarize into either an M2 subtype which acts in favour of the tumour, or into an M1 subtype which exerts anti-tumorigenic properties. This "switching" ability can be exploited and applied in therapies targeting TAMs reprogramming or inhibition of the M2 subtype (Ireland et al., 2016).

15.2. Tumour-associated neutrophils

More recently, the polarisation of neutrophils in tumours has been described where, similarly to macrophages, they polarise to a pro-tumour or anti-tumour phenotype depending on the chemokine milieu of the microenvironment (Fridlender et al., 2009; Granot and Jablonska, 2015). In pancreatic cancer patients, a high ratio of infiltrating neutrophils to lymphocytes is associated with poor prognosis (Templeton et al., 2014). Tumour cells can also prime neutrophils to release their granules containing proteinases such as neutrophil elastase and metalloproteinase-9, to exert pro-tumorigenic effects. Neutrophil elastase induced degradation of E-cadherin *in vivo* in pancreatic cancer cell lines and upregulated EMT markers, ZEB1 expression and the translocation of β-catenin to the nucleus (Grosse-Steffen et al., 2012). Furthermore, neutrophil extracellular traps (NETs) released from activated neutrophils are implicated in cancer-associated thrombosis (Abdol Razak et al., 2017; Demers et al., 2012; Thomas et al., 2015).

15.3. Platelets

In recent years, the role of platelets in tumour development has expanded considerably. In 2011, Labelle and colleagues showed that the interaction between cancer cells and platelets led to tumour EMT and increased metastasis (Labelle et al., 2011). Thromboembolism is a major complication for pancreatic cancer sufferers, indicating the crucial role of platelets and the coagulation system. Although the exact part platelets play, if any, in pancreatic cancer chemoresistance is not well understood, certain means by which they can sustain it have been identified, and it is interesting that an over-the-counter drug, aspirin, has been shown to positively affect-to some extent-individuals suffering from familial adenomatous polyposis, gastrointestinal cancer, whereas platelet targeted therapy seems to be beneficial in cancer cases with high thrombotic risk (Elaskalani et al., 2017a). A study showed that pancreatic cancer cell lines PANC-1 and BxPC-3, had reduced sensitivity to cisplatin when treated with activated platelets. The chemoresistant effect was found to be mediated by platelet-derived TGF-β1 (Chen et al., 2013). We have recently identified a potential role played by platelets in the association between the development of chemoresistance and EMT (Elaskalani et al., 2017b). Indeed, we found that platelets regulate the expression of hENT1 and CDD in pancreatic cancer cells. In turn, the expression of hENT1 and CDD is regulated by Slug, a mesenchymal transcriptional factor known to be upregulated during EMT. Moreover, we demonstrated that platelet-derived ADP and ATP regulate Slug and CDD expression.

16. Conclusions and future directions

The grim fact about pancreatic cancer is that in a few years it is expected to become the second leading cause of cancer death in the Western world, and by 2030 it will surpass liver, colon, lung and prostate cancers. This is due to both the increase in the incidence of pancreatic cancer and the improvement in the outcomes of other cancers. The persistent intractability of this cancer is demonstrated by the fact that survival rates have not changed in the last 40 years. One of the major challenges that we face in pancreatic cancer is to overcome its resistance to drug treatments. During the last decade, a better comprehension of these mechanisms has promoted our understanding of this grievous disease. The development of treatments currently used in therapy, such as Abraxane, can be seen as the product of this effort. Yet, major improvements are needed, and the strategy to selectively target elements of the tumour microenvironment is promising and may lead to major advancements.

Conflict-of-interest statement

Authors declare no conflict of interests for this article.

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4.3. Antiplatelet agent ticagrelor potentiates the antitumour effect of the chemotherapy drug gemcitabine in pancreatic cancer

Abstract

Experimental data suggest that the tumour microenvironment components can promote chemotherapy resistance in pancreatic ductal adenocarcinoma (PDAC). Thus, overcoming chemo-resistance requires combining multiple inhibitors that simultaneously target intrinsic pathways in cancer cells and extrinsic factors related to the tumour microenvironment. We aimed to determine whether ticagrelor; an inhibitor of the ADP-P2Y12 receptor axis and a well-known antiplatelet drug, could be a therapeutic option for PDAC. First, we explored the P2Y12 expression and its associated signalling pathways in a panel of cancer and normal pancreatic cell lines. We found that PDAC cells express a functional P2Y12 receptor that mediates ADP-induced AKT signalling. P2Y12 was upregulated in a panel of PDAC cell lines compared to the non-cancer pancreatic cells hTERT-HPNE and is positively correlated with epidermal growth factor receptor (EGFR) gene expression in PDAC. Besides, knockdown studies revealed that P2Y12 receptor contributed to EGFR activation and the expression of SLUG and ZEB1, which are epithelial-mesenchymal transcriptional factors implicated in cancer metastasis and chemotherapy resistance. Further studies using genetic and pharmacological inhibitors revealed that the crosstalk between P2Y12 and EGFR enhances cancer cell proliferation. Thus inhibition of P2Y12 signalling significantly reduced EGF-dependent AKT activation and promoted the anticancer activity of anti-EGFR treatment. Importantly, ticagrelor significantly decreased the proliferative capacity of cancer but not normal pancreatic cells. *In vitro*, synergism was observed when ticagrelor was combined with several chemotherapeutic agents (gemcitabine, paclitaxel, and cisplatin). *In vivo*, a combination of ticagrelor with gemcitabine significantly reduced tumour growth, whereas gemcitabine (25 mg/kg per week) or ticagrelor (50 mg/kg, twice a day) alone had a minimum effect. These findings uncover a novel effect and mechanism of action of the antiplatelet drug ticagrelor in PDAC cells and suggest a multi-functional role for ADP-P2Y12 signalling in the tumour microenvironment.

Key Points:

- P2Y12 expression in pancreatic cancer cells is required for proliferation via modulating EGFR-dependent and independent AKT survival signalling
- Ticagrelor displays a strong antiproliferative and proapoptotic effects on pancreatic cancer cells.

Introduction:

Pancreatic cancer has the lowest survival rates which have been mostly unchanged for the past 40 years, where approximately 3% of patients survive five years after diagnosis [370]. PDAC is the most common malignancy of the pancreas, which accounts for more than 90% of pancreatic cancer cases [371]. Late diagnosis, the prevalence of distant metastasis, and chemotherapy resistance account for the strikingly poor survival rates. Pancreatic cancer is also associated with an increased rate of thrombotic complications [128]. The elevated risk is attributed to the high metastatic rate, and the ability of pancreatic cancer cells to activate platelets and the coagulation cascade [51]. The current standard of care for PDAC includes gemcitabine as a single agent or in combination with nab-paclitaxel. Another combination of chemotherapy, FOLFIRINOX (5-fluorouracil, oxaliplatin, irinotecan and leucovorin), has limited use in PDAC due to profound adverse effects [32]. Anticoagulants have been examined as adjuvant therapy in combination with chemotherapy, and despite a significant reduction in symptomatic venous thrombosis, there was no major increase in overall survival in clinical trials [372,373]. Recently, a clinical trial has been initiated to examine clopidogrel (antiplatelet, P2Y12 inhibitor) in combination with gemcitabine in PDAC patients (NCT02404363).

PDAC is one of the most chemotherapy-resistant tumours. Several pathways in cancer cells can cause chemo-resistance including increased drug efflux, reduced drug cellular uptake, accelerated drug deactivation, and activation of alternative oncogenic signalling pathways. Moreover, an activation of epithelial-mesenchymal transition (EMT), a cell developmental programme, in cancer cells further promotes chemotherapy resistance in PDAC [32]. We recently showed that SLUG, an EMT transcriptional factor, regulated the expression of cytidine deaminase (CDD) and equiliberative nucleoside transporter 1 (ENT1) in PDAC cell lines [53]. CDD can deactivate gemcitabine, whereas ENT1 controls gemcitabine influx to the cell. The upregulation of CDD and downregulation of ENT1 contribute to gemcitabine resistance [32]. Our data suggested that platelets and platelet-derived factors, predominantly ADP/ATP, were responsible for the increased in SLUG expression and oncogenic signalling and EMT in PDAC [53]. Platelets, along with immune cell infiltrates, are known to

be enriched in the tumour microenvironment, and they are capable of enhancing chemo-resistance pathways in PDAC cells [32]. Platelet is the main source of tumour growth factor β1 (TGF-β1) in the circulation. Thus, inhibition of platelet significantly reduced TGF β1-mediated EMT in cancer cells and subsequently, metastasis [44]. More recently, it has been shown that platelet-derived TGF-β1 can suppress tumour immune response, promoting primary tumour growth [70].

The receptor P2Y12 has an important role in platelet activation and is the target of several drugs that have shown therapeutic benefits in the treatment of cardiovascular diseases. P2Y12 belongs to a family of purinergic G-protein coupled receptors (GPCRs) and is physiologically activated by ADP [357]. Solid tumours secrete more ADP (and ATP) compared to normal tissues, especially under hypoxic conditions which often occurs in solid tumours as they outgrow their blood supply [102]. ADP released by tumours activates platelet P2Y12 receptor, which in turn, releases growth factors to support tumour growth, and metastasis[63].

Inhibition of platelet P2Y12 receptor has been shown to reduce cancer growth and metastasis in ovarian, melanoma and lung cancer mice models [63,374]. However, the expression and signalling of the ADP-P2Y12 receptor axis in cancer cells are poorly investigated. P2Y12 is primarily expressed in platelets and brain, with some reports showing P2Y12 in glioma, astrocytoma and breast cancer cell lines [375]. Here, we validated the expression of a functional P2Y12 receptor in a panel of PDAC cells. Since several P2Y receptors have been shown to activate oncogenic EGFR signalling, we hypothesised that the pharmacological inhibition or knockdown of P2Y12 may reduce EGFR signalling and, consequently, cancer growth. Our results clearly show that ticagrelor, a clinically available antiplatelet drug and potent P2Y12 inhibitor, exerts an anticancer effect through targeting several oncogenic pathways in PDAC. Ticagrelor downregulated the AKT signalling pathway, suppressed cancer proliferation, increased apoptosis and synergised with chemotherapeutic agents (gemcitabine, paclitaxel and cisplatin). Data obtained from *in vivo* studies of BxPC-3 xenograft mouse model suggest that the combination therapy (ticagrelor/gemcitabine) is more effective at reducing tumour growth than the single drugs.

Methods and materials

Cell Lines

AsPC-1, BxPC-3, MiaPaCa-2, CFPAC-1, PANC1 and hTERT-HPNE cell lines were obtained from ATCC® and tested negative for Mycoplasma (tissue culture facility routine testing). AsPC-1 and BxPC-3 cells were maintained in RPMI 1640 medium. MiaPaCa-2 and PANC-1 were maintained in

DMEM medium. CFPAC-1 cells were maintained in IMDM medium. All culture media were supplemented with 10% Foetal Bovine Serum (FBS, from Bovogen Biologicals, Keilor East, Australia), 2 mM glutamine, 1 mM Sodium Pyruvate and 1 mM non-essential amino acids. hTERT-HPNE cells were maintained in DMEM medium supplemented with 5% FBS, human epidermal growth factor (EGF 10 ng/ml, ThermoFischer Scientific Inc., MA, USA), puromycin (750 ng/ml, ThermoFischer Scientific Inc., MA, USA) and 5 mM D-glucose. Unless specified, all culture media and supplements were obtained from Gibco® Life Technologies Australia Pty Ltd., Mulgrave, Australia.

Reagents

Paclitaxel, ADP, Apyrase, Tween 80 and polyethylene glycol 300 (PEG 300) were obtained from Sigma-Aldrich, (St. Louis, MO, USA). Cisplatin and erlotinib were obtained from Selleckchem (Houston, TX, USA). Gemcitabine was obtained from Eli Lilly (Australia). Ticagrelor was obtained from Sigma-Aldrich, Selleckchem and Pure Chemistry Scientific Inc., MA, USA. PSB 0739 and MRS 2179 were obtained from Tocris Bioscience (Bristol, UK). Cultrex basement membrane Type 3 was obtained from Trevigen, Inc. (MD, USA)

Proliferation assay

Cancer cells were seeded at 2000 cells per well in a 96-well plate. After 24 h, the media were replaced with fresh media (1% FBS) supplemented with different treatments and incubated for a further 72 h. In another set of experiments, and to examine the selectivity of ticagrelor on cancer versus normal cells, AsPC-1 cells were seeded in the same culture media used for hTERT-HPNE (except for puromycin), and both cell lines were treated with ticagrelor under the same experimental conditions. Cell viability was measured by detecting the metabolic activity of live cells using MTT reagent as previously described [376]. The half maximal inhibitory concentration (IC50, μM) of ticagrelor was calculated using GraphPad PRISM 5.0 software (GraphPad Software, Inc, CA, USA) and log (inhibitor concentration) vs normalised response (variable slope). For combination studies, a suboptimal dose of ticagrelor that is clinically relevant (2.5 μM) was chosen based on cell viability studies with ticagrelor on different cell lines. Synergism analysis was performed using CompuSyn Version 1.0 software (ComboSyn, Inc, Paramus, NJ, USA) based on Chou-Talalay's combination index (CI) method [377].

Immunoblotting

The following specific antibodies against SLUG, ZEB1, p-AKT (Ser473), p-ERK1/2 (Thr202/Tyr204), EGFR, p-EGFR (Tyr1068) and α-actinin were obtained from Cell Signalling Technology® (Danvers, MA, USA). Anti- P2Y12 [EPR18611] monoclonal antibody was obtained from Abcam Biotechnology (Cambridge, UK). Anti- P2Y12 [4H5L19] monoclonal antibody was obtained from ThermoFischer Scientific Inc., (Waltham, MA, USA). Anti- P2Y12 (NBP2-33870) polyclonal antibody was obtained from Novus Biologicals (Colorado, USA).

In order to validate P2Y12 expression, pancreatic cells and washed human platelets were lysed in non-ionic detergent (1% n-dodecyl β-D-maltoside, 150 mM NaCl, 25 mM Tris·HCl, pH 7.5) supplemented with protease inhibitors (Cell Signaling Technology®, Danvers, MA, USA). Cell lysates were kept at 4°C for 2 h, then clarified by centrifugation. Twenty µg of protein from pancreatic cell lysates and twenty or ten µg of protein from platelet lysate were loaded per lane. Proteins were analysed by polyacrylamide gel electrophoresis and immunoblotted for P2Y12. It is important to mention that P2Y12 in the positive control (e.g. platelets) and test cell lines were subjected to the same experimental conditions to prepare lysates. P2Y12 can form homo and heterodimers, and each form can be glycosylated, thus it may appear at different molecular weights in immunoblots, depending on experimental conditions, cells tested and antibody used [57,378]

To investigate P2Y12 signalling, cancer cells (1.5×10^5 per well) were seeded in a 12-well plate. After 24 h, cells were serum starved for 6 h. Different treatments were added, and the plate was incubated for the specified time. Cells were lysed in sample loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approx. 6.8) supplemented with protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology®, Danvers, MA, USA). Lysates were then analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted for the relevant protein.

P2Y12 Knockdown

AsPC-1 cells were seeded at 5×10^4 cells per well in a 12-well plate and maintained for 24 h. P2Y12 siRNA (Hs_P2RY12_4 FlexiTube Predesigned siRNA directed against human P2RY12, (NM_022788, NM_176876)) was obtained from Qiagen Pty Ltd, Australia. Silencer® negative control (sequence:

proprietary, catalogue #AM4635) was obtained from Ambion®, ThermoFisher Scientific Inc., Waltham, MA, USA. P2Y12 and Silencer siRNA (25 nM) were prepared using DharmaFECT1 transfection agent (1 µl per well) (GE Dharmacon, Lafayette, CO, USA) in serum/antibiotic-free media. The transfection mixture was then added to the adherent cells in serum-free media. For BxPC-3, cells were seeded at 1.5×10^5 cells per well in complete media in a 6-well plate. P2Y12 siRNA and Silencer (50 nM) were prepared using DharmaFECT1 transfection agent (1 µl per well) in serum/antibiotic-free media. The transfection mixture was then added, and the plate was incubated (reverse transfection). After 24 h, the media (containing the transfection mixture) were then replaced with complete media and the plate was further incubated for 48 h. Cells were washed in PBS and then lysed with RIPA buffer (supplemented with protease inhibitors).

ADP secretion assay

Cells were seeded at 7.5×10^5 cells per well in a 6-well plate and grown overnight (12 h). After removing media and washing cells, PBS (250 µl) was added, and cells were incubated for 15 min. PBS was then collected in ice-cold microcentrifuge tubes and then spun at 300 g for 2 min at 4°C. Supernatants were transferred to a black 96-well plate. ADP was included to ensure the selectivity of the assay. ADP was measured using ADP colourimetric/fluorometric assay kit (Abcam Biotechnology (Cambridge, UK) according to the manufacturer's instructions. Fluorescence was measured at Ex/Em 535/587 nm using a plate reader (EnSpire Multimode, PerkinElmer®, Waltham, MA, USA).

Apoptosis assay

Cells were seeded at 3000 cells per well in a 96-well plate. After 24 h, ticagrelor was added (1, 5, and 10 µM) to the cells and incubated for 12 h. Apoptosis was evaluated using Amplite fluorometric Caspase-3/7 Assay Kit (AAT Bioquest, CA, USA) according to the manufacturer's instructions. Fluorescence increase at Ex/Em = 350/450 nm was measured using a plate reader (EnSpire Multimode, PerkinElmer®, Waltham, MA, USA). NucView® 488 Caspase-3 Assay Kit (Biotium, CA, USA) was used to detect caspase 3 activity within intact live cells. The caspase 3 substrate used in this kit is also a non-functional DNA dye that stains the nucleus with bright green only after cleavage by caspase 3.

In vivo tumour growth

Female NOD-SCID mice aged 5-6 weeks were obtained from the Animal Resources Centre (Perth, Western Australia) and maintained under specific pathogen-free conditions. Animals were maintained, at 4 mice/cage, in a standard light/dark cycle at Curtin University animal facility. All experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes as per Curtin University Animal Ethics Committee (Approval number ARE2018-34). BxPC-3 xenograft model was established by subcutaneous injection at a total cell number of 2.5×10^6 in 100 µl RPMI/Cultrex basement membrane Type 3 (1:1) in the right flank of mice. When tumours became palpable (50-100 mm^3) animals were randomly divided into four groups (vehicle control, ticagrelor, gemcitabine, ticagrelor plus gemcitabine). Ticagrelor (50 mg/kg) was prepared in the following vehicle system: 4% DMSO, 30% PEG + 5% tween 80 + ddH2O. Gemcitabine (25 mg/kg) was prepared in 0.9% NaCl. Mice were given either ticagrelor or the vehicle system via oral gavage (200 µl) twice a day every 12 h, 5 days a week (Monday-Friday) in addition to either gemcitabine or 0.9% NaCl via intraperitoneal injection (IP, 150 µl) once a week. Tumour diameters were monitored with a surgical calliper every three days. Tumour volumes were calculated by the formula = (width^2 × length)/2.

Statistical Analysis

Data were analysed using GraphPad PRISM 8.0 software (GraphPad Software, Inc, CA, USA). Results are expressed as the mean ± standard error (SEM). One-way ANOVA with post-hoc Sidak's Multiple Comparison test was used to examine the significance of the mean. Differences were considered significant at *p*-value less than 0.05.

Results

P2Y12 expression across PDAC cell lines

The expression of P2Y12 in PDAC cell lines and in the normal pancreatic epithelial cell line, hTERT-HPNE, with platelets as a positive control, was initially analysed using a P2Y12-specific antibody (Anti- P2Y12 [EPR18611] monoclonal antibody, ABCAM) and immunoblotting techniques. As shown in Figure 1A, P2Y12 in PDAC cell lines was detected at the same molecular weight as P2Y12 in

platelets. The results were confirmed using additional two different anti-P2Y12 antibodies (Anti-P2Y12 [4H5L19] monoclonal antibody, ThermoFischer Scientific Inc. and Anti- P2Y12 (NBP2-33870), Novus Biologicals) (Supp. Data 1A). P2Y12 protein expression level was measured relative to the housekeeping protein α-actinin (Fig. 1B). P2Y12 is considered overexpressed (> 5 fold difference) in the PDAC cell lines AsPC-1, BxPC-3, MiaPaCa-2, PANC-1 when compared to hTERT-HPNE, the non-tumour cell line of the same origin. Across PDAC cell lines, the P2Y12 expression level was the lowest in the well-differentiated cell line CFPAC-1 compared to poorly differentiated (AsPC-1, MiaPaCa-2, PANC1) or moderate to poorly differentiated BxPC-3 cell line. EGFR expression level was also examined to compare with P2Y12 expression level. EGFR was highly expressed in all PDAC cell lines compared to hTERT-HPNE (Fig. 1C). Correlation between P2Y12 and EGFR expression was investigated using the Gene expression profiling interactive analysis (GEPIA) web-based tool which analysed the data by The Cancer Genome Atlas (TCGA) and GTEx projects[379]. Pearson correlation coefficient was calculated and indicated a positive correlation between P2Y12 and EGFR expression in PDAC (R = 0.49, p-value = 2^{e-12}) (Supp. Data 5A).

Using the selective P2Y12 inhibitor, ticagrelor, we investigated P2Y12 signalling in the PDAC cell lines AsPC-1 and BxPC-3. ADP is known to activate AKT through P2Y12 receptor in platelets and glioma C6 cells[357,380]. Figure 1D shows a dose-dependent inhibition of ADP-induced AKT activation by ticagrelor in AsPC-1 and BxPC-3 cell lines, confirming the expression of a functional P2Y12 receptor. The result corroborated with the data of another P2Y12 inhibitor, PSB 0739 (Supp. Data 1B). As ADP can also activate P2Y1, another purinergic receptor that is highly expressed in PDAC [381], we assessed the selectivity of ADP-P2Y12-AKT signalling axis by pretreating AsPC-1 cells with ticagrelor (5 μM) or MRS 2179 (P2Y1 inhibitor, 0-20 μM), before stimulation with ADP (100 μM). Ticagrelor, but not MRS 2179, reduced ADP-induced AKT activation (Supp. Data 1C). Since ADP is the major physiological agonist of P2Y12, we measured the level of extracellular ADP secreted by PDAC cells and normal pancreatic cells hTERT-HPNE. Figure 1C shows variable ADP secretion by both tumour (AsPC-1, BxPC-3, MiaPaCa-2) and non-tumour pancreatic cells (hTERT-HPNE) (Fig. 1E). ADP, ATP and adenosine are known to induce transactivation of the mitogenic receptor EGFR [102]. Therefore, we hypothesised that ADP may induce prosurvival signals in tumour cell lines AsPC-1 and BxPC-3, but not in the normal pancreatic cell line hTERT-HPNE which express a relatively low level of P2Y12 and EGFR. To test this hypothesis, AsPC-1, BxPC-3 (high levels of P2Y12 and EGFR) and hTERT-HPNE (low levels of P2Y12, EGFR) were treated with ADP (0-100 μM). As a result, activation of EGFR, AKT and ERK was significantly enhanced in AsPC-1, BxPC-3 in a dose-dependent manner, but not in hTERT-HPNE. ADP appeared to reduce AKT activation in hTERT-HPNE, while EGFR activation was minimal (Fig. 1F).

These data suggest that PDAC cells express a functional P2Y12 receptor which mediates ADP-induced AKT activation in PDAC cells.

