Curtin Medical School

Mechanisms of Cancer-Associated Thrombosis: Role of Cancer-Derived Exosomes

Norbaini Binti Abdol Razak

0000-0002-3133-9790

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Declaration

To the best of my knowledge and belief, I declare that this thesis titled: **'Mechanisms of cancer-associated thrombosis: role of cancer-derived exosomes'** has been written by myself and contains no material previously published except where due acknowledgement has been made.

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). Human research ethics approval was obtained from the Curtin University Human Research Ethics Committee, Approval Number HR54/2014.

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 proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number ARE2018-34.

Norbaini Binti Abdol Razak

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Abstract

Cancer-associated thrombosis is a serious complication that significantly impacts the quality of life of cancer patients and is linked to poor prognosis. It is the second leading cause of death in cancer patients. Different cancer types have different risk rates for the development of thrombosis, and it is particularly high in pancreatic cancer. Since the first description and association of cancer with thrombosis by physician Armand Trousseau in 1865, it is now well established that thrombosis commonly affects cancer patients, however the specific mechanisms which predispose cancer patients to increased thrombotic complications are not well understood.

So far, several mechanisms have been proposed, such as the expression of procoagulant proteins by cancer cells such as tissue factor or podoplanin. More recently, the role of cancer-derived microvesicles expressing tissue factor has been recognised as contributors to the activation of coagulation, platelet aggregation, and thrombosis in cancer. In addition, the role of neutrophil extracellular traps (NETs) released from activated neutrophils has gained interest in the pathogenesis of thrombosis. Studies from our lab was one of the first to show the potential contribution of pancreatic cancer cells to cancer-associated thrombosis through the direct stimulation of neutrophils to release NETs. NET release was shown to be induced by both pancreatic cancer cells and the conditioned medium. This study stimulated the investigation of identifying the specific mediators through which pancreatic cancer cells stimulate neutrophils to release NETs.

A potential mediator for pancreatic cancer-induced NET is the secretion of exosomes by pancreatic cancer cells. Exosomes are small extracellular vesicles that are released from all cell types, and in greater amounts from cancer cells. In recent years, exosomes have attracted great interest due to their reported roles in mediating cell-cell communication, and their ability to mediate functional changes in the recipient cell. For this thesis, the role of pancreatic cancer-derived exosomes was assessed in the function and activation of different cell types that are known to have important roles in thrombosis, these include neutrophils, platelets and endothelial cells.

This thesis reports several findings that shed light on the mechanism of thrombosis in cancer which can be induced by PDEx. First is that pancreatic cancer-derived exosomes (PDEx) were able to directly activate neutrophils to release NETs. On the other hand, PDEx did not appear to induce direct activation of platelets as assessed by platelet aggregation. Interestingly, however, there was a priming effect in platelets that was induced by PDEx that resulted in the stimulation of NETs. In addition, the results suggested that PDEx may carry functional ectonucleotidase CD73 which may contribute to the dampening effect seen in platelets when stimulated with PDEx. CD73 cleaves ATP, a

platelet agonist that can be released from activated platelets and may explain the absence of activation seen in platelets stimulated with PDEx. Endothelial cells were assessed for permeability, upregulation of cell adhesion molecule ICAM-1 and neutrophil adhesion. PDEx induced a trend of upregulation ICAM-1 in endothelial cells, and accordingly an increase in the adhesion of neutrophils.

The last chapter of the thesis assessed the effect of targeting a key player in thrombosis, platelets, in tumour outcome and angiogenesis in a xenograft model of pancreatic cancer. This study was carried out to determine the additional effect to the inhibition of platelets in cancer. The antiplatelet, ticagrelor resulted in reduced tumour growth and disruption of tumour vasculature.

Together, the results demonstrate that PDEx have a range of effects on neutrophils, platelets and endothelial cells. Overall, they can modulate the function of these cells to have procoagulant properties, suggesting that PDEx has a significant function in promoting thrombosis and can potentially be targeted for therapeutic intervention of cancer-associated thrombosis.

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List of publications

The following research articles and reviews have been published during the course of this thesis. The following list includes both first and co-author publications. These research articles and reviews include components that are relevant to the thesis. Each will be referred to throughout the thesis in the relevant chapters.

1. **Abdol Razak** NB, Jones G, Bhandari M, Berndt MC, Metharom P. Cancer-Associated Thrombosis: An Overview of Mechanisms, Risk Factors, and Treatment. Cancers (Basel). 2018 Oct 11;10(10):380. doi: 10.3390/cancers10100380.

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Attribution statements

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, Norbaini Abdol Razak, contributed as first author to the writing and manuscript preparation for the review publication entitled, "Cancer-Associated Thrombosis: An Overview of Mechanisms, Risk Factors, and Treatment"

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I, Gabrielle Jones, as a co-author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signed: [redacted] Date: 05/02/2024

I, Mayank Bhandari MD, as a co-author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signed: [redacted] Date: 2/02/24

I, Michael Berndt PhD, as a co-author, endorse that this level of contribution by the candidate indicated above is appropriate.

Signed: [redacted] Date: 25/2/2024

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

Signed: [redacted] Date: 29 January 2024

To whom it may concern:

I, Norbaini Abdol Razak, as a co-author contributed to the writing for the review publication entitled, "*Small Extracellular Vesicles in Pancreatic Ductal Adenocarcinoma immunity.* Seminars in Cancer Biology" In particular, the writing on the section under "3.5 Neutrophils"

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I, Jordan Fyfe, as the first author, endorse that this level of contribution by the candidate indicated above is appropriate. Signed: _____ [redacted] _____ Date: _____12 February 2024_____
I, Danielle Dye PhD, as a co-author, endorse that this level of contribution by the candidate indicated above is appropriate. Signed: _____ Date: _____
I, Pat Metharom PhD, as senior corresponding author endorse that this level of contribution by the candidate indicated above is appropriate. Signed: _____ [redacted] ___ Date: 29 January 2024 _____
I, Marco Falasca PhD, as senior corresponding author endorse that this level of contribution by the candidate indicated above is appropriate. Signed: ____ [redacted] ___ Date: 6/8/2024 _____

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I, Jordan Fyfe, as the first author, endorse that this level of contribution by the candidate indicated above is appropriate. Signed: _____ Date: _____
I, Danielle Dye PhD, as a co-author, endorse that this level of contribution by the candidate indicated above is appropriate. Signed: _____ [redacted] _____ Date: 7 February 2024
I, Pat Metharom PhD, as senior corresponding author endorse that this level of contribution by the candidate indicated above is appropriate. Signed: _____ [redacted] __ Date: 29 January 2024
I, Marco Falasca PhD, as senior corresponding author endorse that this level of contribution by the candidate indicated above is appropriate. Signed: _____ Date: _____

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Chapter 1

Literature Review
Cancer-associated thrombosis: an overview of mechanisms, risk factors and treatment

This chapter includes a published review article, which can be found attached in the Appendix at the end of the thesis.

1.0. Publication

Abdol Razak NB, Jones G, Bhandari M, Berndt MC, Metharom P. Cancer-Associated Thrombosis: An Overview of Mechanisms, Risk Factors, and Treatment. *Cancers (Basel)*. 2018;10(10):380. Published 2018 Oct 11. doi:10.3390/cancers10100380

1.1 Introduction

Thrombotic complications such as venous thromboembolism or ischaemic stroke in cancer patients contribute to increased morbidity and mortality. This role of thrombosis in cancer is referred to as cancer-associated thrombosis (CAT) and was first described in the 19th century [1]. Not only can thrombosis significantly impair quality of life, but cancer-associated thrombosis is regarded as the second leading cause of death in cancer patients, second to the progression of cancer resulting in organ failure [2]. Although both arterial and venous thromboembolism are prevalent in cancer patients, the most common type observed is venous thromboembolism (VTE). About 4-20% of cancer patients experience VTE, however, it has been postulated that this may be largely underestimated as post-mortem examinations have found up to half of the cancer patients have VTE [3].