The expression of P2Y12 regulates the levels of phospho-EGFR, EMT markers SLUG and ZEB1, and proliferation in PDAC cells

Since we observed that ADP induced EGFR activation in PDAC cells, we hypothesised that a reduction in the expression of ADP receptor P2Y12 may affect the level of activated (phosphorylated) EGFR. We first investigated the effects of four different knockdown siRNA sequences (Supp. data 2A) and the best performing sequence (Hs_P2RY12_4 FlexiTube) was selected for all subsequent studies. Immunoblot results show a downregulation of P2Y12 by siRNA significantly reduced the level of phosphorylated EGFR in AsPC-1 and BxPC-3 cells grown in complete media (Fig. 2A-B). EGFR is overexpressed in PDAC and is a known mitogenic activator of cell proliferation [382]. Indeed, our results showed that a decrease in EGFR activation mediated by the P2Y12 siRNA knockdown attenuated the proliferation of AsPC-1 and BxPC-3, compared to control siRNA transfected cells (Fig. 2C). We previously showed that ADP can induce SLUG upregulation in PDAC cells [53]. SLUG is an EMT transcriptional factor and is known to regulate the activity of ZEB1; another EMT transcriptional factor that is important in PDAC metastasis [52,383]. Additionally, SLUG knockdown has been shown to improve gemcitabine sensitivity in PDAC [32]. Therefore, we examined whether downregulation of P2Y12 affected the expression level of SLUG and ZEB1. Immunoblot results show that knockdown of P2Y12 significantly reduced SLUG expression in AsPC-1 and BxPC-3 (Fig. 2A-B). ZEB1 expression was also reduced in AsPC-1 cells after depletion of P2Y12. However, ZEB1 was initially undetectable in BxPC-3 cells (Fig. 2A-B). These results together demonstrate a significant role of P2Y12 in EGFR activation, and SLUG and ZEB1 expression.

Ticagrelor, a P2Y12 inhibitor, reduces EGF-induced AKT activation in PDAC cells

P2Y12 is known to signal through AKT in platelets. As a result, inhibition of P2Y12 reduces AKT activation in response to a variety of platelet agonists[357]. Therefore, we hypothesised that in PDAC cells, the pharmacological inhibition of P2Y12 may reduce AKT activation in response to EGF. As shown in figure 3A and Supp. Data 1D, ticagrelor (5 µM) and PSB 0739 (another P2Y12 inhibitor) reduced AKT and ERK activation in response to EGF (10 ng/ml, 30 min), while apyrase (5 U/ml), an ADP and ATP scavenger, failed to show a significant effect on EGF-mediated AKT or ERK activation

(Fig. 3A). Ticagrelor and apyrase did not show any consistent inhibition of EGF-mediated EGFR phosphorylation.

To further investigate P2Y12-EGFR crosstalk, we examined whether pharmacological inhibition of EGFR may reduce ADP-mediated AKT activation. Erlotinib, a clinically available EGFR inhibitor, at 5 µM markedly reduced ADP-induced EGFR and AKT phosphorylation in AsPC-1 and BxPC-3 (Fig. 3B). Since AKT is downstream of both EGFR and P2Y12, we tested whether ticagrelor can potentiate the anticancer activity of erlotinib. Growth inhibitory studies revealed that addition of ticagrelor (2.5 µM) to erlotinib yielded greater growth inhibition than erlotinib alone (Fig. 3C). A synergism between ticagrelor and erlotinib was evaluated using the CI method developed by Chou and Talalay[377]. The CI values were calculated using CompuSyn software and are summarized in table 1-2. The CI values is interpreted as follows: 0.1-0.3 strong synergism, 0.3-0.7 synergism, 0.7-0.9 moderate to slight synergism, 0.9-1.1 nearly additive, 1.1–1.45 slight to moderate antagonism, 1.45–3.3 antagonism, and >3.3 strong to very strong antagonism[384]. Our results demonstrate that erlotinib and ticagrelor were synergistic with CI values of less than 0.7 at erlotinib concentrations of (0.001-1 µM) in AsPC-1 cells. However, at erlotinib concentration of 10 µM, the combination with ticagrelor shows slight to moderate antagonism (CI = 1.4). In BxPC-3 cells, the CI values show slight synergism to nearly additive (CI 0.8-1.1) at erlotinib concentrations of (0.001-10 µM) (Fig 3C). Immunoblot results show that ticagrelor (2.5 µM) potentiated erlotinib-mediated AKT inhibition in AsPC-1 and BxPC-3 cells grown in non-serum starved media (1% FBS) (Fig. 3D). The above results demonstrate the importance of P2Y12-EGFR crosstalk in PDAC growth *in vitro*.

Ticagrelor suppresses PDAC cells growth *in vitro*

We next investigated the effect of ticagrelor on the proliferation of five PDAC cell lines and the normal pancreatic cells hTERT-HPNE (Fig. 4A). IC50 values were determined in these cell lines 72 h post-treatment (Table 1). Across PDAC cell lines, AsPC-1 displayed the least sensitive response to ticagrelor (IC50, 10.7 µM), whereas BxPC-3 showed the most sensitivity to ticagrelor treatment (IC50, 6.4 µM). Ticagrelor, however, did not show any cytotoxicity on hTERT-HPNE up to 20 µM, confirming the selectivity of ticagrelor towards cancer cells (Fig. 3A). To further validate the selectivity of ticagrelor, AsPC-1 cells were grown in the same culture media used for hTERT-HPNE but without puromycin. Both cell lines were then treated with ticagrelor 10 µM, however, only AsPC-1 cells displayed sensitivity towards ticagrelor treatment, with a 50% reduction in cell viability (Supp. Data 2C). This was a promising result as previous studies have established that ticagrelor plasma concentrations up to 10 µM are clinically tolerated[385]. PSB 0739, another P2Y12 inhibitor, also

showed growth inhibitory effects (Supp. Data 1D). As AKT is downstream of P2Y12 and is a known inhibitor of apoptosis [386], we performed further studies to investigate the effect of ticagrelor on apoptosis and AKT activity. Ticagrelor was found to induce a dose-dependent increase in apoptosis (caspase 3/7 activity), suggesting that ticagrelor can initiate cell death in PDAC cells (Fig. 4B). After treatment with ticagrelor (10 µM) the apoptotic cells appeared with bright green nuclei (Fig 4C). Additionally, in non-serum starved PDAC cells, ticagrelor caused a dose-dependent reduction in the expression level of phosphorylated AKT (Fig. 4D-E). Taken together, these data suggest an anticancer activity of ticagrelor through inhibition of AKT activation.

Ticagrelor synergises with chemotherapy in PDAC cells *in vitro*

After characterising the activity and regulatory mechanism of ticagrelor as a single agent in PDAC cells, and showing a synergistic effect with erlotinib, we proceeded to investigate its antitumour potential in combination with chemotherapeutic drugs used in PDAC treatment *in vitro*. Ticagrelor at a clinically relevant concentration (2.5 µM) [387,388] was combined with different concentrations of the chemotherapeutic agents; gemcitabine, paclitaxel and cisplatin. Dose-dependent growth inhibition was observed in AsPC-1, BxPC-3 and MiaPaCa-2 after 72 h exposure to the chemotherapeutic agents (Fig 5A-C). AsPC-1 cells displayed the least sensitivity to the combined treatment (Fig. 5A-C). Addition of ticagrelor improved the efficacies of the chemotherapeutic agents in all the three tested PDAC cells. The analysis of the drug interaction using the CI method revealed that the combination of ticagrelor with gemcitabine, paclitaxel or cisplatin was largely synergistic, especially at low chemo-drug concentrations (Fig. 5A-C). Collectively, these data demonstrate that ticagrelor can enhance the antitumour activity of chemotherapy in PDAC cells.

The combination of ticagrelor and gemcitabine significantly reduced tumour growth *in vivo*

Gemcitabine alone or in combination with nab-paclitaxel is the standard of care in PDAC therapy [32]. Therefore, we tested the effect of gemcitabine alone or in combination with ticagrelor on the growth of BxPC-3 cells in a xenograft mouse model. BxPC-3 cells were injected subcutaneously in a NOD/SCID mice. After the tumour became palpable (3 weeks), mice were randomly distributed into four groups (vehicle control, gemcitabine, ticagrelor, gemcitabine + ticagrelor). Ticagrelor has a shorter half-life in mice compared to humans, with a high concentration of ticagrelor (30-100 mg/kg) were required to achieve a maximum effect over 4 h post dosing [389]. Therefore, ticagrelor was administered twice a day every 12 h. As shown in Figure 6 A-C, only the combination therapy of

ticagrelor plus gemcitabine consistently and significantly reduced tumour growth. Ticagrelor (50 mg/kg, twice a day) or gemcitabine (25 mg/kg per week) as single agents reduced the tumour volume but had minimal effect on tumour weights. Addition of ticagrelor to gemcitabine did not produce any significant weight loss and the haematological parameters were similar between the gemcitabine and combined therapy groups (Supp data 3B-C). Our *in vivo* data suggest that ticagrelor may have therapeutic benefits as an additional treatment strategy as it potentiates the efficacy of gemcitabine in PDAC therapy.

Discussion

The tumour microenvironment is well-equipped to induce platelet activation. For example, pancreatic cancer cells express and release tissue factor, which can induce platelet activation through thrombin generation [374]. More recently, it has been shown that pancreatic cancer cells can trigger neutrophils to secrete neutrophil extracellular traps (a mesh of DNA, histones and proteases) which can directly activate platelets [6,96,259]. Once activated, platelets secrete several biological factors that can promote cancer growth and metastasis [374], including ATP and ADP, which are known to be released before platelet aggregation [192]. Targeting ATP receptors in cancer cells or the ADP receptor P2Y12 in platelets have attracted an increased interest from researchers recently [102,374]. However, the role of ADP and P2Y12 in cancer cells remains poorly investigated. Through functional and molecular studies, we demonstrated that P2Y12 receptor is expressed in PDAC cells and is required for cancer cell proliferation. Analysis of gene expression data in PDAC patients using the web-based tool GEPIA revealed that high expression is associated with poor survival although the association is statistically nonsignificant (Logrank $p = 0.24$) (Supp. data 4B) [379]. Targeting the P2Y12 receptor with ticagrelor repressed cancer cell growth and its presence synergised with several chemotherapeutic agents *in vitro*. In NOD/SCID mouse model, the combination of ticagrelor and gemcitabine significantly reduced the growth of BxPC-3 xenografts*.

P2Y12 belongs to a family of purinergic (P2) GPCRs [357]. Several P2 receptor subtypes are involved in the transactivation of EGFR. For example, the ADP receptor P2Y1 and the ATP receptor P2Y2 have been shown to mediate oncogenic signalling mostly through EGFR transactivation [318,390]. Here, our data clearly showed that extracellular ADP could induce EGFR activation in PDAC cells. ADP-mediated EGFR activation was minimal in immortalised normal pancreatic cells hTERT-HPNE, which expressed low levels of P2Y12 and EGFR in comparison to pancreatic cancer cell lines. Furthermore,

our knowledge studies revealed that P2Y12 contributed to EGFR activation. There are several possible mechanisms involved in P2Y12-dependent EGFR activation. Firstly, as it is known that P2Y1 and P2Y2 could induce EGFR transactivation through Src and MMP axis [318,391], P2Y12 may similarly mediate Src activation, leading to the increased expression of matrix metalloproteases (MMP) which are responsible for the cleavage and shedding of the heparin-binding EGF-like growth factor (HB-EGF) from the cell membrane. Released HB-EGF can then bind to and activate EGFR[392]. Secondly, P2Y12 could regulate EGFR activation by promoting EGFR association with another member of the EGFR family, HER3. Thus, inhibition of the purinergic receptor could reduce HER3 activation, and subsequently EGFR activation [390,393]. Thirdly, P2Y12 could induce EGFR activation via Src, then the transactivated EGFR forms a multi-receptor complex with P2Y12 leading to an increase in the downstream oncogenic signalling [394]. The specific mechanism of how P2Y12 contributes to EGFR activation remains to be determined in future studies.

ADP is known to elicit platelet activation through its interaction with P2Y1 and P2Y12 receptors. However, only the binding of P2Y12 leads to AKT activation, which is an essential step in platelet aggregation [357]. Our results demonstrated a striking similarity between P2Y12 signalling in platelets and in PDAC cells since only the inhibition of P2Y12, but not P2Y1, reduced ADP-mediated AKT phosphorylation. Interestingly, ADP reduced AKT activation in hTERT-HPNE cells whereas it significantly upregulated the level of phosphorylated AKT in PDAC cells. This opposing role of ADP in PDAC and HPNE cells is documented for several others purinergic agonists (e.g. ATP, adenosine) since the expression level of different P2 receptor subtypes, and the metabolism of the agonists are different between cell types [102].

Previous studies have indicated that P2 signalling promotes cancer invasion and chemotherapy resistance by supporting EMT in cancer cells [395]. Signalling through ATP, for example, can promote the upregulation of the mesenchymal transcriptional factor SNAIL1 in prostate cancer cells [355]. Whereas we previously demonstrated that ADP signalling promotes SLUG upregulation in PDAC cells [53]. SNAIL1 and SLUG (SNAIL2) belong to a family of mesenchymal transcriptional factors which can modulate EMT and chemotherapy resistance, through controlling the expression of cell adhesion proteins (e.g. E-cadherin), other mesenchymal transcriptional factors (e.g. ZEB1) or drug metabolising enzymes and drug transporter proteins (e.g. CDD, ENT1) [32]. Here, our results showed for the first time that P2Y12 mediated the expression of the EMT related transcriptional factors SLUG and ZEB1.

Interestingly, analysis of RNA sequencing expression data from tumours and normal samples from the TCGA and GTEx projects using the web-based tools GEPIA revealed a positive correlation

between the expression of P2Y12 and ZEB1 (Pearson correlation coefficient = 0.76, P < 0.05) in PDAC patients. As recent data indicate that ZEB1 is a critical player in pancreatic cancer metastasis [52], substantially more investigations are needed to elaborate on the role of P2Y12-ZEB1 axis in cancer invasion and metastasis. However, our results established a mechanistic rationale for P2Y12 inhibition with ticagrelor as a potential adjunctive therapy in the treatment of PDAC.

Ticagrelor is recognised to mediate its antiplatelet effect through the suppression of AKT signalling [50]. Our findings showed for the first time that, in PDAC cells, ticagrelor reduced the activation status of EGF downstream effectors AKT and ERK. Inhibition of the ADP receptor P2Y1 also reduced EGF-induced AKT and ERK activation (Supp. data 1E), indicating that both P2Y1 and P2Y12 contribute to EGFR signalling. The inconsistent inhibitory effect of ticagrelor, unlike the results of P2Y12 siRNA knockdown, on the expression level of phosphorylated EGFR (p-EGFR Y1068) could be due to the off-target effects of ticagrelor on ENT1. Ticagrelor is known to increase extracellular adenosine through inhibition of ENT1-mediated adenosine cellular uptake [396]. Extracellular adenosine can also induce EGFR activation [319], thus the effect of ticagrelor on EGFR activation may be disrupted by the presence of adenosine. Apyrase, an ADP and ATP scavenger, also showed inconsistent effects on EGF-mediated oncogenic signalling, which suggested that ADP and ATP breakdown products could facilitate opposing outcomes [102]. Inhibition of EGFR reduced ADP-induced AKT activation in BxPC-3 more profoundly than in AsPC-1. This may be explained by the difference in sensitivity towards EGFR inhibition between cell lines and also suggested that ADP-P2Y12-mediated AKT signalling in PDAC cells can be both EGFR-dependent and independent. Nevertheless, our data showed that P2Y12 is required for EGF-mediated oncogenic signalling and that ticagrelor could potentiate the anticancer activity of erlotinib, an EGFR inhibitor. Therefore, we propose that the inhibition of P2Y12-EGFR crosstalk could provide a better anticancer treatment strategy then selective inhibition of EGFR alone.

In regards to the effects of ticagrelor on cellular functions, we demonstrated that it inhibited PDAC cell proliferation and induced apoptosis in a dose-dependent manner through AKT-mediated pathway. These results are in accordance with a recent study where it was shown that P2Y12 protected platelets from apoptosis via AKT-dependent inactivation of apoptosis regulators Bak and Bax [386]. Furthermore, our results showed that ticagrelor had minimal impact on normal pancreatic epithelial cells, hTERT-HPNE, similar to FDA preclinical data that showed concentrations up to 20 μM of ticagrelor had negligible toxicity on hepatocytes *in vitro* [385]. In addition to examining its effects as a single agent, we also tested whether ticagrelor could enhance the cytotoxic effects of gemcitabine,

the primary treatment option in PDAC. Our results showed the combination therapy of ticagrelor and gemcitabine significantly reduced tumour growth *in vivo*, whereas ticagrelor or gemcitabine as single agents had a much less therapeutic effect. It must be noted that the tumour suppressive impact of ticagrelor may be partly ascribed to its inhibitory effect on platelet function.

In conclusion, our results demonstrated that ticagrelor reduced AKT survival signalling and triggered a strong antiproliferative and proapoptotic effects in pancreatic cancer cells. The combination treatment of ticagrelor and gemcitabine resulted in significant tumour growth inhibition *in vivo.* Given that platelets are increasingly being used as drug targets in clinical trials of solid tumours studies, our findings suggested the addition of ticagrelor, a clinically available antiplatelet agent and potent inhibitor of AKT signalling in cancer cells, as a novel treatment option for pancreatic cancer, and potentially other cancers with similar pathologies and underlying mechanisms.

Conflicts of Interest

Authors declare no conflict of interests for this article

Figures

Figure 1. **The P2Y12 receptor, activated by ADP, trigger AKT activation in PDAC cells.** (A) Immunoblots show the expression of P2Y12 and EGFR in PDAC cells (AsPC-1, BxPC-3, MiaPaCa-2, CFPAC-1 and PANC-1) and the normal pancreatic duct cells h-TERT-HPNE. Platelets were used as a positive control for P2Y12. Cells were seeded at 3×10^5 cells/well in a 6-well plate for 24 h, then washed, lysed and the proteins were collected and quantified. (B-C) Relative P2Y12 and EGFR expression in 5 PDAC cell lines and h-TERT-HPNE cells. The expression level was quantified and normalised to the loading control; α-actinin with automated software Image Lab (version 5.1, BioRad, CA, USA) and represented as columns using GraphPad Prism 5 (GraphPad Software, Inc, CA, USA. (D) The P2Y12 inhibitor, ticagrelor, reduced ADP-induced AKT activation in AsPC-1 and BxPC-3. Briefly, cancer cells were seeded in a 12-well plate, after 24 h, cells were starved for 6 h, then treated with ticagrelor (5 µM) combined with ADP (100 µM) and the cells were further incubated for 30 min in serum-free media. The figure shows a representative blot from three independent experiments. (E) Extracellular ADP release from AsPC-1, BxPC-3, MiaPaCa-2 and h-TERT-HPNE. ADP was analysed in 250 µl of PBS previously incubated with cells for 15 min as described in the methods and materials. The columns represent the mean of relative fluorescence units (RFU) from three independent experiments (E) Western blot analysis of phospho-EGFR Y1068 (p-EGFR Y1068), phospho-AKT S473 (p-AKT S473), phospho-ERK 1/2 (p-ERK 1/2) expression in lysates derived from AsPC-1, BxPC-3 and h-TERT-HPNE treated with ADP (10, 50 and 100 µM), for 30 min. The figure shows a representative blot from three independent experiments.

Figure 2. **P2Y12 regulates EGFR activation, SLUG and ZEB1 expression and enhances PDAC cells viability.** (A) Immunoblots show the expression of P2Y12, p-EGFR Y1068, EGFR, SLUG and ZEB1 in AsPC-1 and BxPC-3 after P2Y12-specific siRNA treatment. Cancer cells were seeded and treated with P2Y12 siRNA or a negative control siRNA as described in the methods and materials. (B) The columns represent the fold change of protein levels (p-EGFR Y1068, SLUG and ZEB1) relative to negative control siRNA treated cells ($n \geq 3$). (C) Cell viability of AsPC-1 and BxPC-3 cells following knockdown of P2Y12 compared with negative control siRNA treated cells (n ≥ 3). Data are presented as mean ± SEM. *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$. siNeg: siRNA negative control.

Figure 3. **Ticagrelor attenuates EGF-stimulated AKT activation and potentiates the anticancer activity of erlotinib.** (A) Immunoblots show the expression of p-EGFR Y1068, p-AKT S473, and p-ERK 1/2 in serum-starved AsPC-1 and BxPC-3 cells treated with EGF (10 ng/ml) combined with ticagrelor (5 µM) or apyrase (5 U/ml) for 1 h. (B) Immunoblots show the expression of p-EGFR

Y1068, p-AKT S473, and p-ERK 1/2 in serum-starved AsPC-1 and BxPC-3 cells treated with ADP (100 μM) combined with erlotinib (5 μM) for 30 min. EGF +/- erlotinib was used as a control for EGFR activation. (C) Cell viability of AsPC-1 and BxPC-3 following treatment with erlotinib (0.001-10 μM) with and without ticagrelor (2.5 μM) for 72 h. Data are presented as mean ± SEM. Two-way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean. N = 5, *** $p < 0.0001$, * $p < 0.05$. Table 1 and 2 show the combination index (CI) values calculated using Chou-Talalay's method and can be interpreted as follow: CI < 1, synergism, CI > 1, antagonism, CI = 1, additive. (D) Immunoblots show the expression of p-EGFR Y1068, p-AKT S473, and p-ERK 1/2 in non-starved AsPC-1 and BxPC-3 cells treated with erlotinib (5 μM) combined with ticagrelor (2.5 μM) for 30 min. Immunoblots in A, B and D are representative samples of at least 3 independent experiments.

Figure 4. Ticagrelor treatment reduces PDAC cell viability and enhances apoptosis through attenuating AKT activation in PDAC cells. (A) Relative cell viability in AsPC-1, BxPC-3, MiaPaCa-2, PANC-1, CFPAC-1, and h-TERT-HPNE cells upon treatment with ticagrelor (0-20 μM) for 72 h (n ≥ 3). Table 3 shows (IC50, μM) of ticagrelor calculated using GraphPad PRISM 5.0 software (GraphPad Software, Inc, CA, USA) and log (inhibitor concentration) vs normalised response (variable slope). (C) AsPC-1 and BxPC-3 cells were treated with ticagrelor (0-10 μM) for 12 hours and analysed for apoptosis (caspase 3/7 activation). The columns represent fold change in the level of activated caspase 3/7 relative to control vehicle-treated cells, measured as described in the methods and materials. Data are presented as mean ± SEM. One-way ANOVA with post-hoc Bonferroni's Multiple Comparison Test was used to examine the significance of the mean. N ≥ 4, *** $p <$ 0.0001, ** $p < 0.001$ * $p < 0.05$. (C) Detection of caspase 3 activity in live cells treated with vehicle or ticagrelor 10 μM) for 12 hours. The caspase 3 substrate, once cleaved by caspase 3, forms a DNA dye which stains the nucleus bright green (D) Immunoblots show the expression of p-AKT S473 in non-starved AsPC-1 and BxPC-3 cells treated with ticagrelor (0-10 μM) for 1 h. (E) The columns represent the fold change of p-AKT S473 relative to control vehicle-treated cells (n ≥ 3). Data are presented as mean ± SEM. *** $p < 0.0001$, * $p < 0.05$.

Figure 5. Ticagrelor synergises with chemotherapy in PDAC cells *in vitro.* (A-C) Relative cell viability in AsPC-1, BxPC-3 and MiaPaCa-2 upon treatment with gemcitabine (0-100 μM), paclitaxel (0-100 nM), and cisplatin (0-100 μM) as single agents or in combination with ticagrelor (2.5 μM) for 72 h. The significance of the difference between viability of cells treated with chemotherapy alone or in combination with ticagrelor was tested using two-way ANOVA with post-hoc Bonferroni's Multiple

Comparison Test, $n \geq 3$, *** $p < 0.0001$, ** $p < 0.001$ * $p < 0.05$. Tables (4-12) show CI values calculated using Chou-Talalay's method, and can be interpreted as follow: CI < 1, synergism, CI > 1, antagonism, CI = 1, additive.

Figure 6. Combined treatment of ticagrelor and gemcitabine reduced tumour growth in vivo. (A) Representative image of subcutaneous xenografts transplanted with BxPC-3 cells treated with vehicle control, gemcitabine, ticagrelor or gemcitabine plus ticagrelor. Effect of different treatments on tumour weight (B) and volume (C) in each mouse bearing BxPC-3 tumours. The significance of the difference between tumour weight and volume in mice bearing BxPC-3 tumours treated with vehicle control or gemcitabine, ticagrelor, gemcitabine plus ticagrelor was tested using ordinary one-way ANOVA with post-hoc Sidak's Multiple Comparison test, $n = 8$ for vehicle, $n = 8$ for gemcitabine, $n = 7$ for ticagrelor, $n = 7$ for gemcitabine plus ticagrelor. P value is calculated above each column to represent the significance of the difference between vehicle and different treatment.

Supp. Data 1. (A) Verification of P2Y12 expression using two different anti-P2Y12 antibodies. Platelets were used as a positive control (two different amounts of platelet lysates (20 and 10 µg of protein lysate per lane). As discussed in the methods and materials, platelet P2Y12 was detected mostly as oligomers (> 100 kDa), while in PDAC cells P2Y12 was mostly detected as monomers (39-60 kDa). (B) ADP-stimulated AKT activation was reduced by another P2Y12 inhibitor; PSB 0739. Immunoblot shows the expression of p-AKT S473 in BxPC-3 cells treated with ADP (100 µM) combined with PSB 0739 (20 µM) for 30 min. (C) Inhibition of P2Y12, but not P2Y1, attenuated ADP-stimulated AKT activation. Immunoblot shows the expression of p-AKT S473 and p-ERK 1/2 in AsPC-1 cells treated with ADP (100 µM) combined with the P2Y1 inhibitor, MRS 2179 (0-20 µM) or ticagrelor (5 µM) for 30 min. (D) Inhibition of P2Y12 with PSB 0739 reduces EGF-stimulated AKT activation. Immunoblots show the expression of p-AKT S473 in BxPC-3 cells treated with EGF (10 ng/ml) combined with PSB 0739 (20 µM) or ticagrelor (5 µM) for 30 min. (E) Inhibition of P2Y1 with MRS 2179 reduces EGF-stimulated AKT and ERK activation. Immunoblot shows the expression of p-AKT S473 and p-ERK 1/2 in AsPC-1 cells treated with EGF (10 ng/ml) combined with MRS 2179 (20 µM) or ticagrelor (5 µM) for 30 min. Immunoblots (A-E) are representative samples of 3 independent experiments with similar results. Plt: platelet
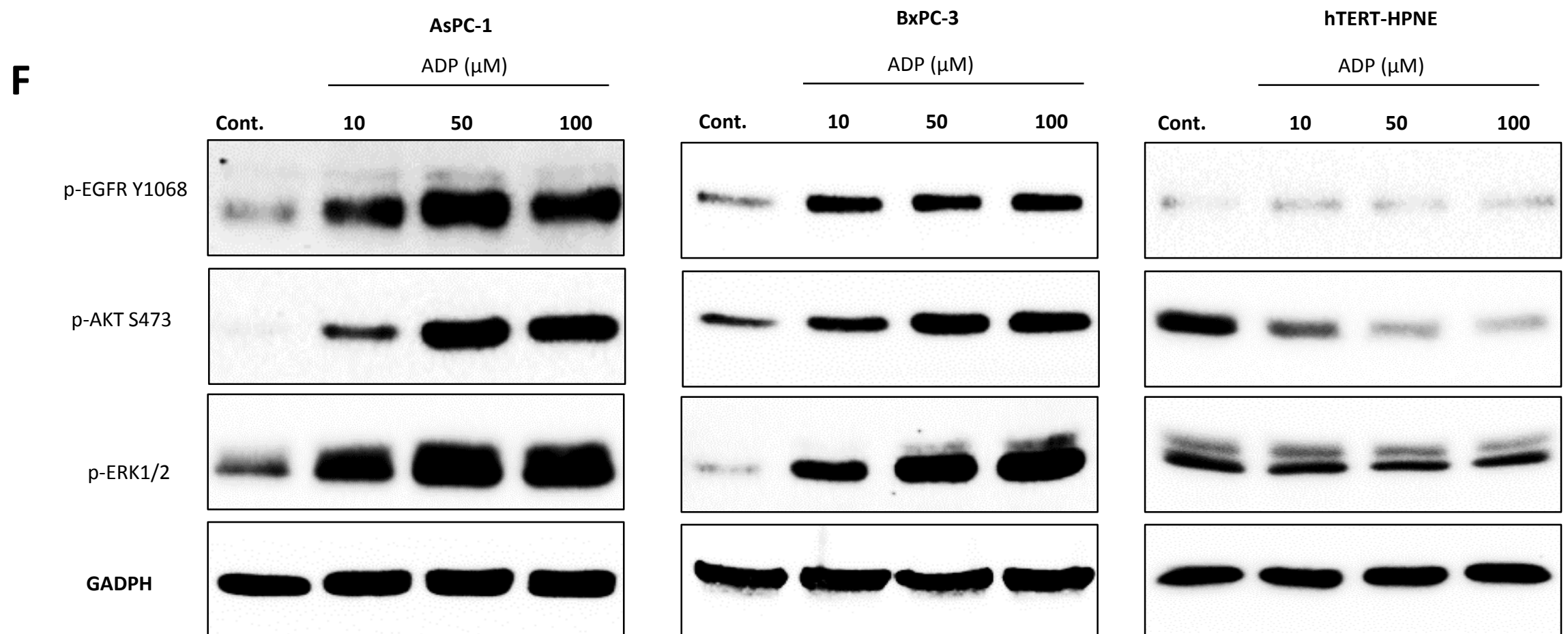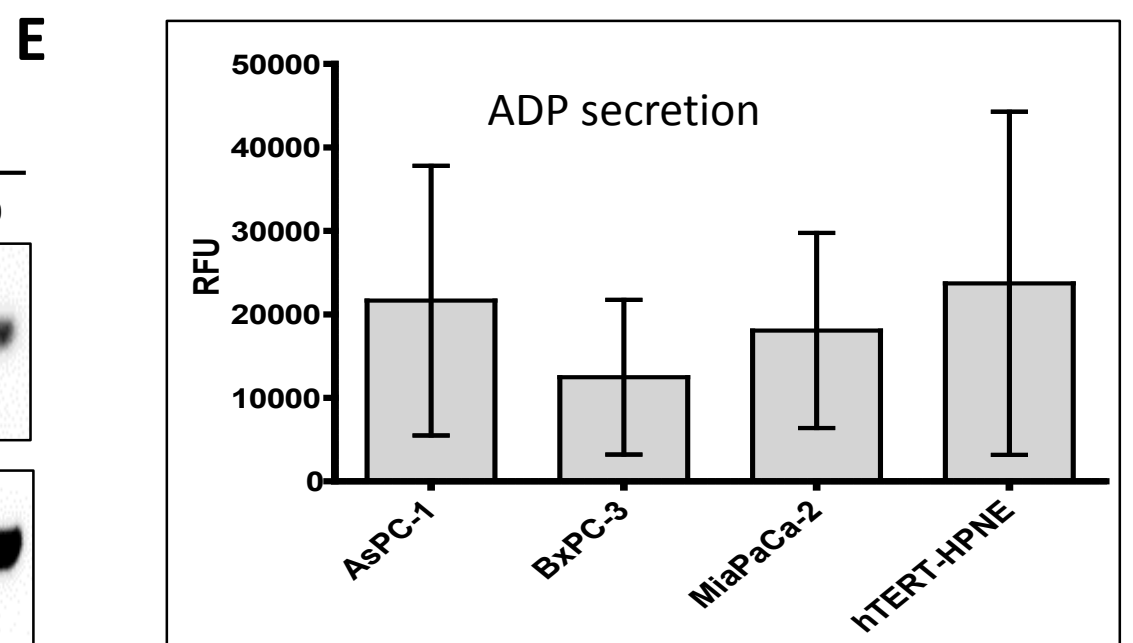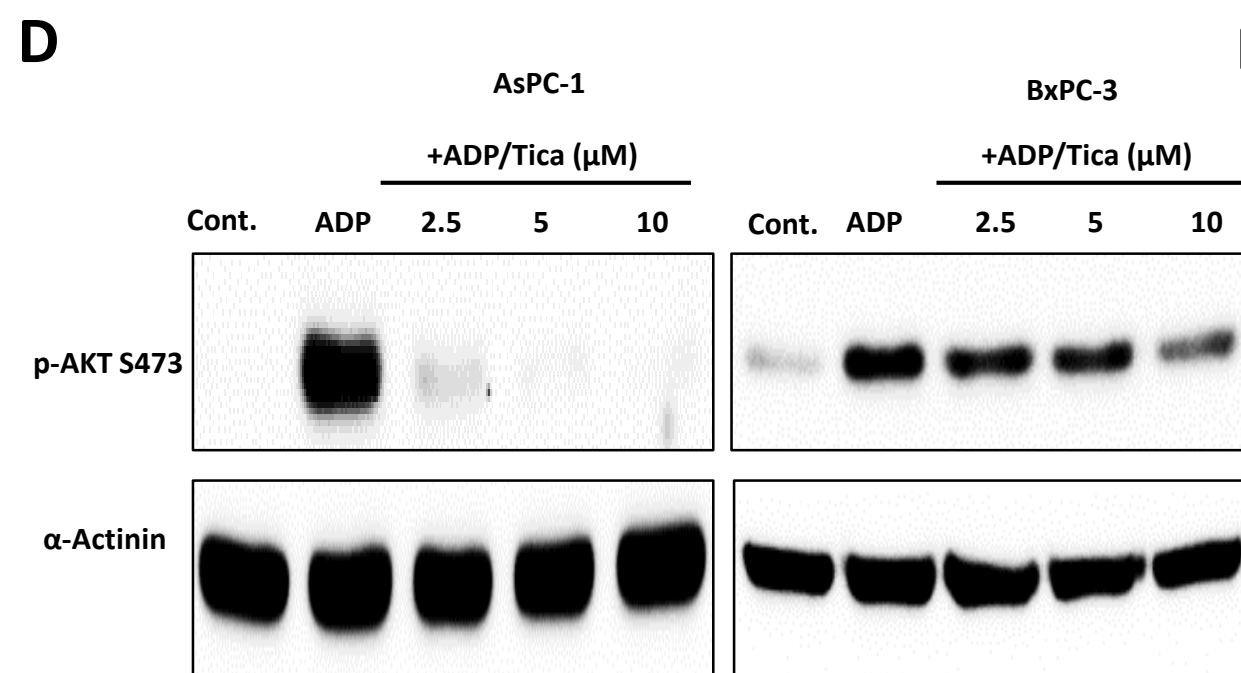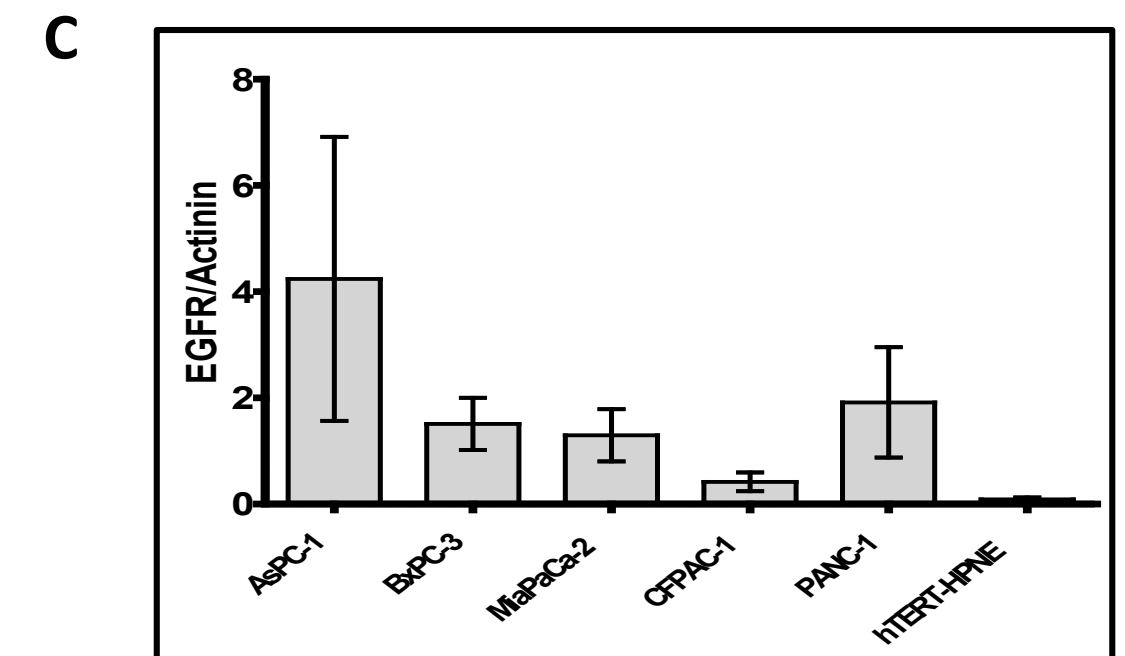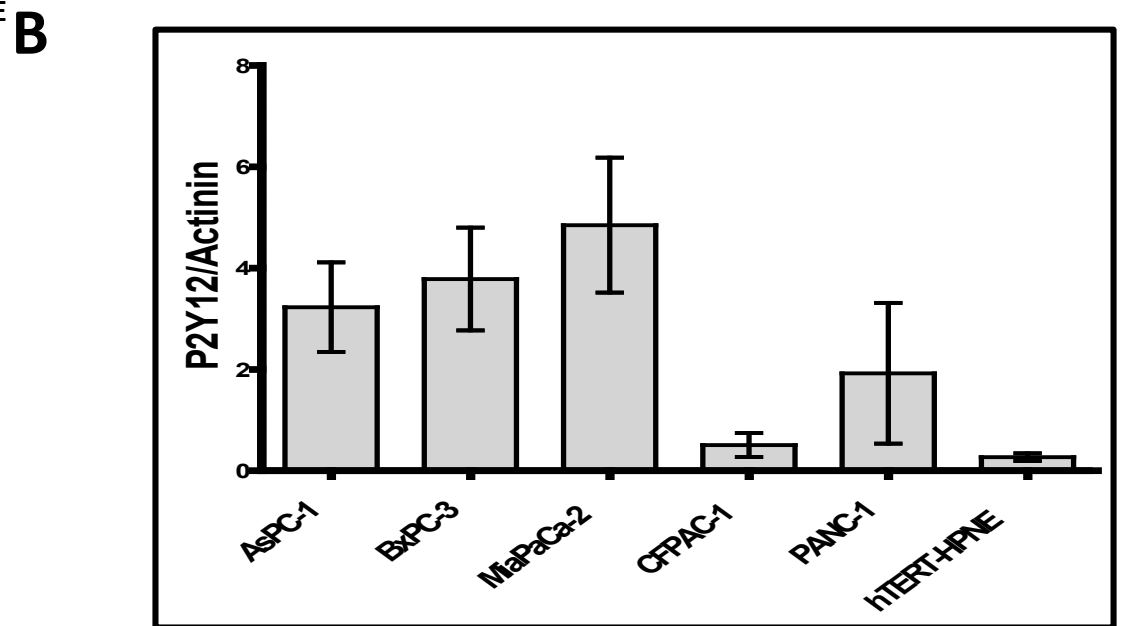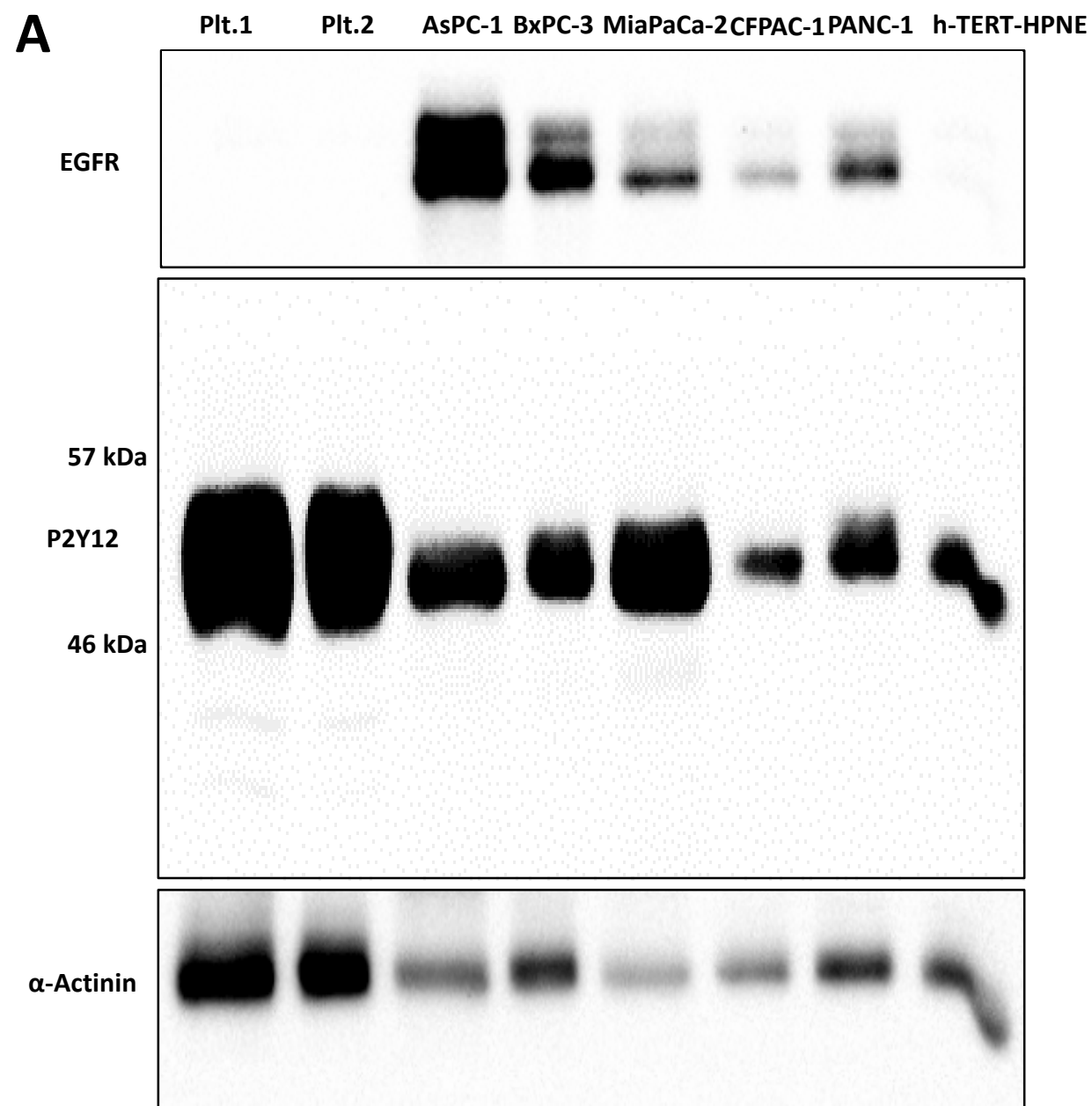
Supp. Data 2. (A) Verification of P2Y12 knockdown and its effect on the downstream target SLUG using four different siP2Y12 sequences. FlexiTube GeneSolution (four different P2Y12 siRNAs) was obtained from Qiagen Pty Ltd, Australia, and the knockdown in AsPC-1 was progressed as described
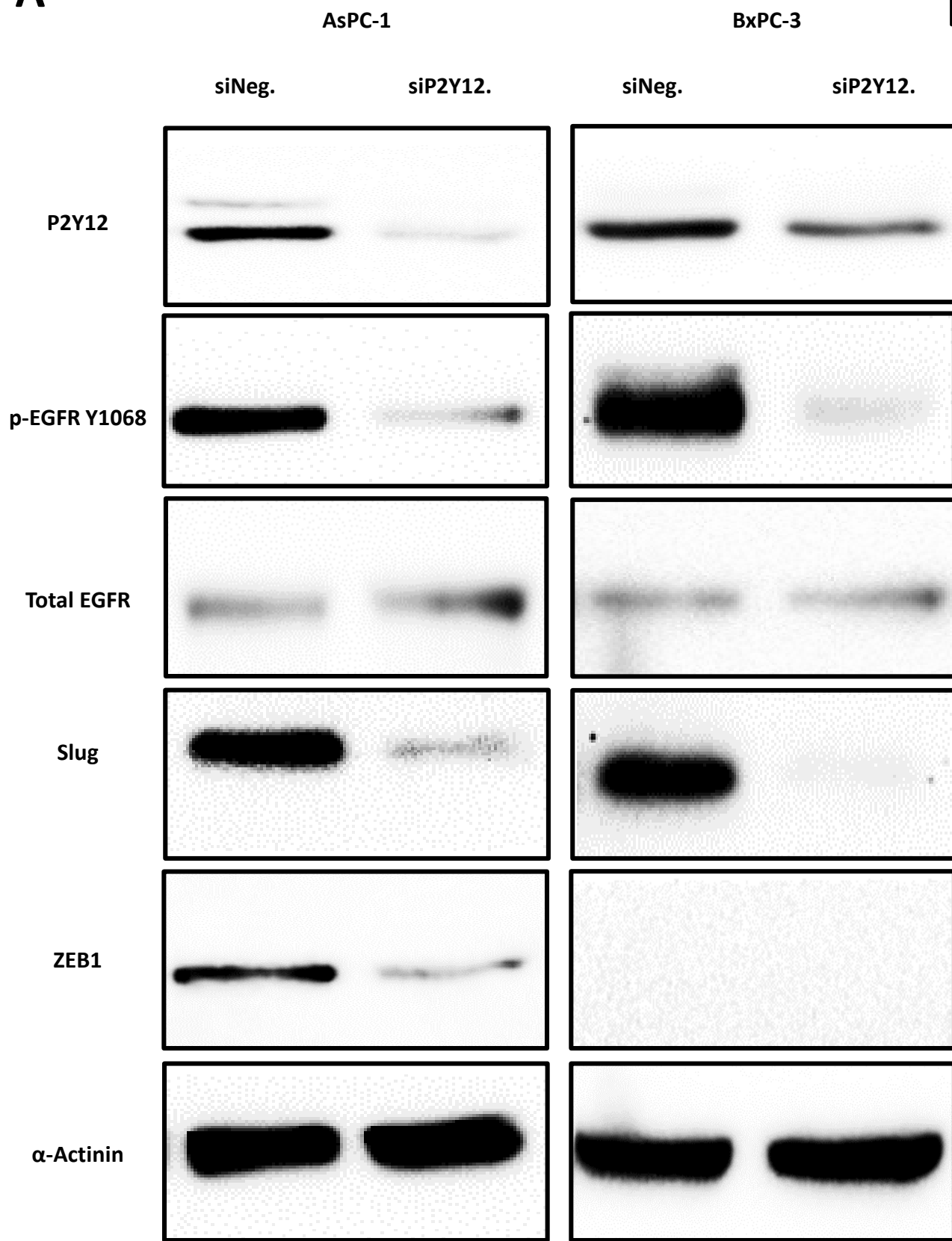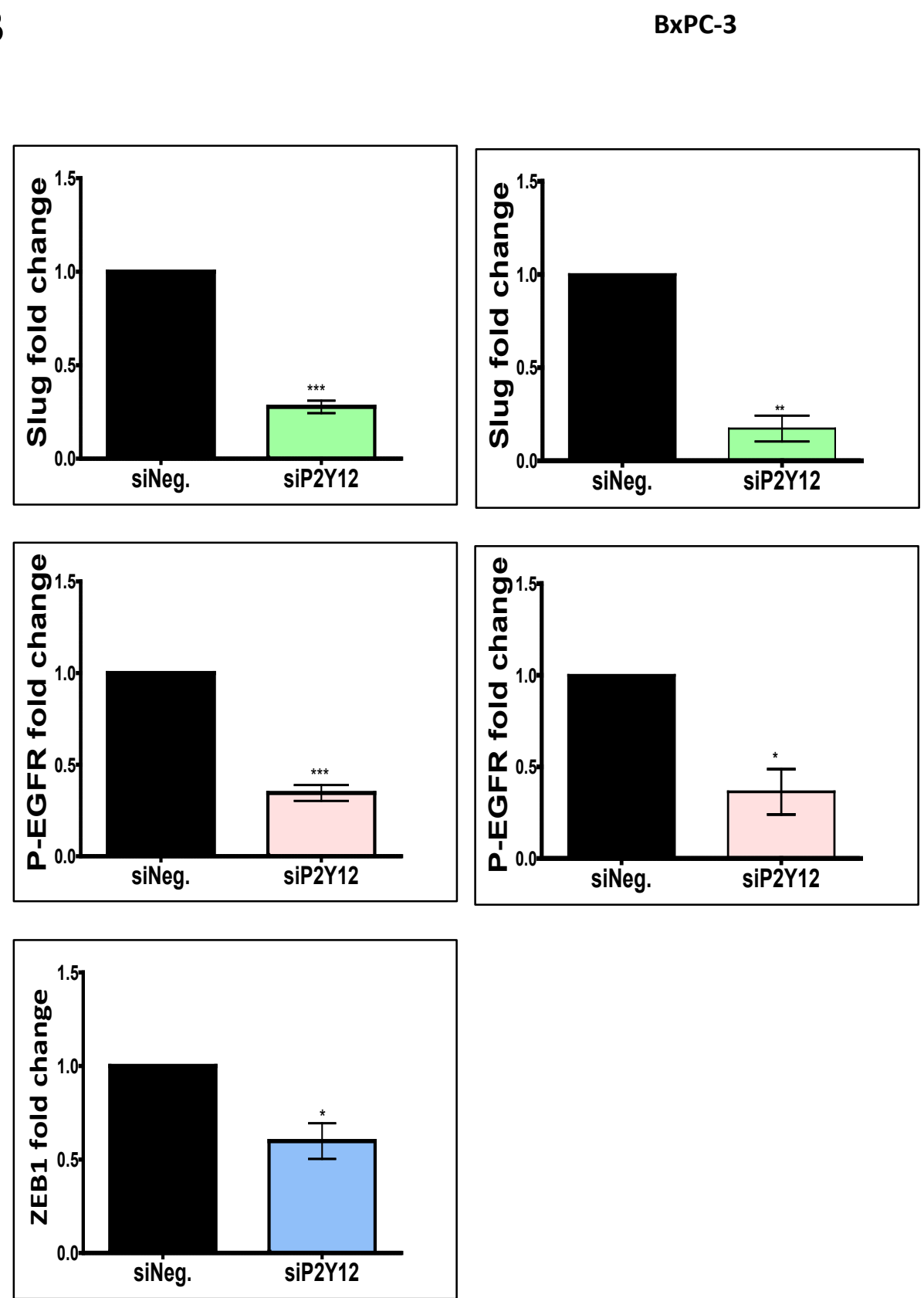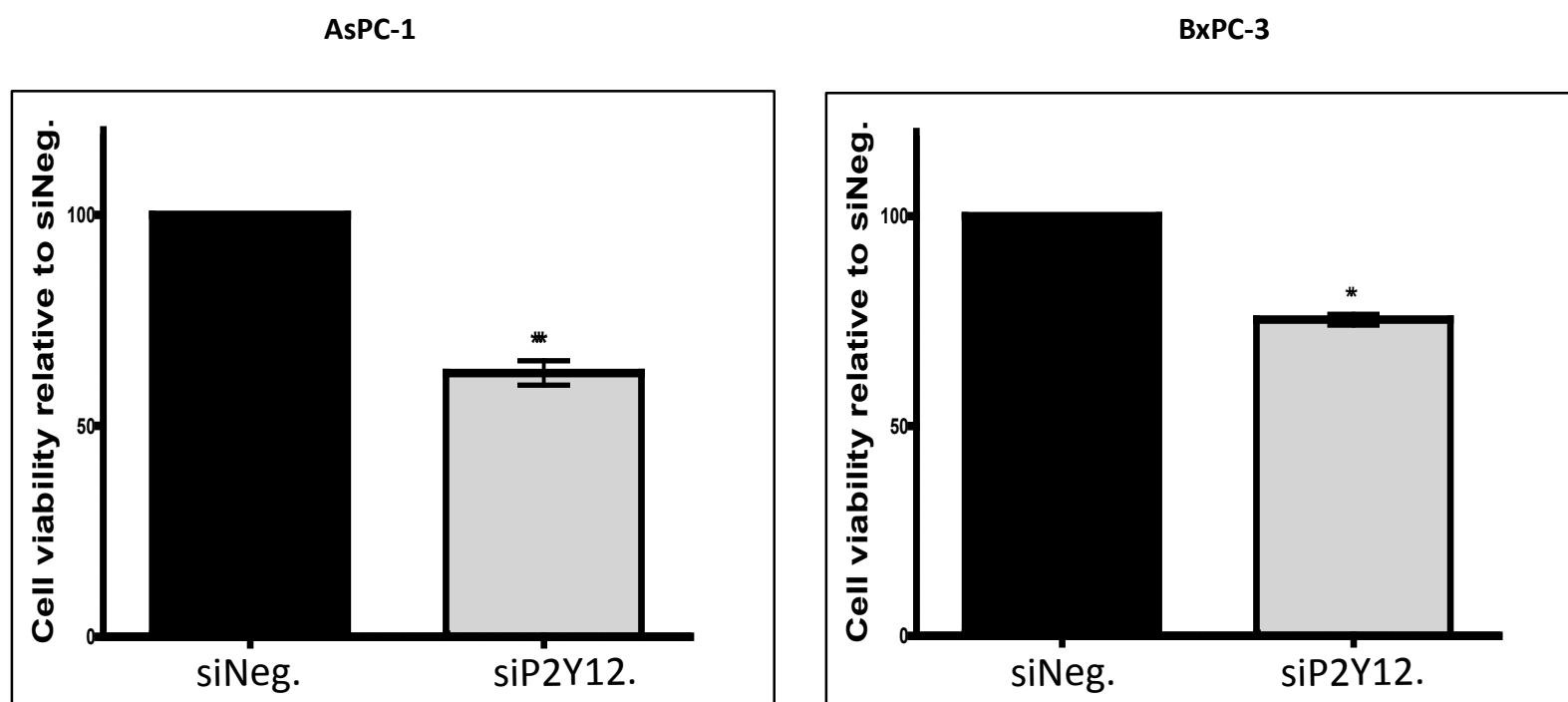
in the materials and methods section. (B) Fold change in P2Y12 and SLUG protein expression in AsPC-1 treated with four different P2Y12 siRNAs compared to siNeg transfected cells. (C) Ticagrelor has minimal cytotoxicity on normal pancreatic cells. AsPC-1 and h-TERT-HPNE were seeded in a 96-well plate in the same culture media, except for the selection antibiotic puromycin which is required for h-TERT-HPNE growth. After 24 h, cells were treated with ticagrelor (10 µM) for 72 h. Cell viability was measured as discussed in the methods and materials. Data are presented as mean ± SEM. The unpaired t-test was used to examine the significance of the mean. N = 5, *** $p < 0.0001$. (D) PSB 0739 reduced cancer cell proliferation. Relative cell viability in BxPC-3 upon treatment with PSB 0739 or ticagrelor (0-20 µM) for 72 h (n = 3).
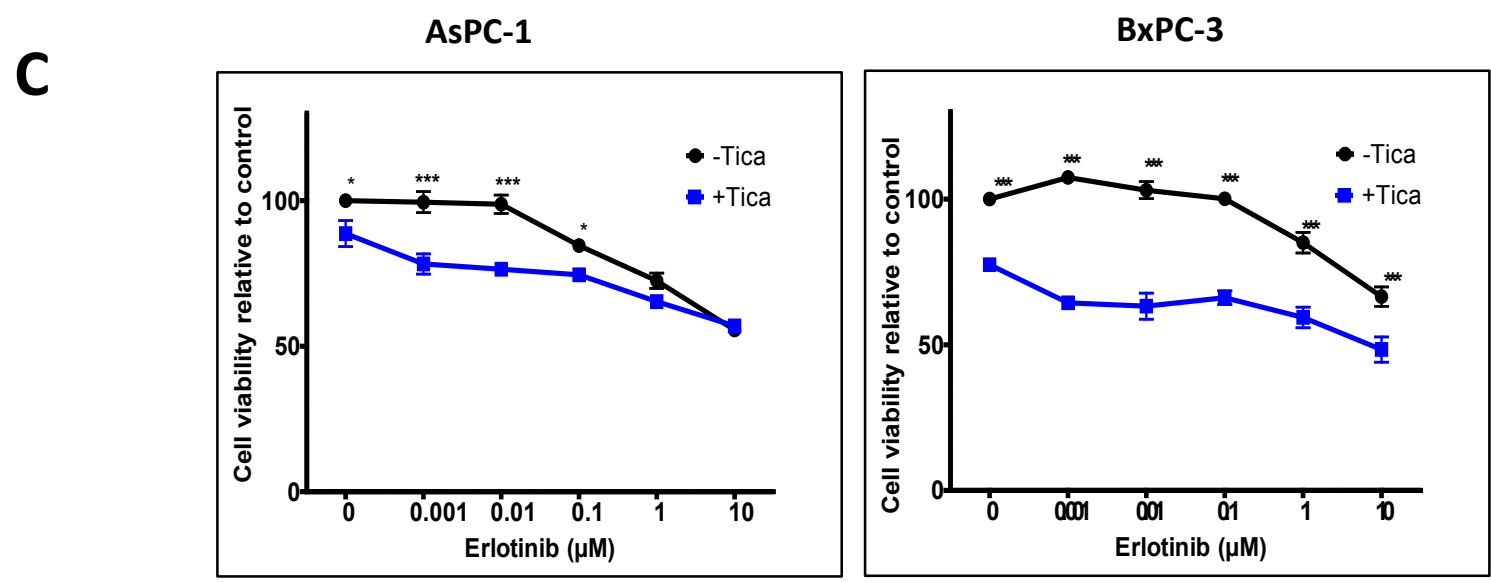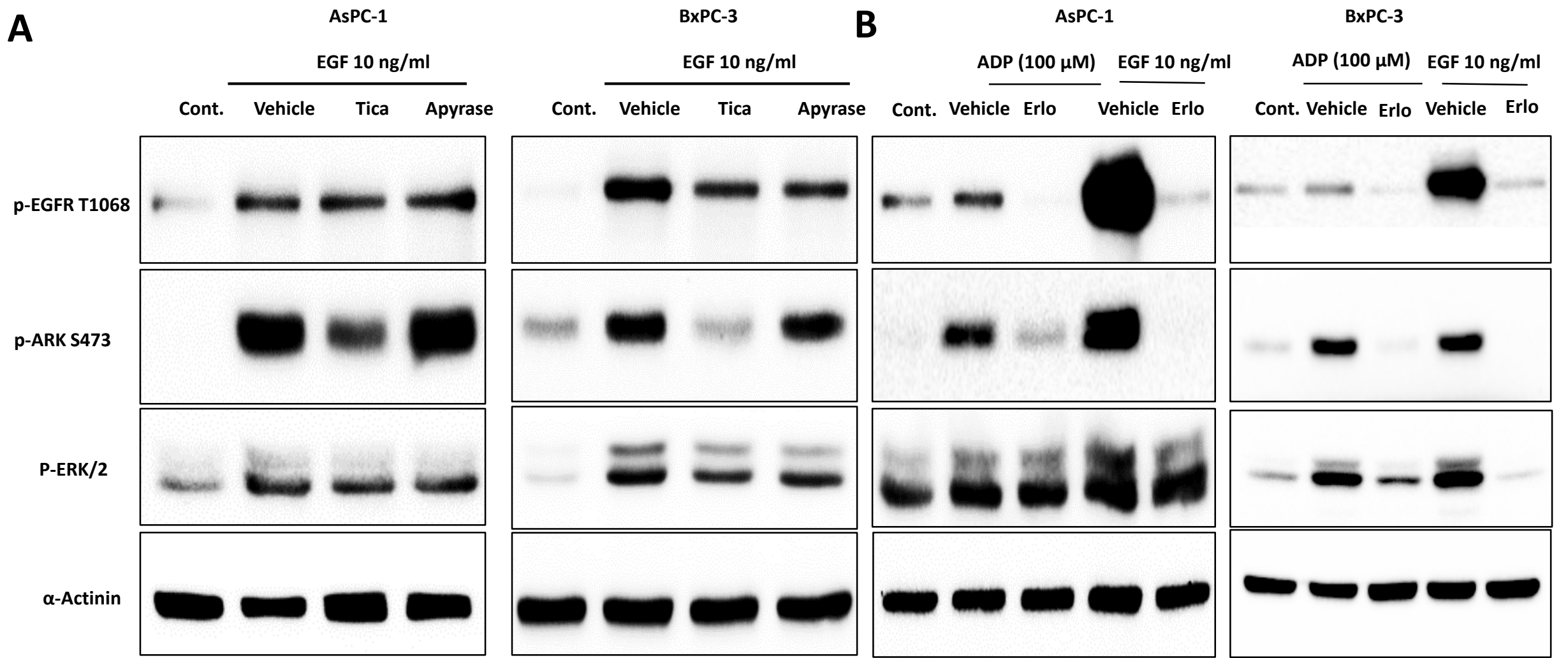
Supp. Data 3. (A) Unmodified image of subcutaneous xenografts transplanted with BxPC-3 cells treated with vehicle control, gemcitabine, ticagrelor or gemcitabine plus ticagrelor. (B) Ticagrelor did not increase gemcitabine toxicity on different blood cells. Columns represent the number of white blood cells, platelet, monocytes, granulocytes, red blood cells and lymphocytes in mice bearing BxPC-3 tumours treated with vehicle control, gemcitabine, ticagrelor, and gemcitabine plus ticagrelor. The significance of the difference between the number of different blood cells in mice treated with different treatments was tested with ordinary one-way ANOVA with post-hoc Sidak's Multiple Comparison test, $n = 8$ for the vehicle, $n = 8$ for gemcitabine, $n = 7$ for ticagrelor, $n = 7$ for gemcitabine plus ticagrelor. P value is calculated above each column to represent the significance of the difference between vehicle and different treatment. (C) Fold change in mice weight before and after treatment