VTE comprises of deep vein thrombosis (DVT) and pulmonary embolism. DVT occurs when clots form in the deep veins of the lower limbs. A pulmonary embolism is a life-threatening complication arising from DVT, in which the clot breaks loose and travels to the lung, causing a blockage in the pulmonary artery. There are many consequences of VTE in cancer patients, including increased morbidity, bleeding complications, increased risk of recurrent VTE and delays in cancer treatment [4]. Despite the well-known association between cancer and thrombosis, the mechanisms leading to thrombosis in cancer patients are poorly understood. The potential mechanisms described thus far, and current treatment for CAT, are discussed in the review entitled, 'Cancer-associated thrombosis: an overview of mechanisms, risk factors and treatments' which was published by this author in Cancers 2018 (paper included at the end of the chapter). Since the publication of the attached review, several additional studies have shed light on new mechanisms of thrombosis in cancer, which will be briefly discussed below. In addition, the role of activated platelets in cancer will also be discussed.

1.2 Tumour cell-induced platelet aggregation

Cancer cells can directly activate platelets through physical contact or by releasing factors that serve as agonists for platelet activation [5-7]. This activation by cancer cells has been documented in several cancers such as lung, breast, pancreatic and ovarian cancers. The mechanism of cancer cells inducing platelet activation and aggregation is referred to as tumour cell-induced platelet aggregation (TCIPA). The best-described mechanism of TCIPA by cancer is through the podoplanin/CLEC-2 pathway. Podoplanin is a mucin-type protein highly expressed on cancer cells, which can trigger platelet secretion, activation and aggregation, via the CLEC-2 receptor on platelets [5, 6]. In addition to the expression of molecule, cancer cells can also secrete molecules such as ADP and thrombin which interact with P2Y12 and PAR1/2 receptors on platelets leading to platelet

activation and aggregation [8]. Furthermore, this activation of platelets in cancer has been documented to contribute to the progression and metastasis of cancer. For example, blocking PDPN with MS-1 (an anti-PDPN antibody) led to a significant reduction in tumour metastasis and tumour growth of lung cancer *in vivo* [9, 10]. With the roles of platelets in coagulation and thrombosis, the activation of platelets in cancer by podoplanin has led to the study of the podoplanin/CLEC-2 pathway in CAT. Studies in mice showed that podoplanin-bearing tumours had increased thrombosis which was dependent on the podoplanin/CLEC-2 axis [11, 12]. In a first study observing the composition of thrombi from cancer patients, 26% of thrombi contained cancer cell clusters which also expressed tissue factor and podoplanin, and were surrounded by aggregated platelets and fibrin [13]. This study provided further evidence of cancer cells expressing factors which contribute to thrombogenesis in CAT.

1.3 Updates in the mechanisms of VTE in cancer

In a mouse model of pancreatic cancer, Sano and colleagues found the role of vascular cell adhesion molecule-1 (VCAM-1) in the contribution of both VTE and cancer progression [14]. VCAM-1 is highly upregulated on activated endothelial cells during inflammation and facilitates the adhesion of leukocytes [15]. VCAM-1 has also been found to be expressed by pancreatic cancer cells [16] however its role in cancer progression is not known. The study by Sano et al. used the PKF mouse model of pancreatic cancer which developed spontaneous cancer-associated thrombosis. VCAM-1 was found elevated in activated endothelial cells, thrombi, and in the plasma of both PKF mice and pancreatic cancer patients with thrombosis [14]. Blockade of VCAM-1 using anti-VCAM-1 antibody led to inhibition of tumour growth, infiltration of leukocytes, angiogenesis and the progression of CAT. Mice survived over 4 times longer than those without antibody therapy. This unique study highlights the potential of a new therapeutic strategy of targeting VCAM-1 to improve prognosis while also reducing associated thrombosis.

While the published review focused on mechanisms of VTE in pancreatic cancer, it is worthwhile to note additional mechanisms investigated in other cancers. Nickel et al. studied the mechanisms of VTE in prostate cancer and focused on the role of prostasomes, extracellular vesicles originally found in seminal fluid [17]. However, earlier studies have reported that prostasomes are also released by prostate cancer cells [18]. Nickel and colleagues found that prostasomes express polyphosphate, a linear unbranched polymer, which has previously been shown to be released from activated platelets resulting in a net potent procoagulant effect [19]. A novel role of polyphosphate, derived from both the surface of prostasomes and prostate cancer cells, was found in driving the intrinsic pathway of coagulation via factor XII activation [18]. Prostasomes induced pulmonary

thromboembolism in mice but not in those that were FXII-deficient, indicating a FXII-dependent pathway. Targeting polyphosphate or FXII in mice both significantly protected mice from pulmonary embolism, without increasing bleeding times. This study highlighted a novel mechanism of prostate cancer-associated thrombosis through a polyphosphate/factor XII pathway, and anticoagulant therapy without the associated risk of increased bleeding.

Furthermore, the role of podoplanin (PDPN) in CAT has been further investigated in mouse models. Lee et al. used OECM-1, a human oral squamous cell carcinoma cell line known to have high expression of PDPN, to study CAT in a mouse model of oral cancer [20]. Tumours grown in the neck of nude mice after inoculation with OECM-1 cells had a higher level of intratumoural platelet infiltration, compared to those with PDPN gene knockdown. Greater levels of plasma thrombin-antithrombin (TAT; biomarker of coagulation activation) and fibrin in small vessels of the lungs and kidneys, was also observed compared to mice bearing PDPN-knockdown tumours. Interestingly, mice bearing PDPN+ tumour showed decreased overall survival, despite the lack of gross appearance of distant metastasis. Another study found that anti-PDPN antibody administered to mice reduced thrombosis in mice-bearing C8161 tumours [21]. Another study investigated the role of PDPN+ extracellular vesicles (EVs) released from cancer cells. The incidence of venous thrombosis was higher in mice injected with PDPN+ EVs derived from the wild type human ovarian cancer cell line HeyA8 cells, compared to the PDPN- EVs from HeyA8 PDPN knockdown [22].

1.4 Microvesicles and thrombosis in cancer patients

An area of study that has gained great interest in the contribution to thrombotic diseases are microvesicles. Microvesicles (MVs), also referred to as microparticles or ectosomes, are a subclass of extracellular vesicles. MVs are generated by the shedding of vesicles from the plasma membrane of a cell and thus enclosed within a lipid bilayer. A more comprehensive description of extracellular vesicles will be discussed in the next chapter. The study of the procoagulant activity of MVs in cancer has had a large focus on the surface expression of tissue factor, the major trigger of the coagulation cascade. The correlation of TF-positive MV with the development of thrombosis has been identified in pancreatic cancer patients [23]. However, this correlation is inconsistent and does not appear to be the case in other cancer types [24]. It is interesting to note that in one study, a large majority of cancer patients who developed VTE during chemotherapy had normal MV-TF activity. [25] Thus, there is a need for a greater understanding of the mechanisms through which microvesicles contribute to thrombosis in cancer [26]. In addition, it seems that the mechanisms of thrombosis, in particular venous thrombosis, in cancer differs markedly from what is thought to underlie thrombosis in a non-malignant condition. This was suggested in the study by Stark et al. which

investigated the mechanisms by which pancreatic cancer cell-derived MVs promote thrombosis in mice [27]. Identifying the key differences in mechanisms of malignant and non-malignant thrombosis may in turn lead to and require more specific treatment of thrombosis in cancer patients that is distinct from those used in non-malignant thrombosis.

The inconsistency of TF-positive MV and its correlation with VTE in cancer patients have led investigators to evaluate the functional capacity of TF found in the plasma of patients with progressive cancers. Ender et al. found both higher levels of MVs and TF activity associated with MVs in the plasma of cancer patients [28]. This study suggested that TF activity, rather than the quantification of MVs, should be used as a marker for risk for VTE in patients with cancer.