Supp. Data 4. (A) Expression of P2Y12 in PDAC tissue compared to matched TCGA normal and GTEX data. (B) Survival curves for patients with PDAC based on the expression of P2RY12 from TCGA data. P2Y12 was normalised by the housekeeping gene POLR2L which has minimal variability in expression between normal and PDAC tissues as previously described [397]. (C) The expression level of different purinergic receptors (P2Y12, P2Y1, P2Y2 and P2X7) in PDAC tissue compared to matched TCGA normal and GTEX data. *For A-B, data were analysed using the web-based tool GEPIA

Supp. Data 5. (A-C) Correlation of P2Y12 with EGFR (A), ZEB1 (B) and SLUG (C) in PDAC tissues from TCGA data were calculated using the Pearson correlation coefficient. P2Y12 was normalised by the housekeeping gene POLR2L. *For A-B, data were analysed using the web-based tool GEPIA
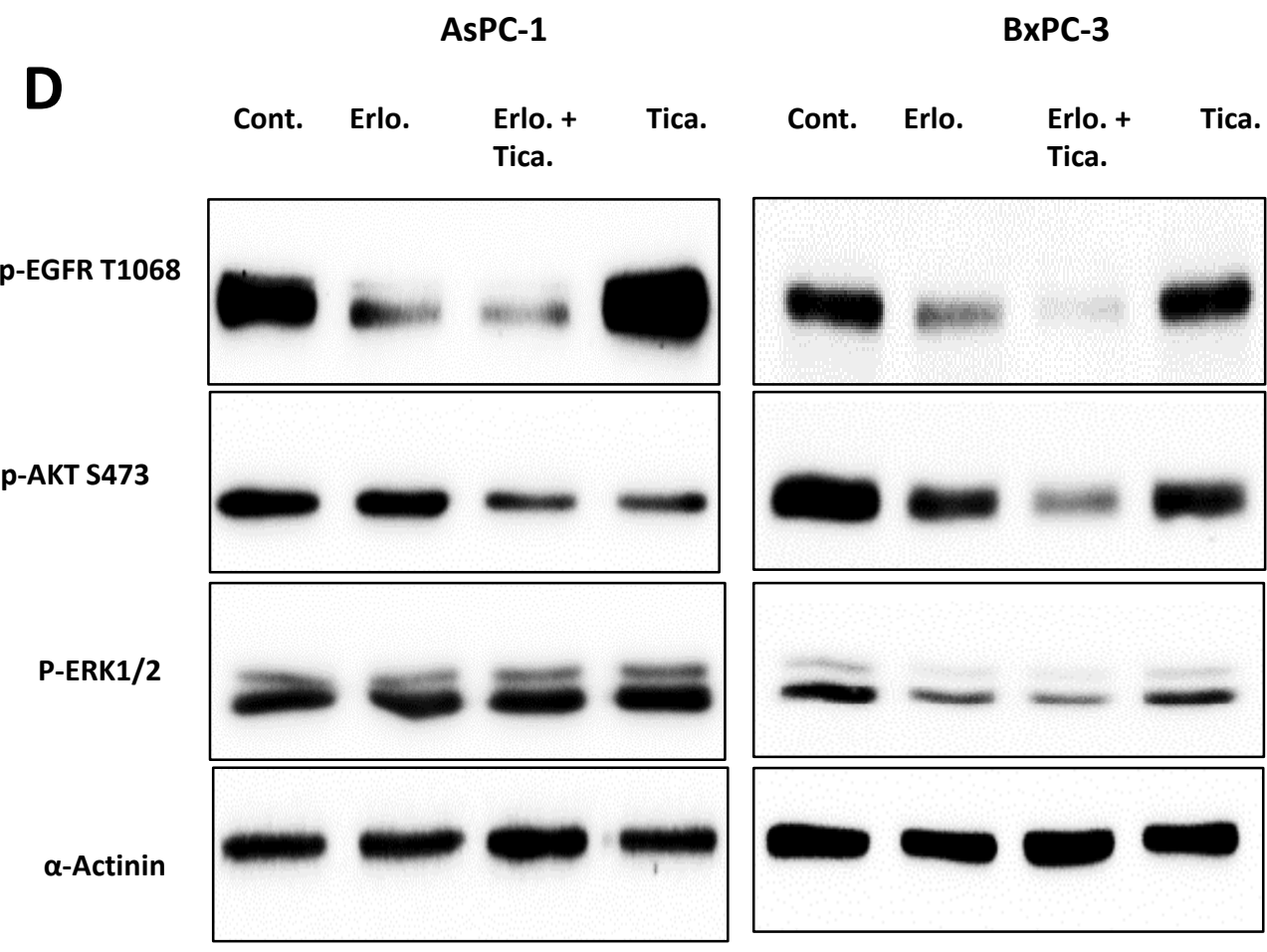
A

EGFR

P2Y12

57 kDa

46 kDa

α-Actinin

Plt.1 Plt.2 AsPC-1 BxPC-3 MiaPaCa-2 CFPAC-1 PANC-1 h-TERT-HPNE

B

P2Y12/Actinin

AsPC-1 BxPC-3 MiaPaCa-2 CFPAC-1 PANC-1 hTERT-HPNE

C

EGFR/Actinin

AsPC-1 BxPC-3 MiaPaCa-2 CFPAC-1 PANC-1 hTERT-HPNE

D

AsPC-1   BxPC-3

+ADP/Tica (µM)   +ADP/Tica (µM)

Cont. ADP 2.5 5 10   Cont. ADP 2.5 5 10

p-AKT S473

α-Actinin

E

ADP secretion

RFU

AsPC-1 BxPC-3 MiaPaCa-2 hTERT-HPNE

F

AsPC-1   BxPC-3   hTERT-HPNE

ADP (µM)   ADP (µM)   ADP (µM)

Cont. 10 50 100   Cont. 10 50 100   Cont. 10 50 100

p-EGFR Y1068

p-AKT S473

p-ERK1/2

GADPH

2

148

A

	AsPC-1		BxPC-3	
	siNeg.	siP2Y12.	siNeg.	siP2Y12.

P2Y12

p-EGFR Y1068

Total EGFR
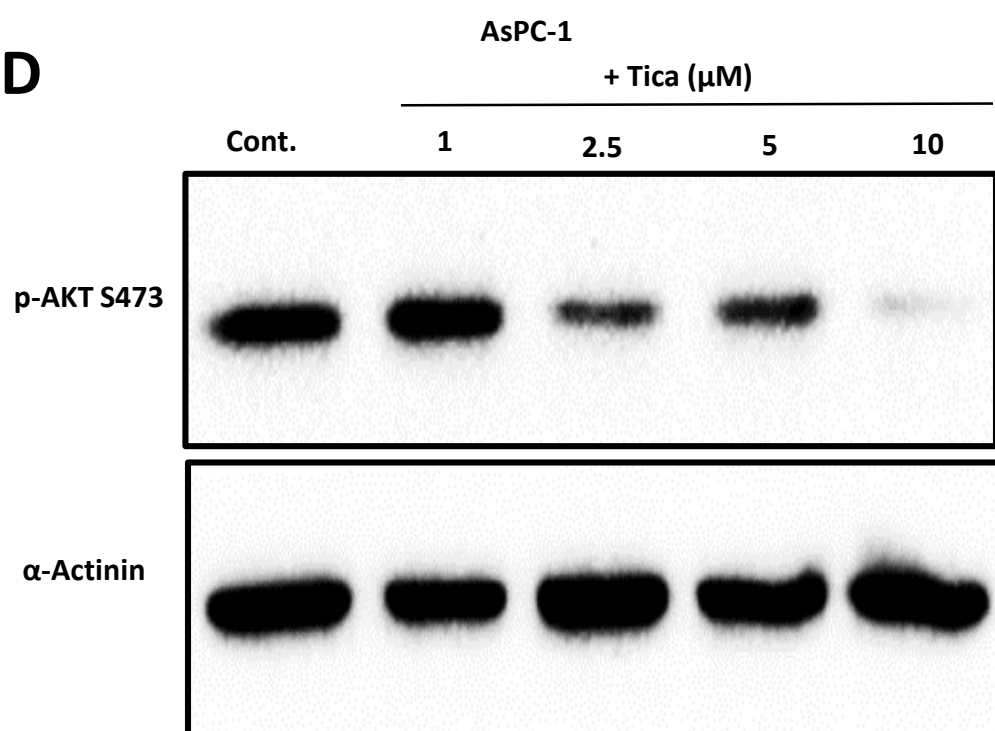
Slug

ZEB1

α-Actinin

B

BxPC-3

A

Table 3: IC50 of ticagrelor in PDAC and hTERT-HPNE cells

Cell line	IC50 (μM)
AsPC-1	10.71
BxPC-3	6.4
MiaPaCa-2	6.98
PANC-1	7.75
CFPAC-1	7.2
hTERT-HPNE	Above 20 μM

B

C

D

E
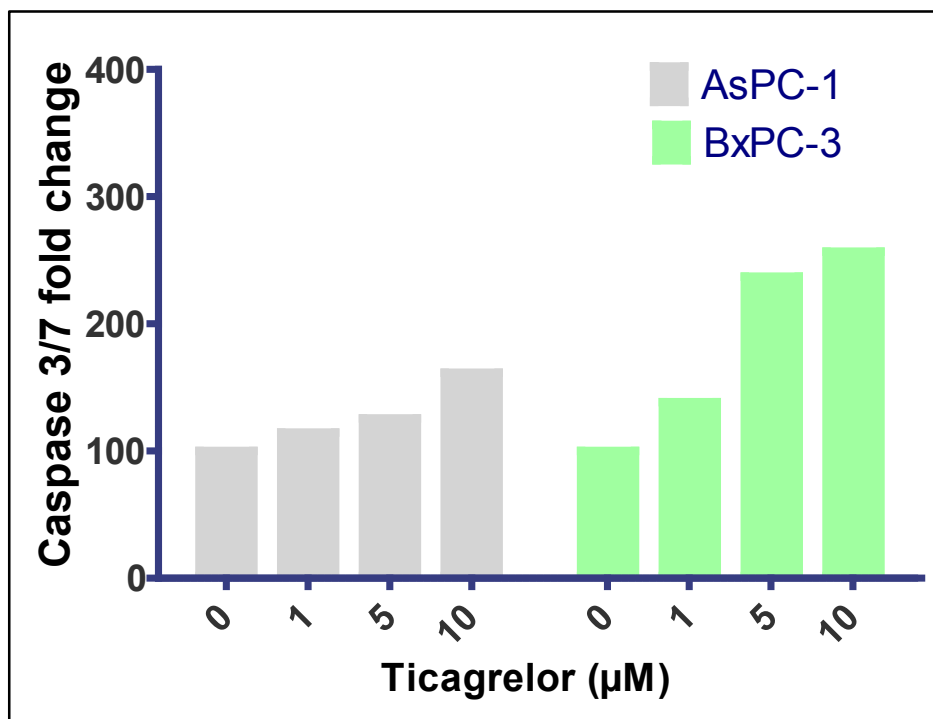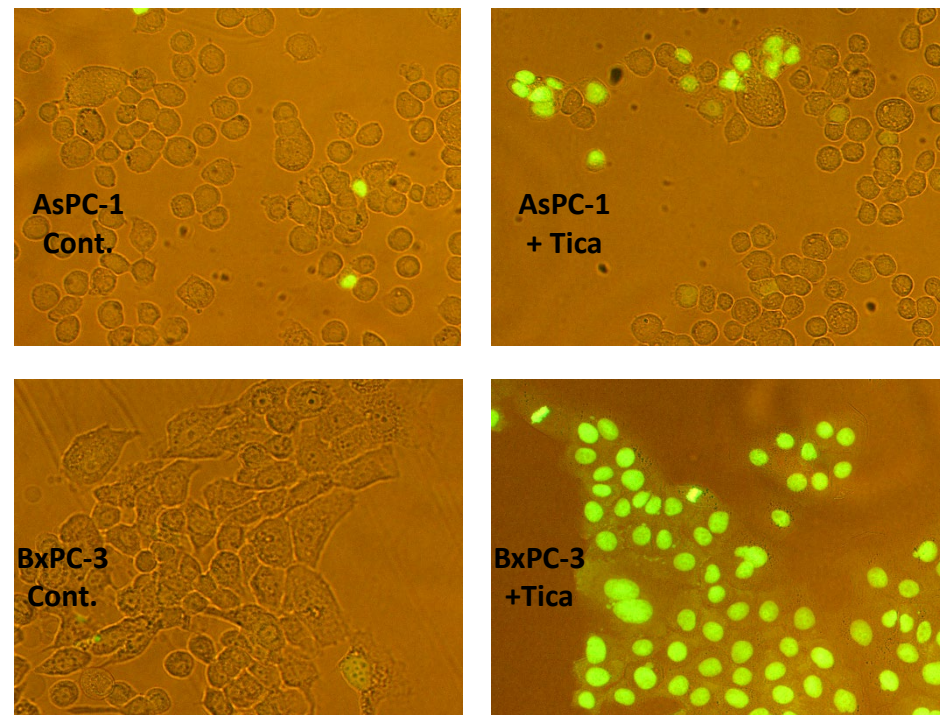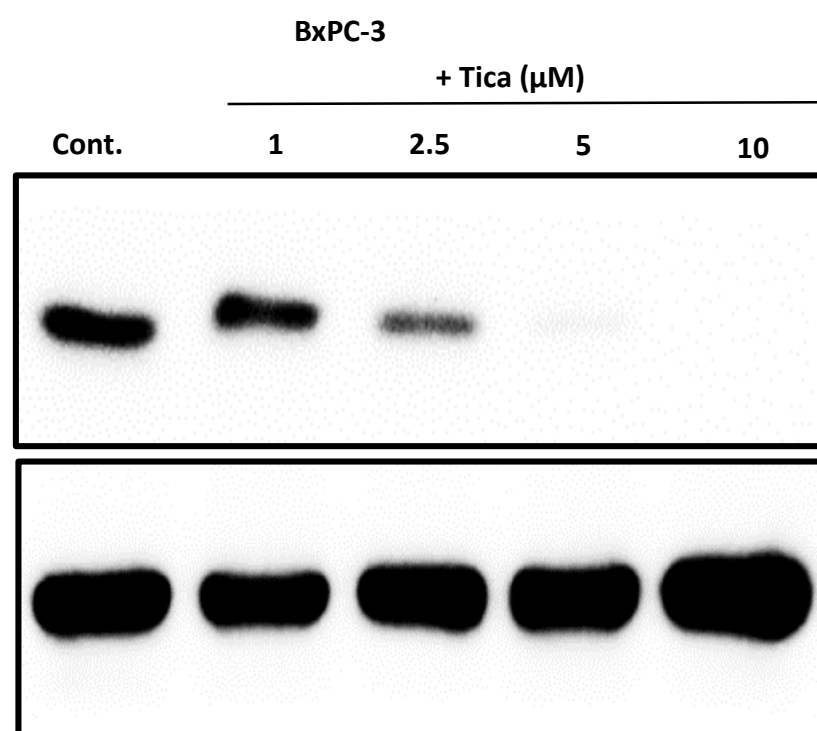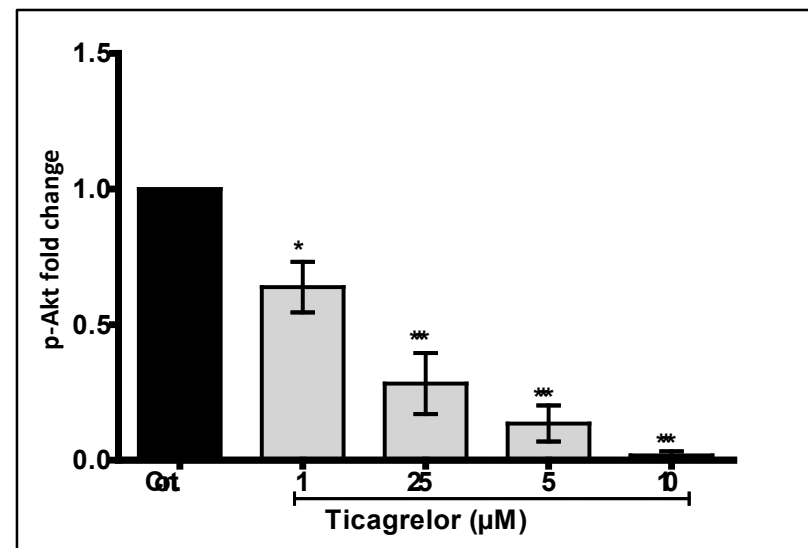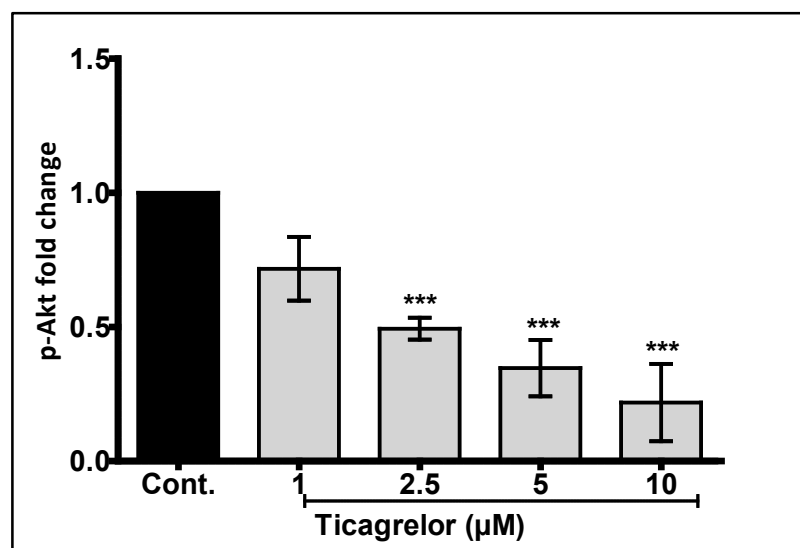
AsPC-1 **BxPC-3** **MiaPaCa-2**
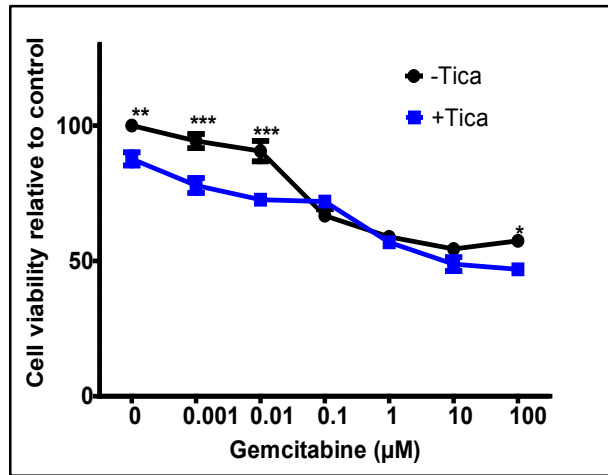
Gem (µM)	CI
0.001	0.43
0.01	0.39
0.1	0.53
1.0	0.38
10.0	0.5
100.0	2.12
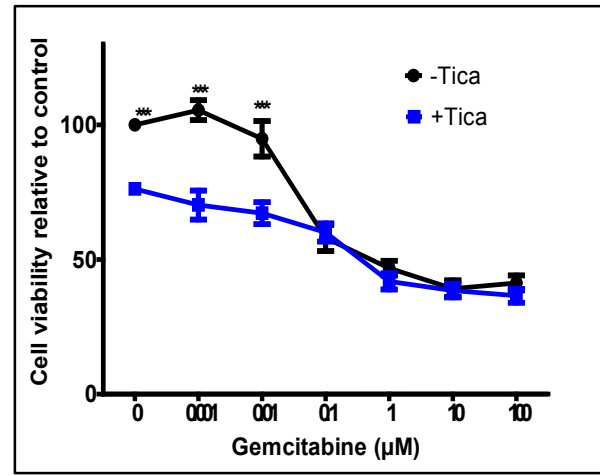
Gem (µM)	CI
0.001	0.92
0.01	0.98
0.1	0.86
1.0	0.58
10.0	1.2
100.0	6.5
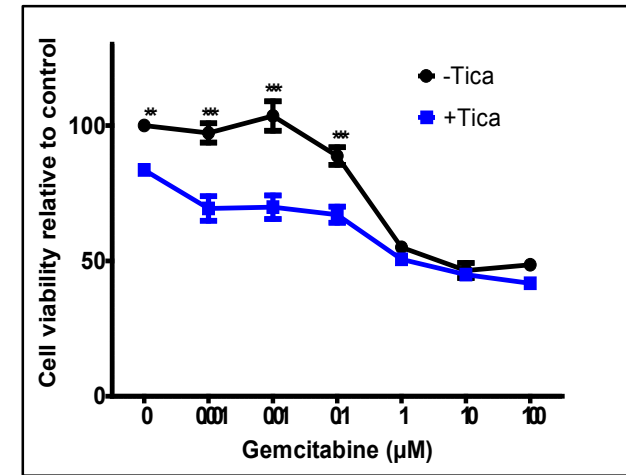
Gem (µM)	CI
0.001	0.8
0.01	0.78
0.1	0.76
1.0	0.58
10.0	0.93
100.0	4.3

B
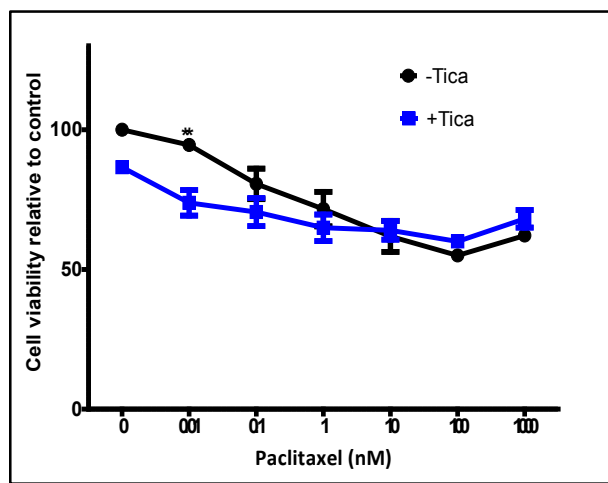
PAC (nM)	CI
0.01	0.41
0.1	0.39
1.0	0.37
10.0	0.6
100.0	1.4
1000.0	7.2
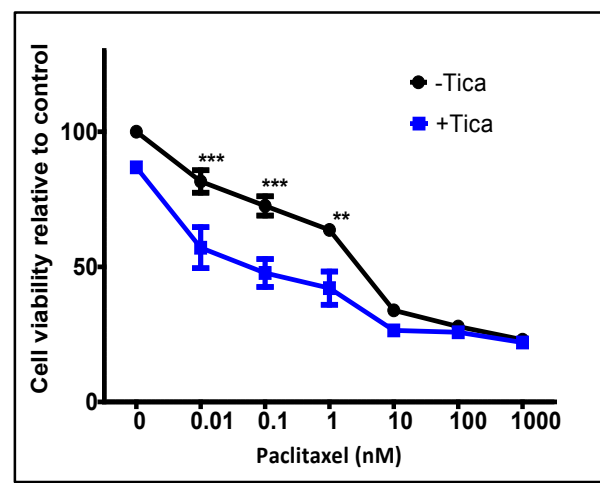
PAC (nM)	CI
0.01	0.68
0.1	0.56
1.0	0.55
10.0	0.44
100.0	1.1
1000.0	1.7
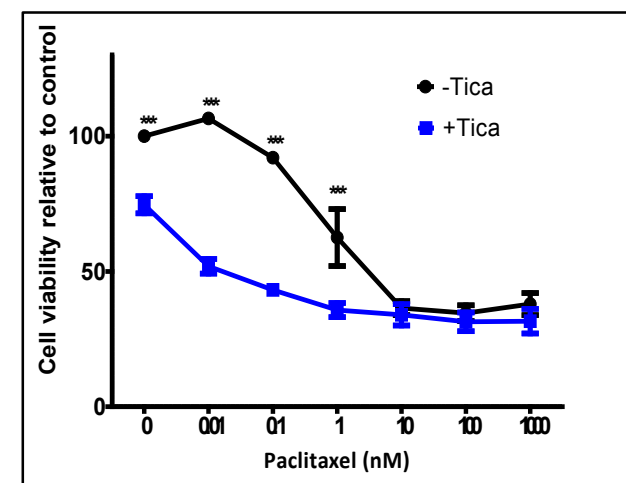
PAC (nM)	CI
0.01	0.55
0.1	0.45
1.0	0.39
10.0	0.46
100.0	1.2
1000.0	9

C
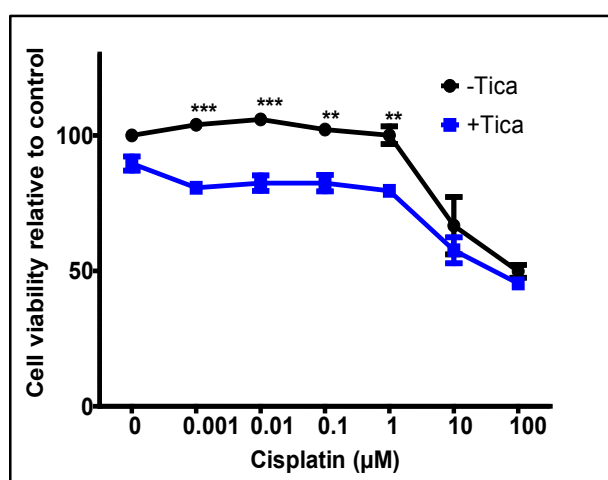
Cis (µM)	CI
0.001	0.46
0.01	0.48
0.1	0.49
1.0	0.49
10.0	0.47
100.0	0.93
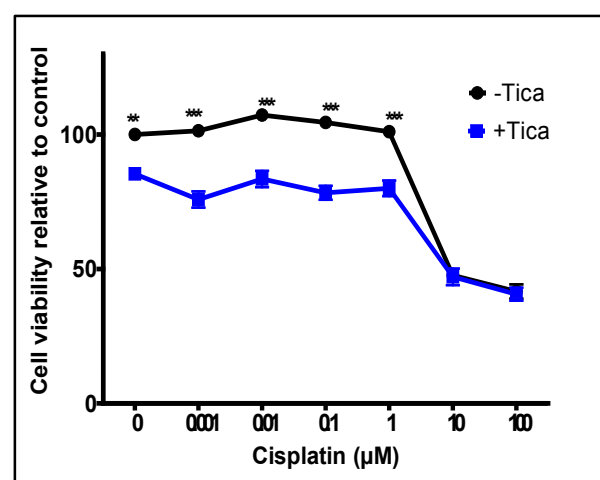
Cis (µM)	CI
0.001	0.63
0.01	0.72
0.1	0.69
1.0	0.74
10.0	0.45
100.0	0.48
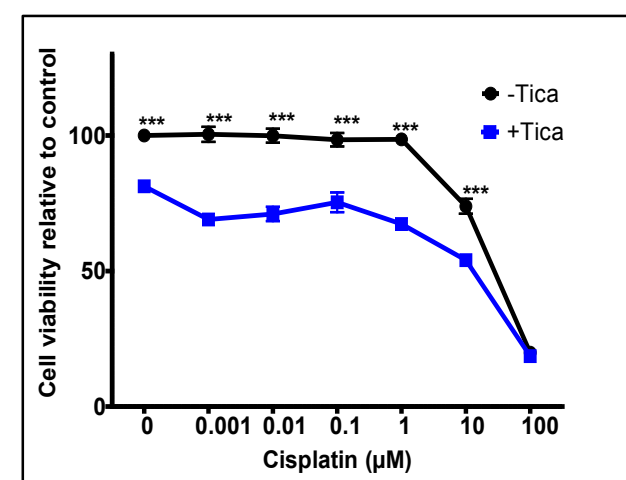
Cis (µM)	CI
0.001	0.78
0.01	0.82
0.1	0.94
1.0	0.83
10.0	0.9
100.0	0.62

*Table 4-12. Combination index (CI): Synergism (CI < 1), Additive Effect (CI = 1) and Antagonism (CI > 1)

Ticagrelor potentiates gemcitabine activity in vivo

A

Vehicle: 1350-1, 1355-1, 1358-4

GEM: 1354-1, 1349-3, 1353-3

Tica: 1351-2, 1354-2, 1357-3

GEM + Tica: 1358-1, 1349-1, 1351-3

B

Tumour weight (mg)

0.1015 (GEM), 0.3926 (Tica), 0.0279 (GEM + Tica)

Vehicle, GEM, Tica, GEM + Tica

C

Tumour volume mm^3

0.0039 (GEM), 0.0005 (Tica), 0.0001 (GEM+Tica)

Vehicle, GEM, Tica, GEM+Tica

A

AsPC-1   BxPC-3   MiaPaCa-2   h-TERT HPNE   Plt2

P2Y12 oligomers

P2Y12 Monomers

100 kDa
57 kDa
32 kDa

GAPDH

P2Y12/P2RY12 Antibody (1C2A9), NOVUSBIO
Expected band at ~ 39 kDa

AsPC-1   BxPC-3   MiaPaCa-2   h-TERT HPNE   Plt1   Plt2

P2Y12 Antibody (4H5L19), ThermoFisher
Expected band at ~ 60 kDa

B   BxPC-3

Cont.   ADP.   ADP/PSB0 0739.   PSB 0739.

P-AKT S473

α-Actinin

C   AsPC-1

ADP/MRS 2179 (µM)

Cont.   ADP   5   10   20   ADP/Tica

P-AKT S473

P-ERK1/2

α-Actinin

D   BxPC-3

Cont   EGF   EGF/Tica   EGF/PSB 0739

P-AKT S473

α-Actinn

E   AsPC-1

Cont.   EGF   EGF/MRS 2179   EGF/Tica

P-AKT S473

P-ERK 1/2

α-Actinin

A

+ siP2Y12

| siNeg | Seq1 | Seq2 | Seq3 | Seq4 |

P2Y12

SLUG

GADPH

AsPC-1

B

P2Y12 knockdown reduces SLUG expression

■ P2Y12   ■ SLUG

C

Cell viability relative to control

hTERT-HPNE   AsPC-1

Ticagrelor (10 µM)

***

D

BxPC-3

Cell viability relative to control

PSB 0739
Ticagrelor

Concentration (µM)

A

B

White blood cells

Platelets

Monocytes

Granulocytes

Red blood cells

Lymphocytes

C

A

PAAD
(num(T)=179; num(N)=171)

B

Overall Survival

Low P2RY12/POLR2L TPM
High P2RY12/POLR2L TPM
Logrank p=0.24
n(high)=89
n(low)=89

Percent survival

Days

C

PAAD

P2RY12 (T)
P2RY12 (N)
P2RY1 (T)
P2RY1 (N)
P2RY2 (T)
P2RY2 (N)
P2RX7 (T)
P2RX7 (N)

A

B

P2Y12 : ZEB1

C

P2Y12 : SLUG

Chapter 5:

General discussion, conclusion, limitations and future directions

5.1. General discussion and conclusion

5.1.1. Platelets, a direct downstream target of NETs in cancer-associated thrombosis.

The ability of cancer cells to directly activate platelets or coagulation is the generally accepted mechanism of cancer-associated thrombosis (CAT). Indeed, pancreatic cancer cells express and release tissue factor which can directly activate coagulation, resulting in the generation of thrombin which is a potent platelet agonist [51]. Recent studies by our lab and others have shown a novel mechanism of CAT mediated by neutrophil extracellular traps (NETs) [6,40,96]. Equipped with histones, neutrophil proteases and double-stranded DNA, NETs are capable of initiating thrombosis [48]. An overwhelming number of research articles have recently shown the production of NETs in cancer [256,398,399]. Whether production of NETs in cancer is associated with symptomatic thrombosis (e.g. venous thrombosis or pulmonary embolism) needs further investigation.

It is well-established that the process of NETs production (NETosis) in response to PMA takes 2-4 hours and reactive oxygen species (ROS) dependent [293]. However, a rapid (less than 30 min) and ROS-independent NETosis triggered by pancreatic cancer cells was recently demonstrated by our lab[6]. Moreover, our group showed NET-induced thrombus formation in blood under a venous shear condition and NET-activated platelet spreading under a static condition[6]. Surprisingly, a mechanistic study by Noubouossie et al., concluded that neutrophils-derived DNA and histones, but not intact NETs expelled from activated neutrophils, can activate coagulation [49]. Our experiments (Chapter 2), however, further demonstrated that intact NETs could actively induce platelet aggregation independent of plasma proteins, DNA and histones, which indicated that platelets are direct targets of NETs in thrombosis [259].

Studies have shown that NETs were able to induce procoagulant platelets, a subpopulation of activated platelets that externalise phosphatidylserine (PS) (Chapter 2) [259,400]. These platelets provide the negatively charged membrane for the assembly of the prothrombinase complex (Factor Xa and factor Va) in the presence of calcium, promoting downstream coagulation reactions. Chapter 2 and the recent mechanistic study by Noubouossie et al., together demonstrate that platelets are the main downstream target of NETs in thrombosis, and suggest that targeting the molecular drivers of procoagulant platelets represent a novel approach in the management of NET-associated thrombosis in cancer patients.

5.1.2. Platelets, a potential target for reducing gemcitabine resistance in pancreatic cancer

The prominent role of platelets in cancer metastasis is increasingly recognised [305]. Through secretion of TGFβ1 or direct contact with cancer cells, platelets can induce EMT in cancer cells [44]. EMT can promote metastasis and chemotherapy resistance in pancreatic cancer [52,338]. EMT is regulated via several transcriptional factors. For example, SLUG is a mesenchymal transcriptional factor that can reduce the expression of the cell adhesion protein, E-cadherin, thus inducing cell invasion and migration and its knockdown can improve gemcitabine sensitivity [187,401,402]. Our study in chapter 3, reported that platelets could significantly induce SLUG upregulation in pancreatic cancer cells and demonstrated for the first time that SLUG modulated the expression of CDD, an enzyme responsible for intracellular gemcitabine deactivation. Through the release of ADP/ATP or via direct cell to cell contact, we showed that platelets could significantly upregulate the expression levels of SLUG and CDD in pancreatic cancer cells [53]. Our data are corroborated by results of *in vivo* studies of pancreatic cancer mouse models which showed that downregulation of CDD improved gemcitabine sensitivity while its upregulation promoted gemcitabine resistance [336,343]. Additionally, analyses of publically available data on The Cancer Genome Atlas (TCGA) revealed that CDD and SLUG are significantly upregulated in pancreatic cancer patients and predict poor survival [379]. Whether platelets may be considered an additional therapeutic target to reduce SLUG and CDD expression and gemcitabine resistance in pancreatic cancer require further investigation.