While TF-positive MVs were the first subtype of extracellular vesicles (EVs) to be recognised as contributors to thrombotic diseases, the role of exosomes (the smaller subtype of extracellular vesicles) may be greatly underestimated and understudied. Recent studies have pointed towards exosomes involved in cellular communication, cell signalling and delivery of content between blood cells [29] (more detail in the following chapter). One study has shown the remarkable capacity of 'education' of bone marrow cells by melanoma-derived exosomes to facilitate metastasis [30]. Similar to this concept, it is hypothesised that cancer-derived exosomes facilitate thrombosis through the education and/or activation of other cells which have important roles in thrombosis and will be investigated in this thesis.

1.5 Mouse models of cancer-associated thrombosis

Several studies have attempted to delineate the mechanisms of VTE in cancer through mouse models. While these mechanisms may not accurately depict those occurring in humans due to differing clotting parameters between animals and mice strains [31], it has directed interest to potential key players, which certainly warrant further investigation.

Different models used to study cancer-associated thrombosis differ by mice strain, cancer type, and thrombosis developed. The type of mice strains used largely depend on the cancer cells used. Immunocompetent mice, most commonly C57BL/6 and BALB/c, are used for allograft models with murine cancer cells [32]. The use of immunocompetent mice serves as a great strength as they can mount a full immune response to the tumour, which may, in turn, contribute to thrombosis. However, only a limited number of murine cancer cell lines are available. The three most commonly used cell lines are 4TI, Lewis lung carcinoma and Panc02 [32]. On the other hand, immunodeficient mice are used for xenograft models, which is useful to investigate a range of human cancers. However, a great weakness in immunodeficient mice is the inability to mount a full immune

response, which could potentially mask any effect the immune response may play in thrombosis [32].

There are four thrombosis models which have been used to study cancer-associated thrombosis in mice, most of which utilise the inferior vena cava (IVC) [33]. Studies with models using the IVC are advantageous as high-frequency ultrasonography can be used for non-invasive monitoring of thrombus formation. IVC models can be divided into stenosis or stasis models. The IVC stasis model involves complete ligation of the IVC and side branches using non-reactive sutures [34]. In comparison, the stenosis model involves partial ligation of the IVC with a spacer that is subsequently removed, achieving approximately 90% stenosis [33]. Another method used to induce thrombosis is the ferric chloride application, which results in thrombosis in the IVC and small saphenous veins [34]. This method applies oxidative damage to the blood vessels, which can be altered with different concentrations and application times, depending on the severity to be tested. The fourth method is the use of nitrogen laser, which can induce endothelial activation to generate clots at the cremasteric microvessels [33, 35].

Some studies have suggested a role for TF-positive MVs in the generation of thrombosis through the direct injection of TF-positive MVs into mice. Geddings and colleagues found that TF+ MVs isolated from pancreatic cancer cells activated platelets and enhanced thrombosis in two mouse models of venous thrombosis [36]. Platelet activation was important for TF+ MVs-induced thrombosis, as thrombosis was reduced in mice that were treated with antiplatelet clopidogrel and in PAR4-deficient mice [36]. Wang et al investigated thrombosis using the IVC stenosis model in mice with a xenograft model of pancreatic cancer. They found that mice injected with exogenous TF-positive MP were able to increase thrombosis [37]. However, the number of MPs that were needed to increase thrombosis resulted in MP levels in the plasma that were 40 to 100 times higher when compared to injection of pancreatic cancer cells that did not increase thrombosis compared to control. Thus cancer-derived TF+ MVs may contribute to platelet activation and thrombosis in cancer, however, it seems it is needed in excessive amounts.

In another recent study investigating thrombosis in nude mice with pancreatic cancer, Hisada et al. found that neutrophils and neutrophil extracellular traps (NETs) were important for propagating thrombosis [38]. NETs are web-like structures of protein and DNA that are expelled upon neutrophil activation [39]. NETs have recently been shown to have roles in thrombosis and platelet activation [40-42] Hisada et al. found elevated NET markers in tumour-bearing mice and that neutrophil depletion and DNAse I treatment in mice bearing pancreatic cancer resulted in a reduction of thrombus size [38].

1.6 Recent clinical trials comparing VTE treatment in cancer patients

Anticoagulant therapy in cancer patients with VTE should only be administered after careful risk assessment due to the high risk of life-threatening bleeding complications. The balance between bleeding risk and thrombosis must be weighed up before offering treatment. There is ongoing debate as to whether the use of direct oral anticoagulants (DOACs) is advantageous over low molecular weight heparin (LMWH). LMWH is the standard treatment for at least six months for acute VTE in patients with cancer [43]. The use of LMWH is considered inconvenient as it requires daily subcutaneous injections; thus, the use of DOACs has become more attractive to patients and clinicians. So far, few clinical trials conducted have given evidence to support the use of DOACs apixaban, edoxaban and rivaroxaban, in cancer patients with VTE. Since the publication of the review, results of the ADAM VTE and Caravaggio study trials have further supported that DOACs are non-inferior to LMWH in the treatment of VTE in cancer patients. Both the ADAM VTE and Caravaggio trials concluded that patients treated with apixaban had lower rates of recurrent VTE and experienced less major bleeding, than those patients receiving dalteparin [44, 45]. Despite the favourable results shown so far in clinical trials, more studies need to confirm these observations and the bleeding risk for patients when choosing anticoagulant treatments in cancer patients. The risk should be individually assessed for each patient, with careful consideration of factors such as clinical history, cancer type, status, and other concomitant medications.

1.7 VTE in pancreatic cancer

Aggressive cancers tend to have a greater risk and higher incidence of developing thrombosis. Pancreatic ductal adenocarcinoma (PDAC) has the greatest risk and incidence of developing VTE [46]. PDAC is the seventh most common cause of cancer-related deaths worldwide and is well known as a highly aggressive cancer with a dismal 5-year survival rate of just 8%. The first striking report showing the relationship between pancreatic cancer and thrombosis dates back to 1938 [47]. This study reviewed autopsies of cases with thrombosis and found almost 60% prevalence of venous thrombosis in patients with pancreatic cancer (particularly of the body or tail of the pancreas) while the rate in other cancers was less than 25%. Now, studies with retrospective cohorts have reported rates of VTE in pancreatic cancer varying between 5%-41% [48-51]. A recent study by Frere and colleagues was the first prospective study following a cohort of pancreatic cancer patients, which found a cumulative incidence of 19% at 12 months. This study also reported an association between the onset of VTE and reduced progression-free and overall survival [52]. This contradicts earlier retrospective studies which showed no association between VTE in pancreatic cancer patients and

reduced overall survival [49, 53]. However, in these retrospective studies, patients had metastatic disease which is expected to have short life expectancy.

Once described as a coincidence, the relation between malignant tumour and venous thrombosis is now well documented. Armand Trousseau was one of the earliest physicians to stress the relationship of malignant tumours with venous thrombosis. Since then, it has been established that pancreatic cancer, among other aggressive cancers, has an unusual ability to induce a hypercoagulable state [54]. Despite the long-known association between pancreatic cancer and thrombosis, the underlying pathological mechanism and interactions with various pathways that may potentially contribute to thrombosis remain poorly understood [54]. Given the high rates of thrombosis in pancreatic cancer, it serves as a good model to investigate the mechanisms of thrombosis. Thus, this thesis will focus on the mechanisms of thrombosis in cancer with a focus on pancreatic cancer-derived exosomes as a key mediator.

1.8 COVID-19 and CAT risk

Coronavirus disease-19 (COVID-19), caused by SARS-CoV-2, has impacted more than 100 million people across the globe, resulting in millions of deaths due to severe complications such as respiratory failure, septic shock, and multiple organ failure. Cancer patients are susceptible to infection due to their immunocompromised state. Early in the pandemic, it became evident that cancer patients are at high risk of developing severe COVID-19 with adverse outcomes [55, 56]. A study in Wuhan found that the SARS-CoV-2 infection rate in cancer patients was two times greater than in the general population [57]. Particularly, cancer patients who have undergone major surgery and immunotherapy prior to COVID-19 had greater rates of death and ICU admission [58]. In addition, patients with severe COVID-19 are at high risk of developing thrombotic complications, such as pulmonary embolism and deep vein thrombosis [59]. Hence, the combination of cancer and COVID-19 may greatly amplify thrombotic complications. The COVID-19-associated coagulopathy from SARS-CoV-2 infection has been attributed to complex interaction between immune cells, coagulation pathways and the vascular endothelium [60].