5.1.3. Ticagrelor, an antiplatelet medication with anticancer activity.

For the first time, our research study showed that antiplatelet drug ticagrelor, a clinically available P2Y12 inhibitor, reduced cell proliferation and facilitated an apoptotic response in pancreatic cancer cells, via the AKT-signalling pathway. In addition to validating the presence and function of the

P2Y12 receptor on various pancreatic cancer cell lines, we investigated the signalling pathway downstream of the receptor. P2Y12 controls the expression level of phosphorylated EGFR, ZEB1 and SLUG. Inhibition of the P2Y12 receptor with ticagrelor or siRNA significantly reduced cell proliferation. Ticagrelor significantly attenuated EGF-mediated oncogenic signalling and synergised with EGFR inhibition, which suggests that P2Y12 is required for EGFR signalling. Ticagrelor synergised with several chemotherapeutic agents *in vitro* and reduced tumour growth in vivo when combined with gemcitabine.

Our results suggest an important role of P2Y12 in cancer and the data merits further exploration in regards to the role of P2Y12 in cell invasion and metastasis. P2Y12 is shown to regulate the expression of ZEB1. A recent study has demonstrated that ZEB1 is essential for pancreatic cancer metastasis [52]. Therefore, targeting P2Y12 in pancreatic cancer may reduce cancer metastasis, not only through reducing platelet-mediated EMT [53,221], but by directly affecting ZEB1 expression in cancer cells. P2Y12 was also required for EGFR signalling. Therefore, the molecular pathway that mediates P2Y12-EGFR crosstalk requires further investigation, especially in types of cancers where EGFR inhibition constitutes the main therapy (e.g. lung cancer).

It must be noted that the FDA preclinical data reveal several off-target effects for ticagrelor that might be relevant to cancer growth. For example, receptor binding assays indicated that ticagrelor has an affinity for phosphodiesterase 5 (PDE5) with IC50 for inhibition of 0.482 µM which is clinically achievable [385]. Encouragingly, Booth et al. showed that inhibition of PDE5 with sildenafil (2 µM) potentiated chemotherapy activity in pancreatic cancer cells [403]. Therefore, it is possible that the anticancer activity of ticagrelor is the result of a combined effect of P2Y12 and PDE5 inhibition.

Interestingly, Rachidi et al., have recently demonstrated that inhibition of platelet activity with aspirin and clopidogrel has no effect on primary tumour growth in the absence of T-cells [70]. Since the mouse model employed in chapter 4 (NOD/SCID) lacks T-cell activity, the effect of ticagrelor in combination with gemcitabine on cancer growth is likely explained by the mechanisms shown in our preceding *in vitro* studies (inhibition of AKT activity in cancer cells). However, the effect of ticagrelor on platelet interaction with immune cells other than T-cells or with cancer cells cannot be ruled out. Moreover, ticagrelor can directly modulate endothelial cells procoagulant activity by reducing tissue factor expression in response to TNF-α through proteasome-mediated pathway [404]. Procoagulant endothelial cells that express tissue factor can promote cancer growth [405]. Collectively, the experimental studies within this thesis demonstrated a novel effect of ticagrelor on cancer cells and proposed P2Y12 as a therapeutic target in pancreatic cancer.

5.2. Limitations

Our studies relied principally on blood isolated from healthy volunteers; however, it is not clear if NETs production in pancreatic cancer patients is associated with platelet hyperactivation, metastasis, or inadequate response to chemotherapy. The study utilised pancreatic cancer cell lines; however, it is not clear if platelet deposition in pancreatic cancer tissues is associated with EMT and high CDD expression in pancreatic cancer.

In **chapter 2**, we utilised cell-free NETs isolated from PMA-activated neutrophils to investigate NET-platelet interaction. This method was chosen for the following reasons; 1) we previously found that NETs produced from neutrophils activated by cancer-conditioned media and PMA similarly initiated thrombus formation under ex vivo shear flow [6], and 2) PMA produced a much more robust and consistent NETs. However, the differences between NETs produced by PMA and AsPC-1 conditioned media were not closely examined. PMA-induced NETs are ROS dependent, unlike pancreatic cancer cells-induced NETs which are ROS independent [6]. Therefore, there might be a distinct NETs related markers that can distinguish between NETs released during infection and cancer metastasis.

In **chapter 3 and 4,** the thesis examined the potential role of platelets in chemotherapy resistance and suggested ticagrelor as a novel therapeutic option in pancreatic cancer. However, several limitations have been observed. Firstly, platelet releasate was prepared using crossed-linked collagen-related peptide (xl-CRP)-activated platelets; however, pancreatic cancer-induced platelet activation is mainly thrombin-mediated, via the presence of tissue factor. It is possible that thrombin-activated platelets may produce a different range of growth factors compared to xl-CRP-activated platelets. It has been previously reported that there is a difference in platelet releasate composition between PAR1 and PAR4-stimulated platelets (thrombin receptors) [75,406]. However, there has been no study to demonstrate a difference in platelet releasate composition between thrombin and CRP-activated platelets, especially after long incubation (30 min). In our experiments, xl-CRP, a specific agonist of platelet GPVI receptor, was used to generate platelet releasate, to avoid potentially confounding effects occurring in downstream studies from contaminating thrombin in platelet releasate. Pancreatic cancer cells are known to express thrombin receptors (PAR1 and PAR4) [407-409], and the presence of thrombin could affect cell invasion and migration by modulating the cell division control protein 42 homolog (Cdc42) [409]. Secondly, the intracellular level of gemcitabine active metabolite inside pancreatic cancer cells incubated with platelets was not examined. The expression of CDD in pancreatic cancer cells can reflect gemcitabine concentration inside the cells, and subsequently, gemcitabine anticancer activity [343]. Therefore, the increased expression of CDD in

pancreatic cancer cells incubated with platelets was used to evaluate the potential effect of platelets on gemcitabine resistance. Thirdly, in **chapter 4**, the effect of P2Y12 inhibition on cancer cell proliferation was examined using genetic and pharmacological inhibitors *in vitro*. However, *in vivo*, only pharmacological inhibitor (ticagrelor) was used. A subcutaneous xenograft mouse model of P2Y12 knockout cancer cells could have clarified whether ticagrelor effect on cancer growth is mediated via inhibition of cancer or platelet-P2Y12 or via an off-target effect (e.g. inhibition of PDE5 or tissue factor expression). Fourthly, due to time and financial constraints, the effects of ticagrelor on gemcitabine activity in immunocompetent and spontaneous pancreatic cancer mouse models were not investigated. These models can clarify the role of platelets on primary cancer growth as well as metastasis, and also the role of platelets on the tumour-mediated immune response.

5.3. Future directions

Our growing understanding of the role of NETs in malignancy and associated thrombosis suggests that targeting NETs may be a viable therapeutic strategy [252]. For example, DNase, increasingly being considered for clinical intervention in cancer, can be employed to dismantle the already formed NETs [252]. However, the release of free DNA from dismantled NETs can trigger danger signals in the host through interaction with toll-like receptor 9 which is expressed in several types of immune cells [410]. Furthermore, NETs fragments can activate cytokines production from macrophages leading to T-cells activation [411]. NETs and NETs fragments can also induce platelet activation and secretion of cytokines, promoting thrombosis and inflammation [6,259]. Most recently, a study by Rachidi et al showed that activated platelets are capable of constraining the CD4+ and CD8+ T cell functions, specifically through their ability to generate functional TGFβ1 [70]. Thus, targeting the already formed NETs with DNase requires careful consideration as there may be side effects due to the complex nature of NETs in tumour immunity. More basic research and *in vivo* preclinical studies are needed to examine different mechanisms of NETs generation in cancer (e.g. autophagy [40]) and whether targeting NETs is more useful than antiplatelet strategies since tumour-educated/activated platelets are involved in NET generation, moreover, targeting platelets directly can lower the level of TGFβ systemically, as well as reduce the risk of thrombosis and metastasis

[70,252,305].

Our body of work indicated a role of platelet in chemotherapy resistance and proposed the antiplatelet drug, ticagrelor as a treatment option in pancreatic cancer. To further expand on the results presented here, it would be informative to investigate the infiltration of platelets into pancreatic cancer tissues. Tumour vasculature should also be examined for dysfunction and leakiness as platelets are integral in neoangiogenesis and maintaining blood vessel integrity [7].

Recently, it has been shown that platelets are the main source of TGFβ1 in the circulation and the tumour microenvironment in a colon cancer mouse model [70]. Platelet secretes active TGFβ1 and also express Glycoprotein-A Repetitions Predominant protein (GARP) which can activate latent TGFβ1 in the circulation [70]. Therefore, it is crucial to characterise the role of platelet-derived TGFβ1 as well as ADP/ATP in pancreatic cancer growth and metastasis in a more robust genetically modified mouse models. For example, genetic ablation of platelet P2Y12, TGFβ1 or GARP in the KRAS$^{WT/G12D}$/TP53$^{WT/R172H}$/Pdx1-Cre$^{+/+}$ (KPC) pancreatic cancer mouse model [412] could clarify the role of these specific platelet receptors on cancer growth, metastasis, specific signalling pathways in cancer cells (e.g. PI3K/AKT and SMAD) and the composition of the tumour microenvironment (e.g. platelets and immune cells infiltration). More recently, it has been shown that pancreatic cancer microbiome suppresses tumour immune response and thus promote oncogenesis [413]. Platelets can modulate innate and adaptive immunity [70,414,415], therefore, the interaction of platelet with the microbiome also deserves further investigation.

The processes of blood coagulation and platelet hyperactivation in solid tumour patients are highly interconnected; however, their impact on cancer progression and patients' survival need to be carefully dissected. Hypercoagulability in pancreatic cancer patients is considered to be caused mainly by high tissue factor expression by cancer cells, which can lead to symptomatic venous thrombosis that is routinely treated with anticoagulants (e.g. low molecular weight heparin) [51]. However, several prospective clinical trials failed to show a survival benefit of anticoagulant use in pancreatic cancer patients despite a reduction in thrombotic complications [372,373,416-418]. This may be explained by the lack of the anticoagulant's effect on NETs-mediated platelet activation and platelet-mediated survival signals and EMT in cancer cells (chapter 2-3) [6,53,259]. Encouragingly, a recent meta-analysis has suggested that aspirin use (antiplatelet) can reduce the risk of pancreatic cancer via slowing the tumour development rather than preventing oncogenesis [230]. Finally, it must be mentioned that approaches to targeting platelets in cancer may interfere with platelet physiological function and lead to major bleeding complications. However, the recent FDA approval of an antibody-based ticagrelor reversal agent [419] may encourage ticagrelor use in combination with chemotherapy in solid tumour's patients with a high risk of thrombosis in a clinical trial.

Appendices

Appendix I: Secondary publications

Secondary publication:

The following publication is complementary to the candidacy objective as it formed the basis for chapter two. The author (Elaskalani O) contribution to this publication included:

- Conception and design (in collaboration with the other author and co-author)
- Performing several experiments:

  - Preparation of cancer and primary cells and conditioned media and related optimisation
  - Isolation of platelets, and preparation of cancer or agonist-activated platelets
  - Performing cancer-induced platelet aggregation experiments and characterisation of the related mechanism using several inhibitors and flow cytometry

- Discussion of results, and final revision of the manuscript before submission

Publication 6:

Abdol Razak NB**, Elaskalani O,** Metharom P. *Pancreatic Cancer-Induced Neutrophils Extracellular Traps; A Potential Contributor to Cancer-Associated Thrombosis.* Cell Commun Signal. Int J Mol Sci. 217;18(3

Abdol Razak NB**, Elaskalani O,** Metharom P. *Pancreatic Cancer-Induced Neutrophils Extracellular Traps; A Potential Contributor to Cancer-Associated Thrombosis.* Cell Commun Signal. Int J Mol Sci. 217;18(3

Article

Pancreatic Cancer-Induced Neutrophil Extracellular Traps: A Potential Contributor to Cancer-Associated Thrombosis

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Abstract: Pancreatic cancer (PaCa) is a highly metastatic cancer, and patients are at high risk of developing venous thromboembolism (VTE). Neutrophil extracellular traps (NETs) have been associated with cancer metastasis and cancer-associated thrombosis, but the ability of cancer to stimulate NET release is not known. The release of NETs has been shown to be a slow process and requires reactive oxygen species (ROS) production. Studies suggest that activated platelets are important mediators in the release. Here, we show that PaCa cells can stimulate the rapid release of NETs, independently of ROS production. We further assessed the role of platelets in PaCa-induced NETs and observed a trend of increased the NET release by PaCa-primed platelets. Additionally, NETs promoted thrombus formation under venous shear stress ex vivo. Taken together, our results suggest that PaCa-induced NETs can contribute to the high risk of venous thromboembolism development in PaCa patients, and reveal NETs as a potential therapeutic target.

1. Introduction

Neutrophil extracellular traps (NETs) are web-like structures composed of DNA and proteins that are expelled from activated polymorphonuclear neutrophils (PMNs) in response to bacterial or inflammatory stimuli [1,2]. They were first discovered to serve a protective role in immune defence as they can entrap pathogens, and directly kill bacteria via NET-bound antimicrobial proteins [2]. However, studies have revealed that NETs are not exclusive to bacterial infection, but are also involved in inflammatory and autoimmune diseases such as systemic lupus erythematosus, preeclampsia, rheumatoid arthritis, cystic fibrosis and thrombosis [3–6].

More recently, several links between NETs and cancer have been made. The first study investigating NETs and their association with cancer established that PMNs isolated from tumour-bearing mice are sensitised to form NETs [7]. Furthermore, spontaneous thrombi were found in association with NETs in tumour-bearing mice, suggesting a possible cause of cancer-associated thrombosis [7]. NETs have also been implicated in cancer progression, where cancer cells were found entrapped in the vasculature by NETs, which correlated with increased tumour metastases [8]. Not only have NETs been found in tumour-bearing mice [7,9], but also in tumour samples from cancer patients [9–11]. Despite the association between NETs and cancer, whether or not cancer cells can directly stimulate the release of NETs has not been investigated.

The molecular mechanisms of NET generation are poorly understood; however, several studies suggest that neutrophil–platelet interactions are important. Activated platelets in inflammation or infection play a vital role in the release of NETs, either by mediating interactions with neutrophils [12–15] or by soluble mediators released from platelets [16]. Platelets are also known to become activated and aggregate in cancers and are usually present in high numbers in cancer patients [17]. Thus platelets may also have a major role in NET release in cancer.

Activated platelets in contact with tumour cells can also recruit neutrophils by secreting chemokines (C-X-C motif) ligand (CXCL) 2 and CXCL5 chemokines [18]. Platelets are crucial in recruiting neutrophils in vivo as depleting platelets resulted in complete inhibition of granulocyte recruitment [18]. Thus, there is evidence to support platelets and their role in recruiting neutrophils to tumour sites; however little is known about platelets and their role in activating neutrophils in cancers [19]. Similarly, platelets have been demonstrated to interact with neutrophils and induce NET generation in bacterial infection, though in the presence of cancer, the importance of platelets in NET release has not been studied.

Therefore, given the high incidence and morbidity due to thrombotic diseases and metastasis in pancreatic cancer patients [20,21], this study aimed to investigate the interactions between neutrophils, platelets and pancreatic cancer cells and their potential contribution to the hypercoagulable state that is present with cancer-associated thrombosis. The impact of platelets and cancer cells on NETs generated, as well as the consequences of NETs on platelet activity, were investigated. We report that pancreatic cancer cells can stimulate the release of NETs, and that this is not dependent on neutrophil–cancer cell interactions as the protein fraction of cancer cell-conditioned media also induced NET release. Furthermore, we also show that NETs cause platelet activation and spreading, as well as thrombus formation under venous flow conditions. This improved understanding of these interactions may help explain contributors to the high risk of thrombosis in pancreatic cancer, and NETs or unique signalling events between cells that promote the NET release may serve as novel therapeutical targets.

2. Results

2.1. Pancreatic Cancer Cell Line AsPC-1 Stimulates NET Generation

To determine whether cancer cells can directly stimulate the release of NETs, pancreatic cancer cell and PMN co-cultures were performed. The metastatic pancreatic cancer cell line AsPC-1 was incubated with PMNs for 3 h, and NET release was quantified. To quantify NETs, extracellular DNA (exDNA) was stained with Sytox Green, a cell-impermeable fluorescent dye specific for DNA, and fluorescence was measured in a plate reader. NETs were quantified at 30 min, and at 3 h, as shown in Figure 1. AsPC-1 cells efficiently stimulated the release of NETs after incubation with human PMNs. In addition, NETs were induced by AsPC-1 in a rapid process as a significant 2-fold increase in exDNA release was observed at 30 min. Positive control, phorbyl myristate acetate (PMA)-stimulated PMNs required 3 h for significant NET release to occur. AsPC-1 cells were also incubated without PMNs to determine if AsPC-1 cells produced any exDNA. Sytox Green staining of AsPC-1 cells confirmed that the increase in exDNA was due to PMNs, as AsPC-1 cells did not carry any exDNA.

2.2. AsPC-1-Induced NETs Are Not Contact-Dependent

To determine whether AsPC-1-induced NETs were mediated through cell–cell contact, or via soluble mediators from AsPC-1, NETs generated were examined after incubation of PMNs with conditioned medium from AsPC-1. Similarly, AsPC-1-conditioned medium (AsPC-1 CM) also resulted in a rapid 2-fold increase in exDNA compared to unstimulated control at 30 min, with no further increase at 3 h (Figure 2). To confirm that the AsPC-1 CM induced NETs phenomenon is specific to cancer cells, the conditioned medium of primary mesenchymal stem cells (MSC) served as a human non-malignant control. Indeed, MSC-conditioned medium did not cause PMNs to release NETs (Figure 2). Fluorescent microscopy confirmed the release of NETs, as a stringy-like expulsion of exDNA

can be visualised in AsPC-1 CM stimulated PMNs (Figure 3). At 3 h, PMA-stimulated PMNs show a greater degree of web-like NETs due to PMA being a potent NET activator (Figure 3). To determine if other NET components are present on AsPC-1 CM-induced NETs, neutrophil elastase was also stained. Neutrophil elastase can also be seen extruded on NETs after incubation of PMN with AsPC-1 CM (Figure 3). AsPC-1 CM stimulated PMNs were pre-treated with DNAse I, which resulted in significant decrease in exDNA fluorescence (Figure A1), confirming the specificity of Sytox Green for exDNA.
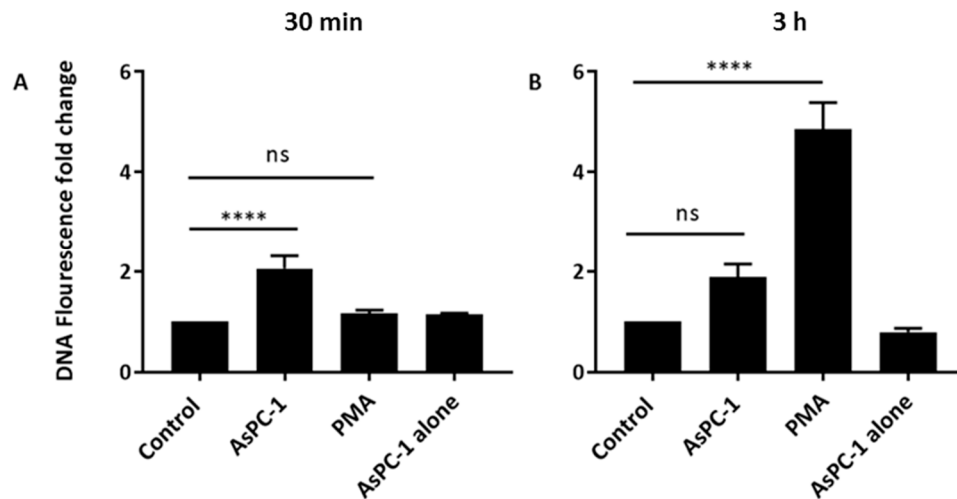
Figure 1. AsPC-1 stimulates the rapid release of neutrophil extracellular traps (NETs) from human neutrophils. Human neutrophils were incubated with AsPC-1 cells at a neutrophil to AsPC-1 ratio of 1:2 and NETs were quantified using Sytox Green (5 μM) in a fluorescence plate reader at (**A**) 30 min and (**B**) 3 h. AsPC-1 induced rapid NET release at 30 min while phorbyl myristate acetate (PMA)-stimulated neutrophils (positive control) required 3 h. Control: unstimulated neutrophils. (n = 12, **** $p < 0.0001$ and ns = non-significant); One-way ANOVA followed by Bonferroni post-test. Data presented as mean ± SEM.
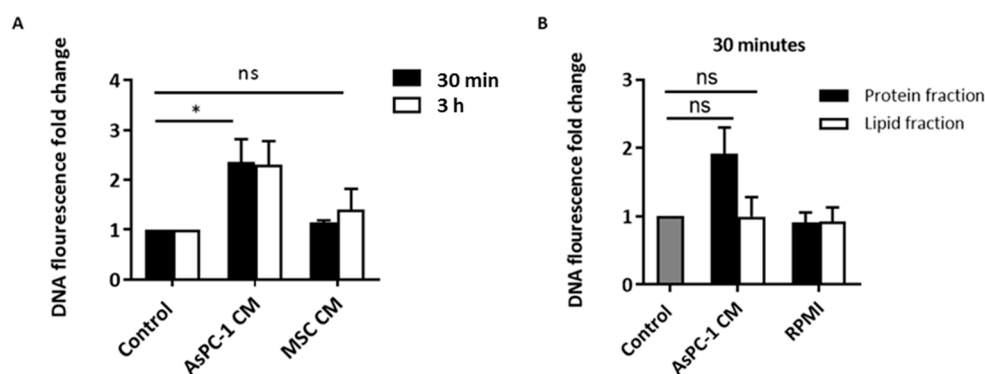
Figure 2. AsPC-1 stimulates NET release via soluble protein mediators. (**A**) The conditioned media (CM) of AsPC-1 cells stimulated the release of NETs. Mesenchymal stem cell (MSC) CM, which served as a control for human non-malignant cells, did not stimulate NET release (n = 3, * $p < 0.01$ and ns = non-significant.); (**B**) AsPC-1 CM was separated into lipid and protein fractions, and each was incubated with neutrophils for 30 min. The protein fraction of AsPC-1 CM stimulated NET release while the lipid fraction did not. Media RPMI fractions served as control (n = 3). Control: unstimulated neutrophils. One-way ANOVA followed by Bonferroni post-test. Data presented as mean ± SEM.
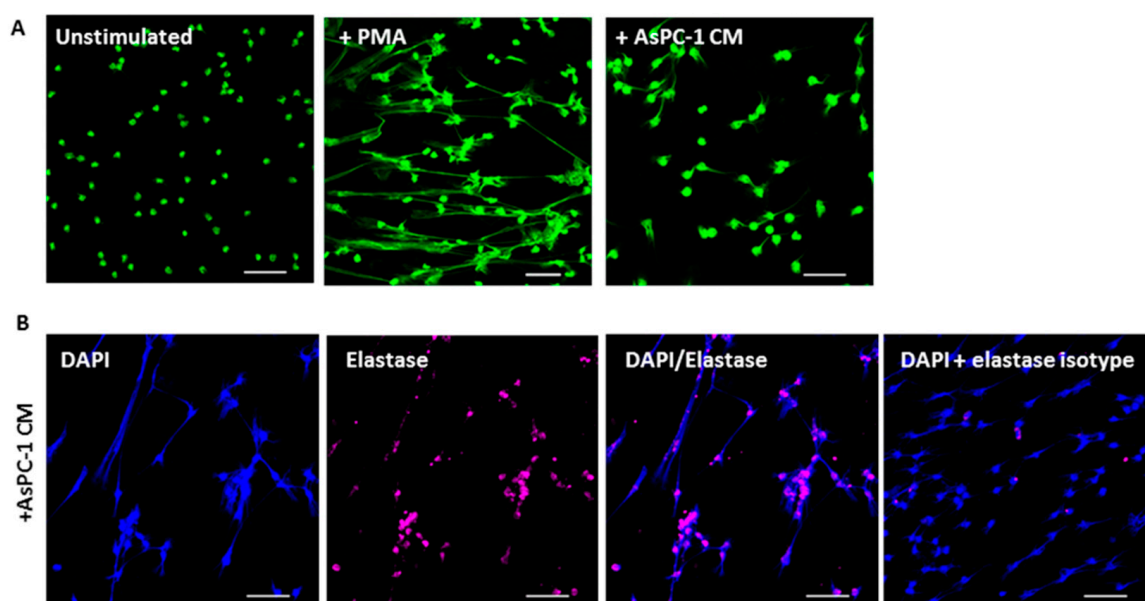
Figure 3. Confocal microscopy images of NETs induced by AsPC-1 conditioned media. (**A**) Neutrophils were incubated on poly-l-lysine coated glass slides and left either unstimulated, or were stimulated with PMA for 3 h, or AsPC-1 CM for 30 min. Neutrophils were fixed with paraformaldehyde and stained with Sytox (green) to visualise extracellular DNA. Images representative of three independent experiments; (**B**) AsPC-1 stimulated neutrophils were also stained with anti-elastase (pink) to visualise elastase bound on NETs. DAPI (blue) was also used as an alternative to visualise DNA. Elastase staining was above isotype control. Images representative of two independent experiments. Scale bar = 50 μm.

Furthermore, to determine the nature of the NET-inducing factor/s, AsPC-1 CM was separated into protein and lipid fractions. As the optimal time for NET release induced by AsPC-1 was observed at 30 min, the lipid and protein fractions of AsPC-1 conditioned media were incubated with PMN for 30 min before measuring NET release. Interestingly, the protein fraction of conditioned media resulted in almost 2-fold NET release, while the lipid fraction-stimulated PMNs did not (Figure 2), suggesting that AsPC-1-induced NETs are mediated by soluble proteins and do not rely on direct contact.

2.3. AsPC-1-Induced NETs Do Not Require Reactive Oxygen Species Production

Although the mechanism of the generation of NETs is poorly understood, several studies have established that the generation of reactive oxygen species (ROS) is required for the release of NETs [1,22,23]. To examine whether ROS production precedes NET generation in PMNs stimulated by AsPC-1, levels of ROS were measured during PMN incubation with AsPC-1 CM using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) indicator dye. Surprisingly, ROS were not detectable in PMNs stimulated with AsPC-1 CM during the 30-min incubation period (Figure 4), the time at which significant AsPC1-induced NETs were observed, while PMA caused a significant accumulation in ROS at 3 h (Figure 4), suggesting that ROS generation is not required for AsPC-1-induced NET. To confirm the ROS-independent release of NETs, a ROS inhibitor, diphenyleneiodonium (DPI), was used to pre-treat PMNs before incubation with AsPC-1 CM. Indeed, AsPC-1 NET release was not affected as exDNA remained unchanged after pre-treatment with DPI (Figure 4). On the other hand, NETs, as well as ROS generation, were significantly reduced to baseline levels in PMA-stimulated PMNs that were pre-treated with DPI (Figure 4).
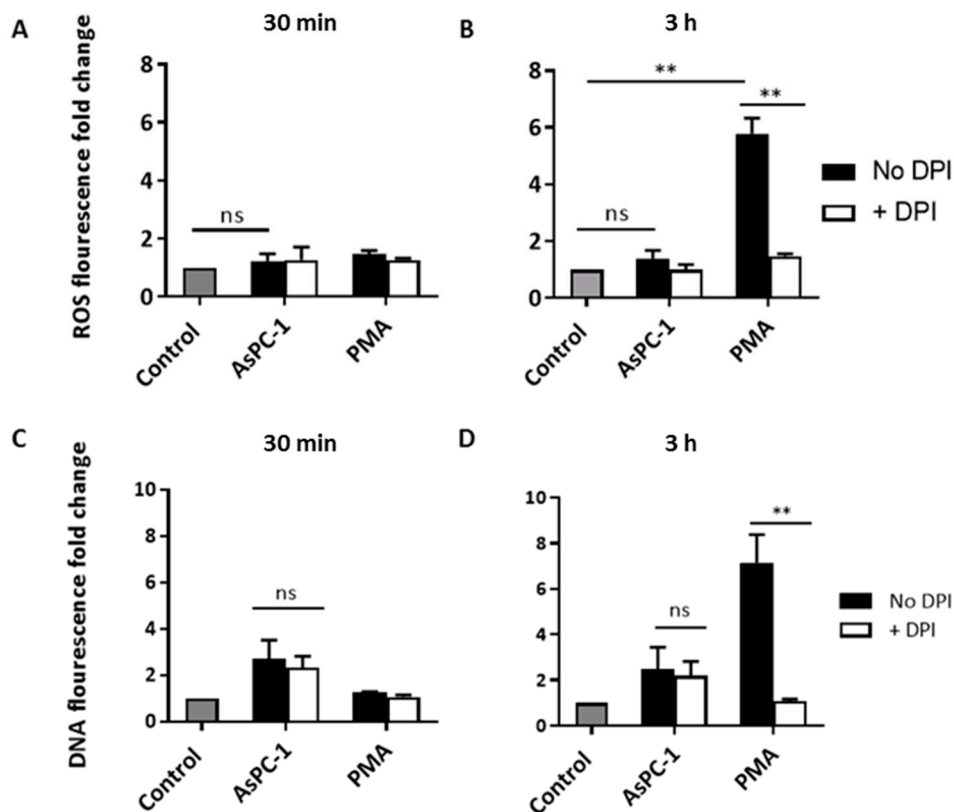
Figure 4. AsPC-1-induced-NET generation is independent of reactive oxygen species production. (**A**,**B**) Quantification of reactive oxygen species (ROS) generated during incubation with AsPC-1 CM and PMA at (**A**) 30 min and (**B**) 3 h using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescent dye. ROS were not significantly generated in AsPC-1 CM-stimulated neutrophils at either time points; (**B**) PMA-stimulated neutrophils had significant ROS generated at 3 h, this was abolished with the addition of non-specific ROS inhibitor diphenyleneiodonium (DPI); (**C**,**D**) The effect of DPI on NET release in AsPC-1 or PMA-induced NETs; (**C**) DPI did not reduce AsPC-1-induced NETs; (**D**) NETs were significantly reduced in PMA-stimulated neutrophils by DPI. $n = 3$, ** $p < 0.001$, ns = non-significant. One-way ANOVA followed by Bonferroni post-test. Data presented as mean ± SEM.

2.4. Platelets Primed by AsPC-1 Stimulate NET Release

To determine whether platelets play a role in AsPC-1-induced NETs and could exacerbate the production of NETs, platelets were added to the PMN and AsPC-1 co-culture, and NET release was assessed. Platelets did not exacerbate NETs generated by AsPC-1, and unstimulated platelets did not cause NET release which was as expected in the context of normal physiological setting (Figure 5).

Since the addition of platelets to the co-culture did not result in a further increase in NETs, we sought to determine if platelets needed pre-activation or priming with AsPC-1 before co-culturing with PMNs to have any effect on NETs formed. Hence, platelets were pre-incubated with AsPC-1 cells for 4 h before incubating with PMNs. Interestingly, platelets that were primed with AsPC-1 cells caused PMNs to release NETs compared to unstimulated platelets (Figure 5). Furthermore, it was a rapid process that occurred in 30 min (Figure 5). On the other hand, collagen-stimulated platelets did not stimulate increased NET release (Figure 5).
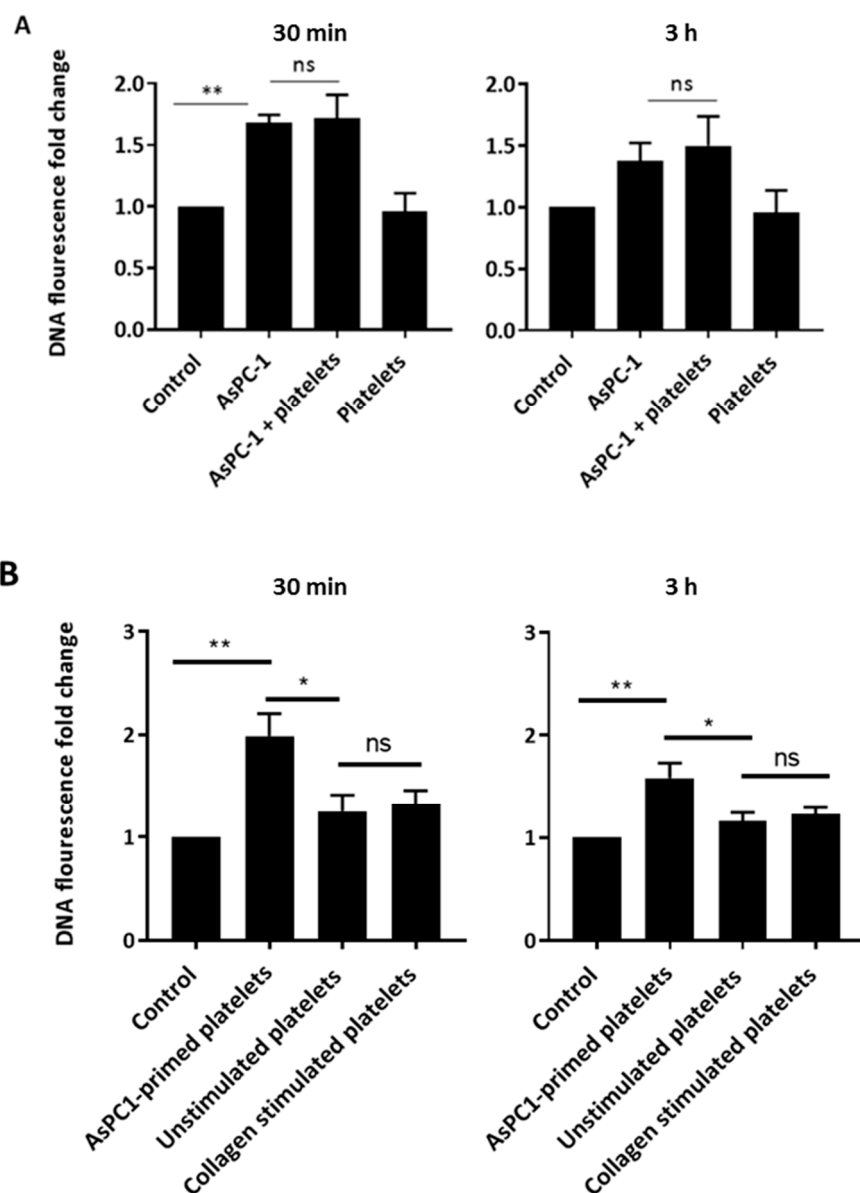
Figure 5. AsPC-1-primed platelets, but not washed platelets, stimulate rapid release of NETs. (**A**) Platelets (1×10^8/mL) were incubated with neutrophils and AsPC-1 cells. Platelets had no effect on AsPC-1-induced NETS; (**B**) the effect of AsPC-1-primed platelets on NET release. Platelets were primed with AsPC-1 cells by incubation with AsPC-1 for 4 h. The supernatant was removed and centrifuged to pellet platelets which were then resuspended and incubated with neutrophils. Primed platelets caused rapid NET release in 30 min. ($n = 4$, * $p < 0.01$, ** $p < 0.001$ and ns = non-significant. One-way ANOVA followed by Bonferroni post-hoc). Control: unstimulated neutrophils. Data expressed as mean ± SEM.

2.5. NETs Promote Static Platelet Adhesion and Activation

NETs have been shown to play a role in promoting thrombosis through studies in animal models with venous thrombosis and through platelet and whole blood perfusion assays on NETs [24–26]. However, whether NETs are able to directly promote platelet adhesion and activate platelets in static conditions has not been shown. To examine if NETs can directly promote the adhesion and activation of platelets, NETs were isolated from PMA-stimulated PMNs and coated on glass slides for platelet spreading assays. Platelets were then placed on a NET-coated slide and incubated for 1 h before staining the F-actin component of platelets with phalloidin-488, to assess the degree of adhesion

and spreading of individual platelets. NETs caused significant adhesion and spreading of platelets as visualised by large spreading of F-actin of platelets across the NET-coated surface, compared to denatured-bovine serum albumin (BSA) coated surface, which served as negative control (Figure 6). Quantification of surface area coverage of platelet spreading also showed a significant increase in spreading on NET-coated slides (Figure 6).
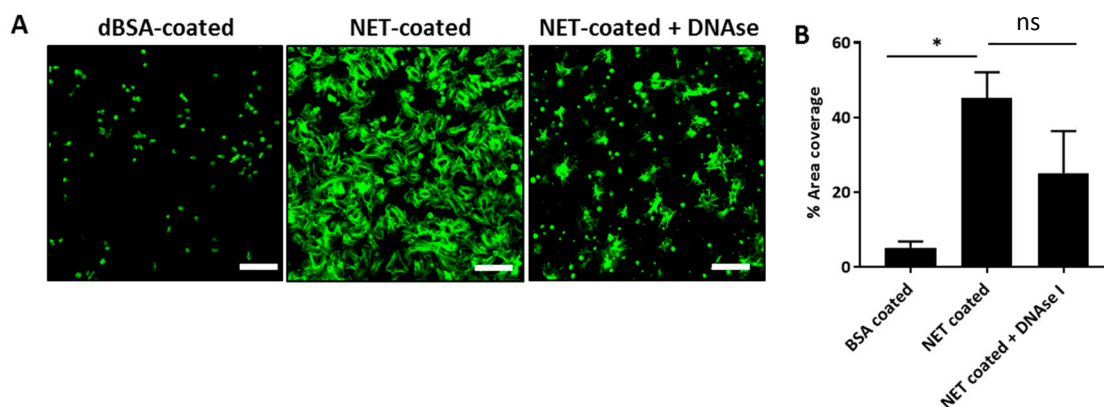
Figure 6. NETs promote static platelet adhesion and activation. (**A**) NETs were isolated from PMA-stimulated neutrophils before coated on glass slides. Washed platelets (2×10^7/mL) were incubated with NETs, and platelet adhesion and spreading were assessed using confocal microscopy. Denatured bovine serum albumin (dBSA)-coated slides served as negative control. NETs caused platelets to adhere and spread. The pre-treatment with deoxyribonuclease I (DNAse I) did not completely abrogate adhesion and spreading. Images representative of three independent experiments. Scale bar = 20 μm; (**B**) quantification of percentage area coverage of platelet adhesion and spreading using Image J analysis software. $n = 3$,* $p < 0.05$ and ns = non-significant. One-way ANOVA followed by Bonferroni post-hoc. Data presented as mean $\pm$ SEM.

To further investigate which NET component was responsible for platelet adhesion and spreading, NET-coated slides were pre-treated with DNAse I before incubation with platelets. Although to a much lesser extent, platelets were still visualised adhering and spreading on NETs that were pre-treated with DNAse I (Figure 6). Analysis on quantification also found no significant decrease in surface area coverage with DNAse I (Figure 6), suggesting that the protein component in NETs are also capable of promoting platelet adhesion, activation and shape-change.

2.6. NETs Are a Scaffold for Dynamic Platelet Adhesion and Thrombus Formation

We next investigated the ability of platelets to adhere and activate on NETs and assessed thrombus formation under dynamic conditions. Since venous thrombosis is a common complication in pancreatic cancer patients, we conducted a whole blood perfusion assay over an NET-coated biochip with a venous shear stress of 10 dyne/cm^2. Whole blood labelled with fluorescent dye DiOC6(3), was perfused over NET-coated channels for 10 min. Platelets were visualised as adhering, and thrombi were formed on NETs (Figure 7, Video S1, available online: https://www.dropbox.com/sh/qlmgmxptzqf4klj/AAA21i7NSjosAG9_zRDPSFxda?dl=0). A greater degree of platelet adhesion and thrombi were formed in collagen-coated channels (positive control), while denatured BSA-coated channels had negligible platelet adhesion (Figure 7; Videos S2 and S3 online). In addition, a significant increase in surface area coverage by platelets was found in NET-coated channels compared to BSA-coated channels (Figure 7). *Z*-stack images also showed 3D visualisation of thrombi that had formed on NETs, similar to that present in collagen-coated channels, while thrombi were absent in BSA-coated channels (Figure 7). Therefore, these data suggest that NETs are able to trap and activate platelets under venous shear stress, and consequently promote thrombus formation.
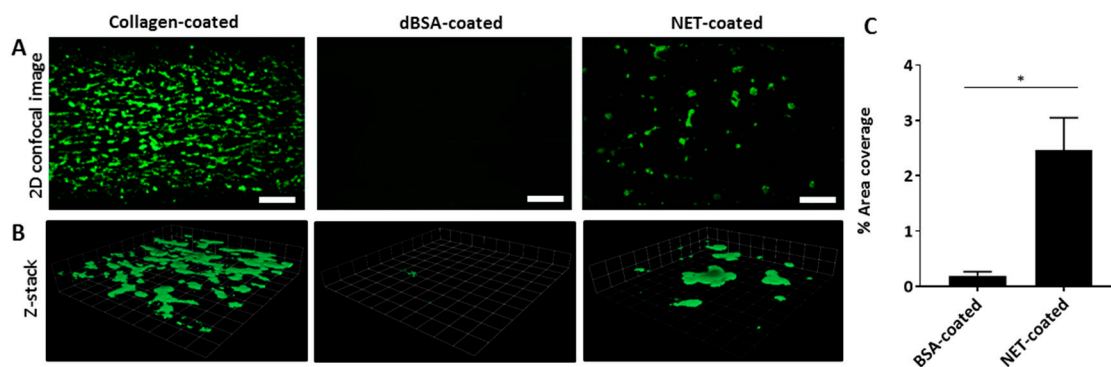
Figure 7. NETs entrap and activate platelets and promote thrombus formation. (**A**) 2D confocal images of platelet adhesion and thrombus formation in collagen-, dBSA-, and NET-coated channels after perfusing DiO6C(3) fluorescently-labelled whole blood for 10 min at 10 dyne/cm^2. Platelet adhesion and thrombi are visualised in NET-coated channels. Collagen- and dBSA-coated channels served as positive and negative controls, respectively. Scale bar = 90 μm; (**B**) Z-stack images confirm the presence of thrombi on NETs, which are similar to those in collagen-coated channels; (**C**) Quantification of surface area coverage of platelet adhesion showed a significant increase in NET-coated channels. *n* = 3, * *p* < 0.05 unpaired *t*-test.

3. Discussion

Pancreatic cancer (PaCa) is associated with high incidence of venous thromboembolism (VTE) [27–29], which suggests a close interplay between PaCa cells and platelets, the latter being a key player in haemostasis and thrombosis [30,31]. VTE in cancer is associated with a low survival rate [32]. The high risk of VTE in PaCa patients is mainly associated with a generation of an intrinsic hypercoagulable state [33]. Factors such as tumour-cell induced platelet aggregation, and increased expression of procoagulant factors including tissue factor and thrombin, promote a prothrombotic state which ultimately contributes to the development of VTE. In addition, platelet-neutrophil interactions can coordinate VTE and various pathological conditions [26,34]. In the context of bacterial infection and inflammation, activated platelets can induce NETs [14,35]. It has been known for years that platelet activation is amplified in cancer [36]. In this study, we examined the interplay between platelets, neutrophils and pancreatic cancer cells with a main focus on their impact on NET generation.

3.1. AsPC-1 Cells Can Induce NET Release

We first examined if pancreatic cancer cells can induce NET release. Here, we show that PaCa AsPC-1 cells can stimulate PMNs to release NETs through direct contact and AsPC-1-derived soluble proteins. Furthermore, we demonstrate that AsPC-1-induced NETs is a cancer specific phenomenon that occurs rapidly and independently of ROS. We have previously shown that AsPC-1 cells in vitro exhibit features that promote coagulation, such as tissue factor expression, and tissue factor-dependent platelet aggregation (Figure A2), which can subsequently contribute to the development of VTE. As NETs have been shown to promote thrombosis, in this study and by others, we propose that NETs that are formed in response to PaCa cells may serve as an additional and novel contributor to the development of VTE.

The release of NETs has been well-established to be a slow process requiring at least 3 to 4 h, however few studies have reported a quick release of NETs occurring within the first hour [14,37]. Similarly, the generation of ROS is thought to be indispensable for forming NETs; however, a few studies have also reported ROS-independent release of NETs [37–39]. The majority of NET-inducers studied thus far, stimulate a NET-releasing mechanism that requires at least 3 h of stimulation and is dependent on the generation of ROS [1,22,23]. Herein, we describe a novel mechanism induced by AsPC-1, that is both rapid and ROS-independent. To date, one other study has described a NET

mechanism that exhibited both rapid and ROS-independent mechanism of NET release, which was induced by the Gram-positive bacteria *Staphylococcus aureus* [37]. Furthermore, Boone, et al. [40] have recently studied NETs in PaCa in vivo, and reported that NETs released in PaCa are mediated through autophagy pathways. Specifically, the receptor for advanced glycation end products (RAGE) which mediates autophagy in PaCa was found to be necessary for NET release, as neutrophils from RAGE knockout mice were less prone to release NETs. Thus, although we found that PaCa-induced NETs did not depend on ROS generation, they may alternatively be dependent on autophagy which will need to be investigated in future studies.

In addition to an early and ROS-independent release of NETs, we have demonstrated that AsPC-1-induced NETs can be mediated by AsPC-1-derived soluble proteins. Potential mediators may be inflammatory cytokines that are known to be increased in the serum of PaCa patients, and are also released from PaCa cells such as interleukin (IL)-1β, IL-6, IL-8, tumour necrosis factor-alpha (TNF-α), and transforming growth factor-β (TGF-β) [41]. The inflammatory cytokines, TNF-α, IL-8 and IL-1β, have been previously shown to stimulate NET release [42]. Thus, these cytokines may collectively contribute to the activation of PMN and release of NETs; however, further studies are required to delineate the specific mediators. Other cytokines, particularly TGF-β may have a role in polarising neutrophils to a pro-tumourigenic phenotype [43]. Recent studies suggest that neutrophils display plasticity in the tumour microenvironment and can polarise from an N1 antitumour phenotype to an N2 protumour phenotype by TGF-β [44]. Whether or not NET generation is a function of N2 protumour, and not N1 anti-tumour neutrophils, remains to be elucidated.

3.2. AsPC-1 Primed Platelets Favour NET Release Compared to Unstimulated Platelets

Neutrophil and platelet interactions are known to exist and play a role in infection and VTE [24]. Likewise, several studies have shown a major role of platelets in mediating interactions or releasing mediators to stimulate NET release [14,15,35,45]. Most studies were carried out in the context of bacterial infection. One study reported that lipopolysaccharide (LPS)-activated platelets were found to mediate NETs, as inhibition of the LPS receptor significantly reduced platelet activation and NET release [14]. Similarly, we observed a trend of increased NET generation when stimulated by platelets that were primed with AsPC-1 cells (or AsPC-1-conditioned media, data not shown), compared to unstimulated platelets. This was not observed when resting platelets were added to the PMN, suggesting that the platelets required priming or pre-activation with AsPC-1 before they could have any effect on PMN activation and NET release. As we have shown that AsPC-1-derived soluble proteins can stimulate NET release, this suggests the potential role of exosomes (small vesicles containing intracellular proteins and RNA) in mediating AsPC-1-induced NETs. Platelets can become 'educated' by tumour cells through the transfer of tumour-associated molecules such as proteins or RNA to platelets [46–48]. Subsequently, we speculate that upon encounter with PMNs, tumour-educated platelets may either transfer their contents to PMNs, or directly interact with PMNs to induce NET release. Further studies are needed to confirm our observations, and to elucidate what factors from AsPC-1-primed platelets are involved in NET generation. Future investigations may include: (1) characterising of exosomes isolated from AsPC-1; (2) transcriptomic and proteomic profiling of cancer-primed platelets; and (3) assessing the NET-stimulating capability of platelets isolated from tumour-bearing mice.

On the other hand, NETs were not expected to be generated by unstimulated platelets as neutrophil and platelets are constantly in contact within the systemic circulation, and spontaneous NET generation would be undesirable, as it could potentially damage endothelial cells [49] and promote unnecessary coagulation. Similarly, as collagen and platelets are known to play a role in wound healing [50], excessive generation of NETs at a wound site due to collagen-activated platelets is also undesirable, as it may hinder the wound healing process. However, Maugeri, et al. [51] showed that collagen-activated platelets were able to induce NET release. The varying results may be due to different NET quantification methods or number of neutrophils used for stimulation.