With the increased risk of COVID-19 among cancer patients, it is of paramount importance to protect these patients from infection and its associated coagulopathy. Cancer patients with COVID-19 should be assessed for the risk of VTE, as with any patient with COVID-19. According to the 2022 clinical practice guidelines by the International Initiative on Thrombosis and Cancer, there is currently no data regarding the benefits or risks of different anticoagulants for the treatment of VTE in cancer patients with COVID-19 [61]. Recommendations for the treatment of VTE in cancer patients with COVID-19 are similar to those without COVID-19.

1.9 Summary

It is important to understand the mechanisms of thrombosis in cancer patients to provide better treatment options and improve patient outcomes. Currently, the use of LMWH and DOACs have a risk of bleeding, which needs to be assessed in each patient. A better understanding can lead to improved treatment outcomes and lower the risk of treatment-associated bleeding in patients. These can be investigated through key interactions that cancer-derived EVs have with known players in thrombosis including platelet, endothelial cells and neutrophil extracellular traps, which will be discussed in the next chapters.

It is imagined that the interactions are highly complex, with factor/s that play key roles yet to be discovered. This thesis will begin to shed light and uncover pathways and mechanisms not yet studied before. It will be of great worth to further understand the role of cancer-derived EVs in cancer, as not only have they garnered interest for their roles in cancer progression and metastasis but also in cancer-associated thrombosis. Exosomes in particular have been overlooked, as more research has identified their functional role in cellular communication. Further investigating their roles as mediators that contribute to the propagation of thrombosis in cancer will highlight the importance of exosomes to both the progression of cancer and cancer-associated thrombosis.

Chapter 2

Exosome Isolation and Characterisation

This chapter will be presented in two parts:

Part 1: Background on exosomes

Part 2: Isolation and characterisation of exosomes

PART 1: Background on exosomes

2.0 Introduction

The secretion of extracellular vesicles (EVs) was initially described as a means to eliminate unwanted cellular components [62, 63]. Subsequent research have now regarded extracellular vesicles as important mediators of cellular communication over short and long distances, both in normal physiological processes and pathological progression [64]. Extracellular vesicles are membrane-bound spherical vesicles that are released from a variety of cell types with size ranging from 30-1000 nm [65]. The two main categories of EVs are exosomes and microvesicles (also known as microparticles) which are primarily characterised by their size and mechanism of release. Microvesicle size ranges from 100-1000 nm in diameter and are formed by outward budding or shedding of the plasma membrane [66]. On the other hand, exosome have a size range of 30-150 nm [67] and they are derived from the endosomal pathway where multivesicular bodies (MVBs) are formed and fuse with the plasma membrane, releasing their contents into the extracellular space as exosomes [68]. EVs have been shown to be capable of transferring their content such as nucleic acids, proteins, lipids and microRNA [69] to other neighbouring cells as a form of intercellular communication [65, 70]. These vesicles are found in all bodily fluids such as blood, urine, and breast milk, thus making them a potential source for prognostic or diagnostic biomarkers.

Initially, exosomes were seen as vesicles for removal of unwanted products from the cell. The first use of the term exosome was in 1981 by Trams and colleagues [71]. This study reported the exfoliation of the cell membranes from various normal and neoplastic cell lines, leading to the release of vesicles ranging from 40 to 1,000 nm in size which possessed 5'-nucleotidase activity [71]. Not long after in 1987, the term exosome was adopted to refer specifically to endosomal-derived vesicles released from the fusion of MVBs with the plasma membrane, which was observed during the study of reticulocyte maturation [62]. In the following decade, exosomes were minimally studied, mostly focusing on the maturation of red blood cells, and viewed as a means to remove unwanted contents. The interest in exosomes was renewed in the late 1990s, when some studies suggested that exosomes released from immune cells may be mediators of intercellular communication [72, 73]. It is now clear that not only immune cells, but all cell types can release exosomes such as neurons [74], epithelial cells [75], Schwann cells, and tumour cells [76].

Since the earlier studies of exosomes being secreted by immune cells, and the first to show their potential role as mediators in immune response, a great number of studies have emerged to show the role of exosomes in immune function (reviewed in [77]). Apart from immune function, the

diversity of the roles of exosomes has expanded and is now recognised for their contribution to various diseases, such as the dissemination of viruses, pathogenesis of neurodegenerative disease, and promoting cancer progression (reviewed in [78]). The focus on this section will be on cancer-derived exosomes and the current studies and effects known so far on immune modulation, and its potential contribution and link to thrombosis. The second section to this chapter will present the methods for isolation and characterisation of exosomes.

An important point to note is the change in terminology used within the EV field of research from 'exosomes' to 'extracellular vesicles' [79]. It is the consensus among the EV international community to use "extracellular vesicles" to describe lipid-bilayer enclosed vesicles released from a cell and a term such as "small EVs" is recommended to describe exosomes [80]. The term "small EVs" is more accurate than "exosomes", as the latter depicts an endosomal origin which is very difficult to demonstrate unless caught in the act of release such as live imaging. However, readers should note that as this thesis was in preparation and writing before this change was noted, the use of the term "exosomes" was used and continued for the remainder of the thesis. In this thesis, the term cancer derived-exosomes in the experimental components are defined as small extracellular vesicles released from cancer cells with a size range of 30 - 150 nm.

2.1 Biogenesis and release of exosomes

The formation of exosomes consists of three steps: biogenesis, intracellular trafficking and release. Exosome biogenesis is initiated from the endosomal pathway which involves double invagination of the plasma membrane, resulting in the formation of early endosomes [78]. Early endosomes will recycle proteins at the plasma membrane and sequester various cargo which are incorporated into intraluminal vesicles (ILVs), leading to the formation of a multivesicular endosome, also known as MVBs [81]. Several pathways can modulate the formation of MVBs and the molecular composition of ILVs. These can occur through dynamic communication of MVBs with other organelles, such as the trans-Golgi network where vesicles are released to or taken up from [82]. Other modes occur through direct contact with the endoplasmic reticulum, mitochondrion or phagosomes [83-85]. Eventually, MVBs are either directed to lysosomes for degradation or fuse with the plasma membrane for release of ILVs into the extracellular milieu as exosomes [86].

The first and best-described mechanism for the generation of exosomes is the endosomal sorting complexes required for transport (ESCRT) machinery [87]. This ESCRT complex is also responsible for the cargo sorting of proteins into ILVs [88]. It is an intricate machinery composed of four multiprotein complexes: ESCRT-0, -I, -II and -III together with accessory proteins Alix, VPS4 and VTA1

[65]. The ESCRT complexes are recruited to the endosomal membrane in a step-by-step process [88]. Firstly, ESCRT-0 is recruited to early endosomes after recognition of ubiquitinated cargo and binding to the endosomal membrane. This facilitates the recruitment of ESCRT-I, which together with ESCRT-II, interact to form ILV buds at the endosomal membrane and combine with ubiquitinated cargo. A cargo-rich area is formed and ESCRT-III is assembled at the neck of the bud, leading to vesicle budding into the MVB. ESCRT-III is disassembled by the VPS4-VTA1 complex [88].

However, some evidence suggests that the formation and MVBs and ILVs can occur in the absence of ESCRT machinery. For example**,** Stuffers et al found that the inactivation of four proteins of the ESCRT complexes did not abolish ILV formation [89]. The authors also found that this resulted in tetraspanin CD63-rich exosomes. Tetraspanins are a family of proteins with four transmembrane domains. Some studies have suggested a role for different tetraspanins in the formation of ILVs. For example, CD9 knockout in mice resulted in fewer exosome release from bone marrow dendritic cells, compared to wild-type mice, while the expression of CD9 led to exosome release containing β-catenin [90]. In a breast cancer model in mice, the release of exosomes carrying Wnt11 from cancer-associated fibroblasts was dependent on CD81 [91]. In addition to proteins, lipids have also been involved in ESCRT-independent exosome biogenesis. This involves the generation of ceramide with the lipid metabolism enzyme neutral sphingomyelinase at the membrane of MVBs, which induces inward budding and gives rise to ILVs independently of ESCRT [92]. Thus, it is evident that exosomes can be secreted in ESCRT-dependent and -independent mechanisms, depending on the cell type and the cargo to be sorted in the given cell.

Once MVBs are generated, the appropriate stimulation will trigger its translocation from the perinuclear cytoplasm to the plasma membrane and will undergo fusion by exocytosis. The intracellular translocation and MVB docking to the plasma membrane process is controlled by the G-proteins Ral1, Rab27a and Rab27b [93, 94]. The exocytotic release of exosomes from cells then involves the soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARES) complex. This complex comprises proteins present on the cell membrane (t-SNAREs) and the membrane of the budding exosomes (v-SNARES) [95]. The SNARES allow for the apposition of the exosome and cell membrane, allowing for membrane fusion and subsequent release of exosomes through endocytosis.

2.2 Factors affecting exosome release

Currently, the factors that regulate the release and levels of circulating exosomes in bodily fluids such as blood remain undefined. Some *in vitro* investigations have suggested some factors which may impact exosome release. Hypoxic conditions in *in vitro* cell culture induced greater release of

exosomes from breast cancer cell lines, compared to those cultured in normoxic conditions [96]. In immune cells, exosome released was not constitutively released by B cells but was triggered in the response to stimulation of CD40 and IL-4 receptors via mitogens and cytokines [97]. Low pH condition, which is a hallmark of malignancy, was reported to cause increased exosome release from human metastatic melanoma cells *in vitro* [98]. Exosome release was significantly downgraded in cancer cells when pH was selectively buffered to counteract acidity [98, 99]. Several studies have also shown that cellular stress increases exosome release [100, 101]. Increased exosome release has also been reported in response to chemotherapy such as cisplatin treatment [101]. It is not clear why cell types increase exosome release in response to stress but it may be a means to remove waste. Another explanation is that exosomes may communicate with neighbouring cells about intracellular stress through an increased secretion. In contrast, the induction of autophagy in cells resulted in decreased exosome release [102]. The investigators of the study proposed that it may be due to the increased fusion of MVBs with autophagosomes with autophagy induction, which directs MVBs to the degradative pathway, resulting in the inhibition of exosome secretion.

2.3 Travel of exosomes in the blood

After the release of exosomes, a fraction will break down and its contents become available for contact with adjacent cells and mediate specific responses. Most exosomes, however, are resistant to breakdown and are highly stable structures in the extracellular fluid due to their lipid bilayer [103]. Some studies have attempted to delineate the route of exosomes once released into the circulation. In these studies, exosomes were biotinylated and either intravenously or subcutaneously injected into mice, and imaged to visualise which organs the exosomes were targeted to [104]. The authors found injection of B cell-derived exosomes had a half-life of 2 minutes, though exosomes were detected in the spleen up to 2 hours later. Another study investigating the efficacy of administering drugs via exosomes found exosomes in the brain and intestine 3 hours later [105]. Both studies found that exosomes were associated with macrophages in the circulation, suggesting macrophages as carriers through which exosomes travel in the blood. A study investigating the vascular distribution of nanoparticles of sizes ranging from 10 to 1000 nm using intravital microscopy and computational modelling, found that particles with exosome size range were randomly distributed within the blood vessel and would occasionally drift laterally to the periphery where leukocytes reside [106].

2.4 Uptake of exosomes

When reaching a target cell, a number of mechanisms have been proposed for the interactions and uptake of exosomes with recipient cells. These include fusion with the plasma membrane, adhesion with cell surface receptors resulting in ligand-receptor interactions, or internalisation via endocytosis or phagocytosis [107].

Direct interaction

A common route for mediating immunomodulatory function in exosomes is through the direct interaction of transmembrane ligands on exosomes binding directly with receptors on the surface of the recipient cell. In exosome and T cell communication, adhesion is achieved via integrins and is vital for communication. Dendritic cell-derived exosomes have been shown to carry major histocompatibility class-peptide complexes which bind to and activate T cells [108]. In exosomes from mature dendritic cells, ICAM-1 is critical to induce T cell responses [109] which is mediated by leukocyte integrin LFA-1 on dendritic cells [110]. This study highlighted the ability of exosomes to induce activation without the need for internalisation and processing of the exosomes. In macrophages, the cell adhesion molecule CD169 was required for the capture of B cell-derived exosomes [104].

Fusion with plasma membrane

Fusion involves the merging of vesicles with the plasma membrane of a cell. In a fusion assay using exosomes labelled with a lipid fluorescent probe, exosomes derived from metastatic melanoma cells were found to fuse with the plasma membrane, which was markedly reduced with filipin, a molecule known to bind to cholesterol and perturb the composition of the cell membrane [98].

Internalisation

Exosomes carry a range of transmembrane and membrane-anchored proteins that can enhance endocytosis within other cells. Alternatively, exosomes express proteins such as CD47, an integrin-associated transmembrane protein that functions to protect cells from phagocytosis [111, 112]. Other membrane-bound glycoproteins CD55 and CD59 that are found on exosomes have also been shown to play a role in limiting their clearance in the circulation [113]. Thus, these exosomes can avoid clearance in the circulation and travel to reach distant cells. Different pathways have been

described for the internalisation of exosomes into recipient cells. These include micropinocytosis, fusion to the plasma membrane or clathrin-mediated endocytosis. The uptake of exosomes can be non-specific, which is shared with all cell types [114]. However, specific targeting of cells would be necessary for the delivery of exosomal contents and exerting functional change. Some studies have suggested that this may be mediated by the surface composition of exosomes. For example, studies investigating the therapeutic potential of exosomes with the integration of nervous system-specific rabies viral glycoprotein on exosomes led to specific targeting to acetylcholine receptors in the brain [115].

The different methods of uptake and the specificity of exosomes for certain cell types add to the complexity of their function in cell-cell communication. For example, the oncogenic KRAS mutation in pancreatic cancer cells resulted in increased uptake of exosomes by micropinocytosis [111]. At the same time, these exosomes avoided phagocytosis by macrophages due to the expression of CD47. In melanoma cells, exosomes were taken up by the process of fusion with the plasma membrane [98]. In these cells, staining and confocal microscopy analysis suggested that exosomes specifically interact with the acidic vesicles within melanoma cells. The uptake of exosomes was also enhanced with low pH, suggesting that an acidic microenvironment may favour exosome-cell interaction and uptake in cellular physiology. In another study, it was reported that exosomes from PC12 cells (derived from rat adrenal medulla tumour), were more readily taken up by clathrin-mediated endocytosis [116]. Evidently, several different mechanisms have been observed for the uptake of exosomes in recipient cells. Though it is not known if different mechanisms of uptake lead to or have an impact on different fates of exosomal cargo. What determines if exosomes become destined for degradation or mediators of cellular signalling within a cell is not clearly understood.

2.5 Role of cancer-derived exosomes in cancer

Intercellular communication and oncogenic signalling are key features of cancer progression and metastasis. An increasing number of studies have demonstrated the role of exosomes in the progression and dissemination of cancer. Tumour-derived exosomes can transfer bioactive molecules between tumour cells and cells within the tumour microenvironment, such as the immune cells, fibroblasts, endothelial cell and mesenchymal stem cells [117, 118]. The transfer of specific microRNA has been shown to increase resistance to chemotherapy in cancer [119, 120]. The initiation of metastasis of cancer is not thought to be a random process. The ‘seed and soil’ hypothesis states that the seed (cancer cell) will only grow on suitable soil (at the metastatic site), meaning that a favourable environment to support the growth of tumour cells must be achieved.