Maugeri et al. [51] measured soluble DNA to quantify NETs which may have been a more sensitive detection method than the one used here, and the authors also used 10-fold more neutrophils than in our study. NETs generated at wound sites may cause a negative impact and become obstructive as new matrix is deposited. Indeed, a study investigating the role of NETs in a diabetic mouse model, known to have impaired wound healing, found excessive NETs being formed compared to normal mice. Furthermore, degradation of NETs in these diabetic mouse models led to improvements in wound healing [52]. Thus, the generation of NETs by activated platelets may be a regulated process which is favoured in severe conditions where the immune response is compromised, such as in cancer or bacterial infection.

3.3. NETs Activate Platelet Dynamics

We have shown for the first time that NETs can directly promote the adhesion, activation and shape change of platelets in static conditions, which corroborates previous findings that NETs are a scaffold for platelet adhesion and aggregation under dynamic conditions [24,25]. The adhesion and activation of platelets was not entirely due to the DNA component of NETs, as the pre-treatment of NETs with DNAse I did not completely abolish platelet adhesion and spreading. This suggests that the protein component of NETs also contributes to platelet adhesion and activation. Histones are the most abundant protein found on NETs [53], and therefore could possibly exert the greatest impact on platelet activation amongst other NET proteins. Extracellular histones have been shown to activate platelets, which in turn promote the generation of plasma thrombin and result in a procoagulant phenotype [54]. Cathepsin G is also present on NETs and has been reported to promote platelet aggregation in vitro [55]. The possible role of these proteins in promoting platelet activation will need to be confirmed by re-assessing platelet adhesion, activation and spreading under the presence of cathepsin G and histone inhibitors, or inhibitors that prevent the mechanism of histone-mediated platelet activation.

We further extended our findings of platelet activation by NETs, and confirmed previous reports by showing that thrombi can form on NETs under dynamic conditions. Our whole blood perfusion assay over NETs was carried out under venous shear conditions, which implicates NETs as a scaffold that can trap platelets and promote thrombus formation within the venous circulation, further corroborating NETs as a mechanism that can contribute to VTE development. We did not investigate the effects of DNAse I on the formation of thrombi under dynamic conditions; however, it will be necessary to determine if DNAse I can serve as a viable drug to reduce VTE risks in pancreatic cancer patients by dissolution of NETs, as we have shown that NETs can be stimulated by pancreatic cancer cells, and can promote venous thrombosis. In addition, heparin may also provide some benefits as it is known to prevent interactions between histones and platelets [56]. As suggested earlier, NET-bound histones may play a major role in platelet activation and subsequent thrombi formation when released in the context of cancer. Thus, heparin may provide additional benefits by reducing or preventing cancer-associated thrombosis.

4. Materials and Methods

4.1. Neutrophil Isolation

A qualified phlebotomist under approved Curtin University Human Research Ethics Committee number HR54/2014 collected blood from healthy volunteers using EDTA (5 mM) as an anticoagulant. Neutrophils were isolated by density gradient centrifugation using PolymorphPrep (Axis-Shield, Oslo, Norway) with minor changes to the manufacturer's protocol. Briefly, blood was layered over PolymorphPrep then centrifuged at room temperature for 35 min at $600 \times g$. The layer containing neutrophils was collected and washed twice at 4 °C in Hank's buffered saline solution (without calcium and magnesium). Contaminating red blood cells were lysed using Red Blood Cell Lysis buffer (Sigma, St. Louis, MO, USA). Neutrophils were resuspended in ice-cold X-VIVO 15 media (Lonza, Basel,

Switzerland) and kept on ice until ready for use in experiments. Cell viability was routinely >99% as assessed by Trypan Blue exclusion. Cell purity was >93% as determined with a haematology analyser (Mindray, Shenzhen, China; BC-VET2800) and flow cytometry staining for Cluster of Differentiation 66b (CD66b) (Figure A3).

4.2. Platelet Isolation

Platelets were isolated using the centrifugation method as previously described [57]. Briefly, whole blood anticoagulated with acid citrate dextrose (ACD) was drawn from healthy volunteers then centrifuged at room temperature for 20 min at $150 \times g$ to obtain platelet-rich plasma (PRP). PRP was collected and platelets pelleted by centrifugation at room temperature for 10 min at $800 \times g$. The platelet pellet was washed three times in Citrate-Glucose-Sodium (CGS) buffer (123 mM NaCl, 33.3 mM glucose, 14.7 mM trisodium citrate, pH 7.0) before resuspending in HEPES Tyrode's buffer (5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5.5 mM glucose, 138 mM NaCl, 12 mM $NaHCO_3$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 2.6 mM KCl, 0.36 mM NaH_2PO_4, pH 7.4) supplemented with 1.8 mM $CaCl_2$ for use in experiments. Prostaglandin I_2 (PGI_2; 0.5 μM) was added to whole blood and each wash to minimise platelet activation during the preparation process.

4.3. NET Quantification Assay

Neutrophils (5×10^4) were seeded in 96-well clear-bottom plate and allowed to adhere for 30 min at 37 °C 5% CO_2 before adding treatments, phorbol myristate acetate (PMA, 25 nM), AsPC-1 cells (10×10^4), collagen (4 μg/mL), platelets (1×10^8/mL), or replacing the supernatant with AsPC-1 conditioned media. After 30 min incubation with treatment, NET release was quantified by staining extracellular DNA with cell-impermeable Sytox® Green (5 μM; ThermoFisher, Waltham, MA, USA) and measurement of fluorescence emission at 523 nm (488 nm laser excitation). Fluorescent measurement was performed with EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). A second reading was obtained at 3 h. Treatments were performed in replicates of three. The amount of NET released was determined by calculating fluorescence fold change from unstimulated control.

4.4. Fluorescence Microscopy and Immunofluorescence

Neutrophils (1×10^6/mL) were seeded on poly-L-lysine-coated glass slides then incubated with AsPC-1 conditioned media for 30 min, or PMA (25 nM) for 3 h at 37 °C. Extracellular DNA was stained with cell-impermeable Sytox® Green (2 μM) before fixing the cells in 100% ice-cold methanol for 5 min at room temperature. Samples were blocked in 10% goat serum for 30 min at room temperature, then incubated with rabbit anti-elastase (1 μg/mL; Abcam #68672, Cambridge, UK) in 1% bovine serum albumin (BSA) overnight at 4 °C. Rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) at the same concentration was used as an isotype control for anti-elastase antibody. Cells were washed three times in phosphate-buffered saline (PBS) before incubating with anti-rabbit Alexa594-conjugated secondary antibody (1/500; Cell Signalling). Samples were mounted with ProLong Gold mounting media with DAPI (Molecular Probes, Life Technologies) and imaged using Nikon A1+ confocal microscope.

4.5. Reactive Oxygen Species Quantification

Neutrophils (5×10^4) were seeded in 96-well black-bottom plate and allowed to incubate for 30 min in 37 °C 5% CO_2, before pre-treating with a cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, 10 μM; Sigma-Aldrich, St. Louis, MO, USA) for 15 min. H_2DCFDA is a non-fluorescent dye that is cleaved by intracellular esterases to a membrane-impermeable H_2DCF, which can subsequently react with a variety of reactive oxygen species to emit a highly fluorescent 2′,7′-dichlorofluorescein (DCF) [58]. Excess dye was removed and replaced with X-VIVO 15 media (Lonza, Basel, Switzerland). Neutrophils were then either left untreated or pre-treated for 15 min with ROS inhibitor diphenyleneiodonium (DPI; 1 μM). Neutrophils were finally treated with stimulants

before measuring ROS fluorescence on an Enspire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) at 523-nm fluorescence emission with 488-laser excitation. The quantity of ROS generated in treated neutrophils was determined by calculated fluorescence fold change from unstimulated control.

4.6. Preparation of Conditioned Media

AsPC-1 cells (purchased from American Type Culture Collection, Mannassasa, VA, USA) were grown in RPMI-1640 medium (ThermoFisher, Waltham, MA, USA) supplemented with 2 mM L-glutamine, 10 mM HEPES (HyClone, ThermoFisher) 1 mM sodium pyruvate (Gibco, ThermoFisher), 4500 mg/L glucose, and 500 mg/L sodium bicarbonate and 10% fetal bovine serum (Bovogen, Victoria, Australia), in an incubator maintained at 37 °C and 5% CO_2. At 80%–90% confluence, the medium was changed to phenol red-free RPMI-1640 without FBS, and cells were cultured for a further 36 h. The supernatant (conditioned medium, CM) was collected and centrifuged at 12,000× *g* for 10 min at 4 °C to remove cell debris. CM was aliquoted and stored at −80 °C. Human primary mesenchymal stem cells (MSC), obtained from Lonza (Basel, Switzerland), were similarly cultured in RPMI-1640 complete medium. Conditioned medium from MSC were prepared as above. Both cell lines were negative for mycoplasma throughout all the studies (routine testing by Curtin Health Innovation Research Institute, Perth, Australia).

4.7. Protein and Lipid Fractionation of Conditioned Media

The protein and lipid fractions were separated as described previously with slight modifications [59]. Briefly, conditioned media were mixed with 100% butanol (1:2) and vortexed before incubating at 4 °C for 90 min. After incubation, protein and lipid phases were separated by centrifugation at 1000× *g* for 10 min. The lipid (upper) and protein (lower) phases were separated and dried using a SpeedVac system (Eppendorf, Concentrator 5301, Hamburg, Germany) at 30 °C for 7 h. The dry pellet was resuspended in PBS to 1/5 of the starting volume.

4.8. Cell-Free NET Isolation

NETs were isolated as previously described [60]. Briefly, neutrophils (5×10^6/mL) were incubated with 600 nM PMA for 4 h at 37 °C. The supernatant was removed and NET monolayer detached from the culture surface with ice-cold PBS, then centrifuged at 400× *g* for 5 min at 4 °C to pellet cell debris. The cell-free supernatant was pooled and centrifuged at 15,000× *g* for 15 min at 4 °C to pellet DNA. DNA pellet was resuspended in PBS to a volume corresponding to 100 μL per 1×10^7 neutrophils to obtain cell-free NETs.

4.9. Platelet Spreading Assay

Glass slides were coated with 1% denatured BSA (dBSA) in PBS or cell-free NET overnight at 4 °C in a humidifier chamber. NET-coated slides were either left untreated or pre-treated with DNAse I (100 U/mL; Stemcell Technologies, Vancouver, BC, Canada) in Hank's buffered saline solution (with calcium). The coated slides were then washed with 1× PBS and blocked with 1% dBSA for 1 h at room temperature, in the humidifier chamber. Denatured BSA was prepared by heating the solution in PBS without calcium or magnesium to 80 °C for 3 min, immediately put on ice until cool, and aliquoted and stored at −20 °C until use.

Platelets (2×10^7/mL) were seeded on the coated slides for 1 h at 37 °C CO_2 in the humidifier chamber. Adherent platelets were washed with PBS before fixing in 4% paraformaldehyde for 10 min at room temperature, permeabilised with 0.1% Triton-X 100 for 2 min, and washed once with PBS. Platelets were then stained with Alexa Flour 488-conjugated phalloidin (diluted 1:100 in PBS, ThermoFisher, Waltham, MA, USA) for 20 min in the dark. Samples were mounted with ProLong Gold antifade reagent (Molecular Probes). Images were taken with Nikon A1+ confocal microscope with 100× objective. Three random fields of view were taken for analysis using Image J software (Version 1.50i, National Institute of Health, Bethesda, MD, USA).

4.10. Perfusion Assay—Ex Vivo Thrombus Formation

Microfluidics biochip (Cellix Ltd., Dublin, Ireland) channels were coated with cell-free NETs, 1% dBSA or 100 µg/mL collagen type I overnight at 4 °C and blocked in 1% dBSA for 1 h. Citrated-whole blood (1:9, 3.8% w/v sodium citrate to whole blood) was drawn from healthy volunteers and fluorescently-labelled with $DiOC_6(3)$ (5 µM; Molecular Probes) for 15 min in the dark. Blood was perfused through the channels at a venous shear rate of 10 dyne/cm^2 for 10 min using a Mirus microfluidics pump (Cellix Ltd.), after which the samples were washed at the same rate for 2 min in PBS. Video live imaging of the perfusion and image snaps of formed thrombi were captured using UltraView Vox spinning disk confocal microscope. Four fields of view were taken after each perfusion assay for surface area quantification using Image J (Version 1.50i, National Institute of Health). Z-stack images were also acquired.

4.11. Statistical Analysis

All statistical analysis was performed on GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). Results are shown as mean ± SEM. The one-way ANOVA with post-hoc Bonferroni was performed to determine statistical significance between means of multiple groups. The unpaired t-test was used to compare means between two groups. $p \leq 0.05$ was considered statistically significant.

5. Conclusions

Taken together, our results demonstrate that pancreatic cancer cells can stimulate NET formation, and in turn NETs produced are a platform for platelet adhesion and thrombi formation. The degradation of NETs by DNAse I, and/or prevention of histone-platelet interaction by heparin, could become a potential new drug option for pancreatic cancer patients if proven effective to reduce venous thrombosis, and may lead to improved survival rates as VTE is linked to poor prognosis in PaCa patients. Moreover, since NETs have also been implicated in metastasis, targeting NETs may not only reduce risk of developing VTE, but it may also attenuate the development of highly metastatic pancreatic tumours. Thus, it will be important to look at the effects of NETs on pancreatic cancer cell survival, proliferation, migration and invasion, to determine if NETs exhibit pro-tumourigenic characteristics, and serve as another reason to be targeted in pancreatic cancer patients.

Author Contributions: Pat Metharom conceived and designed the experiments; Norbaini Abdol Razak and Omar Elaskalani performed the experiments and analysed the data; Norbaini Abdol Razak wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

NETs	Neutrophil Extracellular Traps
VTE	Venous Thromboembolism
PaCa	Pancreatic Cancer

Appendix A
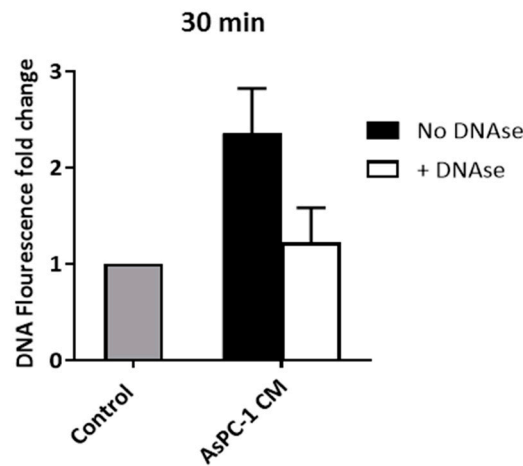
Figure A1. The addition of DNAse I (60 U/mL) abolished extracellular DNA fluorescence to unstimulated control, confirming the specificity of Sytox Green to extracellular DNA (exDNA). Control: unstimulated neutrophils. Data expressed as mean $\pm$ SEM from $n = 3$.
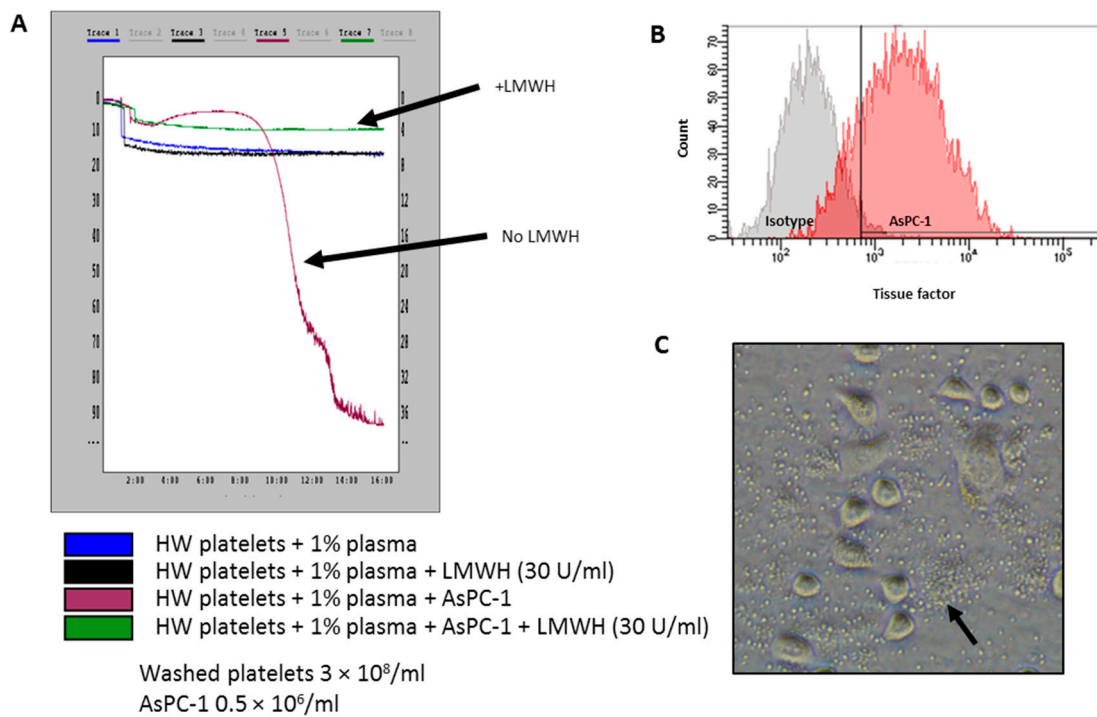
Figure A2. AsPC-1 expresses tissue factor, and causes platelet aggregation (**A**) Light transmission aggregometry showed that AsPC-1-induced aggregation of human washed (HW) platelets, which was tissue factor-dependent, as addition of low-molecular-weight heparin (LMWH) prevented platelet aggregation. The downward-slope represents light transmission approaching 100%, which is indicative of platelet aggregation; (**B**) Flow cytometry analysis of AsPC-1 cells stained with anti-tissue factor show AsPC-1 expressing tissue factor above isotype control; (**C**) Phase-contrast images show platelets aggregating (black arrow) around AsPC-1 cells.
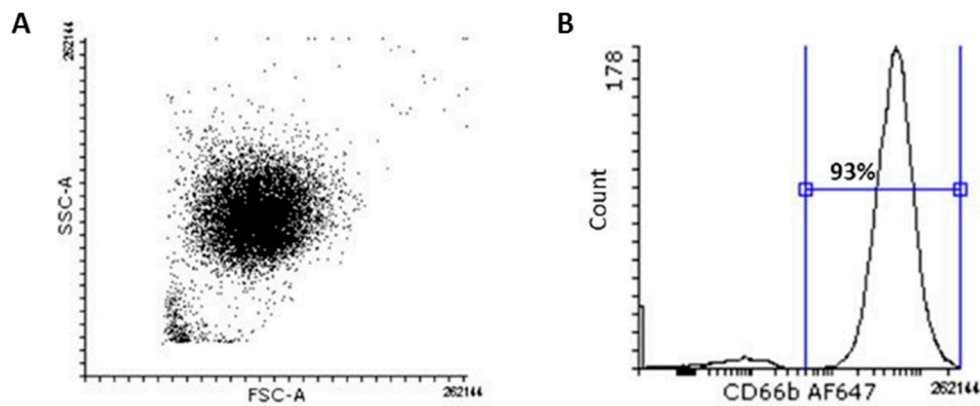
Figure A3. Flow cytometry analysis of CD66b staining on isolated human neutrophils. (**A**) Forward-side-scatter profile of isolated polymorphonuclear neutrophils (PMNs); (**B**) Isolated PMNs were stained with CD66b, a marker for PMNs, to determine purity of neutrophil isolation. A total of 93% of the isolated cell population was positive for CD66b.

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Appendix II: Statement of contribution by others and copyright authorisation

Author and co-author contributions for each publication presented in this thesis have been stated accordingly and indicated in the section below.

To Whom It May Concern:

I, Omar Elaskalani, contributed as a first author to the conception, design, writing and manuscript preparation for publication entitled (**Elaskalani O**, Berndt M.C, Falasca M, Metharom P. Targeting Platelets for the Treatment of Cancer. *Cancers* 2017, *9*, 94)

I, Professor Michael Berndt, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signature ████████████ te ___29/3/2019___

I, Professor Marco Falasca, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

S ████████████ Date ___4/2/2018___

I, Dr Pat Metharom, as the senior corresponding author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signat ████████████ Date ___7/2/19___

To Whom It May Concern:

I, Omar Elaskalani, contributed as a co-author to the study design, experimental procedures, data collection, data analysis and interpretation, and manuscript preparation for publication entitled (**Elaskalani O,** Abdol Razak N, Metharom P. Neutrophil extracellular traps induce aggregation of washed human platelets independently of extracellular DNA and histones. *Cell communication and Signalling* 2018, 16(1) 24).

I, Norbaini Abdol Razak, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signature␣␣␣␣␣␣␣␣␣␣ Date␣␣␣␣ 4/2/19

I, Dr Pat Metharom, as the senior corresponding author, endorse that this level of contribution by the candidate indicated above is appropriate.

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To Whom It May Concern:

I, Omar Elaskalani, contributed as a first author to the conception, design, writing and manuscript preparation for publication entitled (**Elaskalani O**, Razak NB, Falasca M, Metharom P. Epithelial-mesenchymal transition as a therapeutic target for overcoming chemoresistance in pancreatic cancer. *World J Gastrointest Oncol.* 2017;9(1):37-41)

I, Norbaini Abdol Razak, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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I, Professor Marco Falasca, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Sign_____ Date____4/2/2019____

I, Dr Pat Metharom, as the senior corresponding author, endorse that this level of contribution by the candidate indicated above is appropriate.

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To Whom It May Concern:

I, Omar Elaskalani, contributed as a first author to the study design, experimental procedures, data collection, data analysis and interpretation, and manuscript preparation for publication entitled (Elaskalani O, Falasca M., Moran N, Berndt M.C, Metharom P. The Role of Platelet-Derived ADP and ATP in Promoting Pancreatic Cancer Cell Survival and Gemcitabine Resistance. *Cancers* 2017, *9*, 142.)

I, Professor Marco Falasca, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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I, Associate Professor Niamh Moran, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signature ███████ Date __12/4/2019__

I, Professor Michael Berndt, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signature ███████ Date __29/3/2019__

I, Dr Pat Metharom, as the senior corresponding author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signature ███████ Date __7/2/19__

To Whom It May Concern:

I, Omar Elaskalani, contributed as a joint first author to the conception, design, writing and manuscript preparation for publication entitled (Adamska A[*], **Elaskalani O[*]**, Emmanouilidi A[*], Kim M[*], Abdol Razak N.B, Metharom P.; Falasca, M. Molecular and cellular mechanisms of chemoresistance in pancreatic cancer. *Adv Biol Regul* 2017 (68) 77-87)

I, Aleksandra Adamska, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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I, Aikaterini Emmanouilidi, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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I, Minkyoung Kim, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signature ██████ Date 8/ 2/2019

I, Norbaini Abdol Razak, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signature ██████ Date 4/2/2019

I, Dr Pat Metharom, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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I, Professor Marco Falasca, as the senior corresponding author, endorse that this level of contribution by the candidate indicated above is appropriate.

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To Whom It May Concern:

I, Omar Elaskalani, contributed as a co-author to the study design, experimental procedures, data collection, data analysis and interpretation, and manuscript preparation for publication entitled (Abdol Razak N, **Elaskalani O**, Metharom P. Pancreatic Cancer-Induced Neutrophil Extracellular Traps: A Potential Contributor to Cancer-Associated Thrombosis. *Int J Mol Sci.* 2017;18(3))

I, Norbaini Abdol Razak, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.
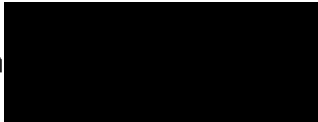
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I, Dr Pat Metharom, as the senior corresponding author, endorse that this level of contribution by the candidate indicated above is appropriate.
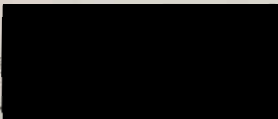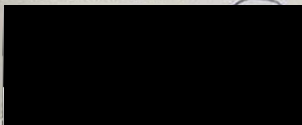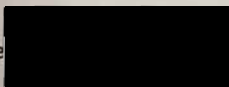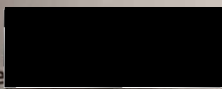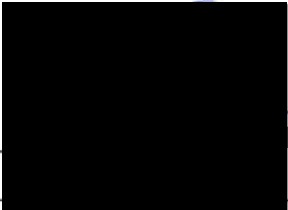
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