This environment in distant organs is referred to as the pre-metastatic niche (PMN). In addition to local signalling within the primary tumour environment, tumours also signal over long distances to distant sites of future metastases to promote PMN formation and support the growth of disseminated cancer cells [121]. While the mechanisms of organ-specific mechanisms are not fully understood, the function of exosomes to carry and transfer molecules has attracted great interest in their ability to regulate metastasis [30, 122]. The importance of exosomes in PMN formation was further supported as the inhibition of exosome secretion, or inhibition of uptake at the site of future metastases resulted in decreased spontaneous metastases in tumour-bearing mice [30, 123].

In 2001, Wolfers and colleagues were the first to report the release of extracellular vesicles of multivesicular origin from human and mouse cancer cells, and suggested that these extracellular vesicles were exosomes [124]. These tumour-derived exosomes were immunogenic and induced potent T cell antitumour effects through the transfer of tumour antigens to dendritic cells. Several other studies have reported tumour-derived exosomes carrying tumour antigens [125-127], thus demonstrating the ability to initiate an anti-tumour response. However, despite the abundance of tumour-derived exosomes being secreted in the tumour microenvironment, it does not seem to induce an effective immunostimulatory role in cancer patients with advanced disease. Studies from the past decade are showing evidence for an immunosuppressive role of tumour-derived exosomes, thus facilitating immune evasion and cancer progression. An outline of the effects of cancer-derived exosomes on modulation of different immune cells and its effect on cancer progression will be presented below. Readers can refer to an extensive review published elsewhere ([128]).

2.6 Exosome levels: healthy vs cancer

It is known that virtually all cell types can produce exosomes, and they are ubiquitous in both healthy and pathological states [78]. However, measuring the rate of exosome production in cells is challenging due to the dynamic process and continuous production and uptake of external exosomes by any given cell [78]. One study compared the amount of shed exosome from noncancerous and cancerous breast epithelial cells [129]. The study used time-lapse monitoring of single cells and captured shed exosomes with a tetraspanin antibody. The secretion rate of exosomes in breast cancer cell lines MCF7 and MDA-MB-231 was 60 – 65 exosomes per hour, while in noncancerous tissue-matched cells it was almost 3 times more [129]. However, other studies have shown that levels of exosomes in the serum of cancer patients is greater compared to healthy individuals [130, 131]. More recent studies have investigated the effect of surgical resection of tumours in cancer patients, which led to a significant decrease in the level of exosomes in the plasma [132, 133]. Correlations have also been made with tumour size and progression. Logozzi et al. correlated levels of exosomes in plasma with tumour size in a mouse model of melanoma [130]. In another study, Liu

et al. investigated 208 non-small cell lung cancer patients and found that exosome levels in plasma correlated with tumour stage progression [134]. Quantification of exosomes from plasma of patients with oesophageal cancer found higher levels of exosomes when compared to patients without malignancy [135]. Thus, it appears that in general, cancer cells secrete greater amount of exosomes compared to non-cancer cells, and it's secretion in cancer patients correlates with tumour size and progression.

2.7 Effect of cancer-derived exosomes in immune modulation

Infiltrating immune cells such as T cells, B cells, macrophages and dendritic cells in the tumour microenvironment play key roles in tumour progression through interaction with the tumour and stroma. Several studies have shown the diverse role of tumour-derived exosomes in modulating immune response, by delivering immune-stimulatory or immune-suppressive molecules. Emerging studies are recognising that tumour cells can secrete bioactive molecules to modulate the immune response. Some of the earliest experiments regarding the functions of exosomes were investigating the impact on immune effector cells [127]. These will be discussed along with more recent studies to provide an overview of the functions of cancer-derived exosomes in modulating immune cell function.

2.7.1 Tumour antigen presentation and anti-tumour immune response

Initiation of anti-tumour responses by T cells is mediated by the uptake and processing of tumour antigens by dendritic cells and presentation on major histocompatibility complex (MHC) molecules. An *in vitro* model showed that exosomes from tumour cells contain and transfer tumour antigens to dendritic cells, which in turn induced potent CD8+ T cell anti-tumour effects [124]. Other studies have reported the roles of HSP70 carried on tumour exosomes along with other tumour antigens that promote migration and cytolytic activity of NK cells, an effect that was abrogated by an anti-HSP70 antibody. Interestingly, further investigation found that the exosome membrane-associated HSP70 induced TNF-α production in activated macrophages, at 260-fold greater than the recombinant protein [136]. These *in vitro* studies are limited and may not completely reflect the immune responses *in vivo.* More recent studies are giving evidence to support that cancer-derived exosomes are assisting cancer cells in tumour progression and metastasis.

2.7.2 Exosomes and modulation of adaptive immune cells

Exosomes derived from cancer cells has shown to exhibit anti-proliferative activity of lymphocytes. The potent proliferative activity of lymphocytes in response to IL-2, could be abolished in the

presence of cancer cell-derived exosomes [127]. Upon separation of T cells into CD8+ and CD4+ fractions, it was found that this effect was not observed in the CD8+ fraction, while it was retained in the CD4+ T cells. This suggested the antiproliferative effect is mediated by exosomes through the CD4+ T cell population. The mechanism of regulatory T cell activation is thought to require transforming growth factor-β (TGF-β) which is expressed on some cancer cell line-derived exosomes. Blocking exosome-derived TGF-β led to abrogated anti-proliferative activity in IL-2 stimulated lymphocytes [127]. Interestingly, the effect of exosome TGF-β when compared to soluble TGF-β at matched concentrations, was more potent from exosomes.

Apart from modulation of regulator T cells, cancer-derived exosomes have also been shown to impact cytotoxic NK cells and CD8+ T cells. Cancer exosomes express NKG2D ligands which trigger a downregulation of surface expression of NKG2D on NK and CD8+ T cells. This resulted in a decrease in IFNγ production and cytotoxic function [137]. Similarly, exosomes from gastric cancer cells were able to modulate CD8+ T cells cytokine secretion and gene expression. These exosomes were targeted to the lungs where they were shown to create an immunosuppressive tumour microenvironment, which promoted gastric cancer lung metastasis in mice [138].

2.7.3 Exosomes and modulation of innate immune response

Macrophages

Macrophages are well known to participate in innate immune responses and have been shown to be polarised into M1 (anti-tumour) and M2 (pro-tumour) phenotypes [139]. Studies have shown that this polarisation can be induced by tumour-derived exosomes, mediated by various molecules. Colorectal cancer-derived exosomes induced polarisation of macrophages to M2 pro-tumour tumour-associated macrophages via exosomal microRNA (miR)-934 which enhanced tumour proliferation [140]. This led to enhanced colorectal cancer liver metastasis through formation of a premetastatic environment with tumour-associated macrophages. The overexpression of miR-940 in ovarian cancer-derived exosome induced M2 polarisation and promoted epithelial cell proliferation and migration [141]. One study showed colon cancer cells with gain-of-function mutant 53 selectively released exosomes enriched with miR-1246, which transferred to macrophages and promoted anti-inflammatory suppression and epithelial-mesenchymal promoting factors, contributing to cancer progression and metastasis [142].

Neutrophils

More recently, neutrophils have also been reported to exhibit two polarising phenotypes, the N1 anti-tumour or N2 pro-tumour phenotype [143]. Zhang et al. identified that tumour-derived

exosomes were responsible for inducing N2 polarisation of neutrophils to promote gastric cancer cell migration [144]. This polarising effect of neutrophils was mediated through the transfer of HMGB1 protein from exosomes and resulted in the activation of the NF-KB pathway via toll-like receptor 4. The release of neutrophil extracellular traps was found in the treatment of mice neutrophils with breast cancer 4T1-derived exosomes [145]. Further details on the effect of tumour-derived exosomes on neutrophil function will be described in the following chapter.

2.8 Inflammation and thrombosis

The interplay of activating inflammation and thrombosis has been well described and is a concept termed thrombo-inflammation (reviewed in [146]). Thromboinflammation occurs in response to inflammation, causing the activation of platelets, recruitment of leukocytes and exacerbation of inflammation, resulting in tissue and endothelial damage [147]. Thromboinflammation is not to be confused with immunothrombosis, as while both involve the activation of platelets, coagulation system and immune cells, the latter was first introduced to describe a host defence response to pathogens, resulting in thrombosis that prevents systemic spread of pathogens [148]. The term immunothrombosis is now broadly used to describe thrombotic events arising from infection as well as sterile-driven inflammation [149, 150]. The main difference between the two is the outcome; immunothrombosis results in thrombosis and blood vessel occlusion, whereas thromboinflammation results in excessive immune cell recruitment, inflammation and ultimately tissue damage [147].

With the increasing evidence of tumour-derived exosomes and their ability to affect immune cell and inflammatory responses, it is hypothesised that tumour-derived exosomes can have various effects on the modulation of the immune system and ultimately influence the system to become pro-thrombotic and promote thrombotic events in cancer. For example, M1-polarised macrophages were found elevated in the blood of deep vein thrombosis patients [151]. These M1 macrophages upregulated the expression of endothelial cell adhesion molecules such as VCAM-1 and ICAM-1. The polarisation of monocytes to macrophages via interleukin (IL)-14 and IL-13 led to increased tissue factor release, as well as increased EV release with increased TF activity [152]. In regard to thrombus resolution, an *in vivo* model of deep vein model thrombosis showed a predominance of M2 macrophages within thrombi, which suggested that polarisation of macrophages to M2 phenotype and its role in impairment of thrombus resolution [153].

Neutrophils are well known to have roles in innate immunity, however their importance in thrombosis has only recently been recognised. The first solidifying evidence of neutrophil's role in

thrombus formation was in 2012. Darbousset and colleagues found that neutrophils expressing tissue factor were the first to accumulate at the site of endothelial injury, even before platelets [154]. In addition, the depletion of neutrophils led to a significant reduction in thrombus formation in a mice model of venous thrombosis [155]. The P2X1 receptor on neutrophils is important in neutrophil activation and fibrin generation, and its expression on both neutrophils and platelets is required for thrombosis. [156].

Apart from macrophages and neutrophils, platelets are known to be essential components of the immune system with roles in interacting with leukocytes and driving thrombosis [157]. In addition, the endothelium is an important regulator of thrombo-inflammation, whereby under healthy conditions it exerts a range of anti-coagulant and anti-platelet properties [158]. However, in response to inflammation, these anti-coagulant properties are lost, leading to a procoagulant endothelial surface [146]. These two components of platelets and endothelial cells and their roles in thrombosis will be described further in the latter part of this thesis.

2.9 Methods of exosome isolation and characterisation

Isolation of exosomes

The isolation of exosomes can be a tedious and costly process, with some methods requiring specialised equipment. Since the increasing interest in exosome research, there have been several isolation methods developed, based on different principles, to achieve exosome purification. Each technique exploits certain properties of exosomes such as density, size, shape and surface protein to aid isolation [159]. While each method has its advantages and disadvantages, the most commonly used methods employed in laboratories include differential centrifugation, density-gradient centrifugation and affinity column [160]. Other traditional methods include size-based isolation (ultrafiltration and size-exclusion chromatography) and precipitation. In the past few years, more research and recent developments have led to new techniques such as microfluidic chips, as well as the integration and innovation of traditional methods [161].

Ultracentrifugation methods

Ultracentrifugation-based isolation is considered the gold standard and is one of the most commonly used technique in laboratories for the purification of exosomes [159]. There are two types of ultracentrifugation-based methods: differential ultracentrifugation and density gradient ultracentrifugation. Differential centrifugation uses a series of centrifugal cycles with different

centrifugal speeds and durations to isolate exosomes, which is achieved based on the differences in size and density of the components. It begins with cleaning steps to remove dead cells, cellular debris, apoptotic bodies and microvesicles [162]. At each step, the pellet is discarded and separated from the supernatant, which will undergo another cycle of centrifugation at a higher speed. The final supernatant is spun at 100,000 – 120,000 x g for 2 hours to obtain a pellet with enriched exosomes [163]. While this method is lengthy, labour-intensive and requires expensive equipment, its greatest advantage is the ability to process large volumes of samples. A variation to this differential ultracentrifugation method is the density gradient ultracentrifugation. This method is more time-consuming due to the layering of the sample over a high-density medium, such as sucrose or iodixanol [159]. This results in a progressively decreasing density from the bottom to the top of the tube. The application of centrifugal force will cause the solute to move through the gradient at a specific sedimentation rate based on their size and mass, facilitating the separation of exosomes from larger vesicles [162]. In comparison to the differential ultracentrifugation, gradient ultracentrifugation results in higher purity [164].

Size-based technique

Ultrafiltration is one of the more popular size-based isolation techniques. It involves the use of a membrane filter with molecular weight or size exclusion limit to isolate particles of a pre-determined size range [165]. This method is faster than ultracentrifugation and doesn't require special equipment, however the use of force to filter exosomes may result in deformation and break up of large vesicles, which may affect results and downstream analysis [166]. Another popular method of size-based isolation is size exclusion chromatography (SEC). SEC uses a porous stationary phase, and a biofluid as a mobile phase [167]. The stationary phase allows for the sorting of molecules and particles based on their size, resulting in differential elution whereby bigger particles elute first, followed by smaller particles. This method is highly efficient and rapid, with an average of 20 minutes processing time per sample, however exosome yields are low [168].

Immuno-affinity capture

The immune-affinity methods rely on the separation of exosomes based on the presence of specific surface proteins. The commonly used antibodies are tetraspanins CD9, CD63 and CD81. This method involves incubation of sample with magnetic beads that are coated with specific antibodies which are targeted to specific surface proteins [162]. This method is commonly used in conjunction with ultracentrifugation to increase the yield of exosomes. Due to the user selecting to target a specific subset of vesicles, the main disadvantage is that it may not reflect all of the exosomes present. While this method can offer high purity and specificity, it is a rather expensive technique as it relies

on high number of antibody-conjugated beads. Thus, it would not be suitable for use with large sample volumes.

Precipitation

The precipitation method is achieved by altering the solubility of exosomes, allowing for exosome aggregates to form which can then be precipitated out of the biological sample. This technique uses a water-excluding polymer, and polyethylene glycol (PEG) is the most commonly used for exosome isolation. This method is easy and fast, and also lacks exosome damage and the need for specialised equipment. However, the main issue encountered is contamination from co-precipitation of various contaminants such as proteins [169].

At the time the work in this thesis was carried out, the gold standard of exosome isolation was ultracentrifugation. It was the most widely used technique while other methods were still new and being developed. Thus, for the work requiring the isolation of exosomes, the method of differential ultracentrifugation was used. Differential ultracentrifugation allows processing of large samples which is ideal for the work in this thesis as it involved isolation from a large volume of cancer cell culture-derived supernatants. This method has disadvantages as it may not have high purity and low yield, however as exosome research further progresses it is expected that new technologies and techniques will allow the isolation of exosomes with greater yield and specificity.

PART 2: Isolation and Characterisation of Exosomes

Part 2 of this chapter is the study component which aims to isolate exosomes from pancreatic cancer cell lines. The pancreatic cancer-derived exosomes were then characterised to ensure that they were within the expected size range and carried markers that are known to be enriched in exosomes. The enrichment of exosomes will be critical for the latter work of the thesis as they will be used to investigate the effect of exosomes on blood and endothelial cell activation.

Cell culture and maintenance

Pancreatic cancer cell lines AsPC-1, BxPC-3 and MIA PaCa-2 were obtained from American Type Culture Collection. AsPC-1 and BxPC-3 were cultured in RPMI-1640 medium (Sigma) supplemented with 10% foetal bovine serum (FBS). MIA PaCa-2 was cultured in DMEM media (Sigma) supplemented with 10% FBS. Cells were maintained at 37°c in the presence of 5% CO_2.

Exosome isolation

Exosomes were isolated from the conditioned medium of pancreatic cancer cell lines AsPC-1, BxPC-3 and MIAPaCa-2 cells. Approximately 3 x 10^6 cells were seeded in one T175 cm^2 culture flask, and a total of three flasks were seeded for each biological replicate. Cells were seeded in their respective medium used for maintenance. After 48 hr when cells reached 60% confluency, the supernatant was removed and cells washed with PBS. The media was switched to their respective media, replacing FBS with exosome-depleted FBS to prevent cross-contamination of serum-derived exosomes. After 48-72 hr when cells reached 80-90% confluency, the conditioned medium was pooled from the three flasks (total of approximately 45 ml) and processed through differential centrifugation to remove cells and cell debris at 480 x g for 5 min and 2,000 x *g* for 10 min, respectively. The conditioned media was filtered through a 0.22 µM filter and stored at -80°C until ready for ultracentrifugation. Exosomes were pelleted at 100,000 x g for 2 hr and washed with PBS. Each tube was washed thoroughly with approximately 1.5 ml of PBS for three cycles. The exosomes in PBS from the washes were pooled and underwent a final centrifugation at 100,000 x g for 2 hr. Exosome pellets were resuspended in filtered PBS and stored at -80°c. Protein concentration of lysed exosomes were quantified using a Micro BCA assay which was used to normalise the amount of exosomes used in subsequent experiments. Exosomes were limited to one freeze-thaw cycle.

Characterisation of exosomes

Exosome characterisation

As the research of extracellular vesicles and exosomes is relatively new and still gaining research advancements, the guidelines for exosome characterisation are continuously being reviewed and evolving. The guidelines are set by an international community of EV experts that have formed a group called the International Society for Extracellular Vesicles (ISEV). In 2018, ISEV proposed the minimal information for studies of extracellular vesicles (MISEV) guideline for the characterisation of EVs, containing a list of categories of markers to be analysed to help standardise isolated EVs for use in research [80]. In the list of guidelines, there are three categories that are recommended for the characterisation of EVs. These are quantification through protein content and particle number, protein-content based characterisation for markers, and single EV characterisation through imaging and non-imaging methods to assess a large number of single EVs such as nanoparticle tracking analysis. The characterisation of exosomes used in this study were western blot analysis for exosomal protein markers, scanning electron microscopy for morphology and size, and nanoparticle tracking for quantification and size.

i. Expression of protein markers

To confirm the enrichment of exosomes, western blot for tetraspanin CD63 and Alix, representative exosomal protein markers [170] was performed. Exosome samples were lysed in RIPA buffer and 5 µg of protein was subjected to western blot analysis. Samples were separated on an SDS-page gel in Laemmli buffer. For CD63 analysis, Laemmli buffer without beta-mercaptoethanol was used to achieve non-reducing conditions. Samples were separated at 200 V for 45 min. Proteins were transferred to a PVDF membrane at 400 V for 90 min. The membrane was blocked with 5% skim milk in TBST-Tween for 1 hr. Membranes were incubated overnight at 4°c with anti-CD63 (1:250, Thermofisher Cat. #10628D) or anti-Alix (1:1000) antibodies diluted in 3% BSA in TBS-T. The following day, membranes were washed three times with TBS-T before incubating with secondary antibody anti-mouse (1:10,000) diluted in 5% skim milk TBS-T. The membrane was washed three times and imaged using the ChemiDoc system.

CD63 and Alix were both present in exosome lysates, confirming the enrichment of exosomes (Figure 2.1a, b). Even with four times greater protein loading of cell lysates (20 µg) than exosome lysate (5 µg), western blot analysis showed a similar amount of CD63 in pancreatic cancer-derived exosomes compared to whole cell lysates, indicating the enrichment of exosomes (Figure 2.1a). Furthermore, other exosome markers Alix and GAPDH were also present in exosome lysates (Figure

2.1b.). The negative marker, GM130 was also analysed, to confirm the absence of membrane-bound vesicles from the Golgi apparatus (Figure 2.1c). On the other hand, GM130 was expressed in the cell lysates as expected. Together, the presence of exosomal markers CD63, Alix and GAPDH and the absence of the negative marker GM130, confirm the enrichment of exosomes from the isolation procedure.

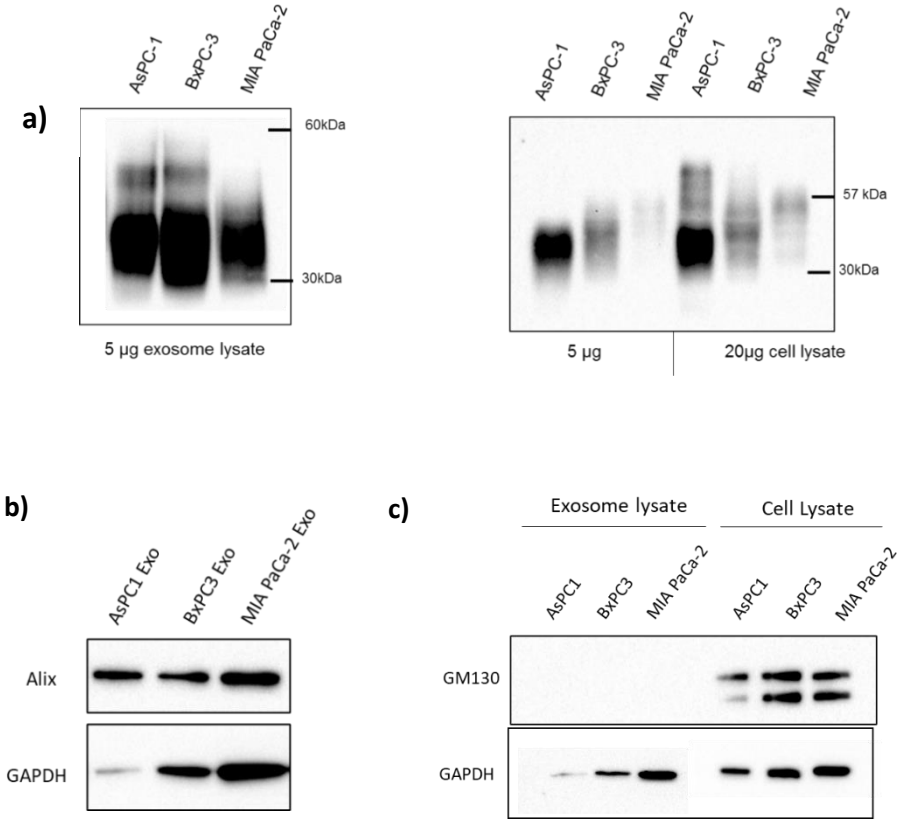


Figure 2.1 Western blot characterisation of exosomes. Exosomes were subjected to SDS-PAGE and proteins were transferred to PVDF membrane for analysis of exosomal marker proteins a) Exosomal marker CD63. Five µg of exosome lysate proteins were loaded. Right: Comparison of the presence of CD63 in exosome lysate and cell lysate (20 µg) indicates enrichment of exosomes as there was four times less protein loaded in exosome lanes. Representative blot of two independent experiments. B) Alix and GAPDH were observed in exosome lysates. Representative blot of three independent experiments. C) The absence of GM130, a negative marker for exosomes was confirmed in exosomes, while present in cell lysates. Blot from two independent experiments. Note: Parts from this figure have been used in a following chapter of this thesis (Chapter 4), as it was written for research article submission.

ii. Damage-associated molecular patterns

In collaboration with co-supervisor Professor Marco Falasca's lab, proteomic analysis of exosomes derived from the same panel of pancreatic cancer cell lines has been obtained. The proteomics data shows that pancreatic cancer-derived exosomes have high upregulation of damage-associated molecular patterns (DAMPS) compared to exosomes derived from non-malignant pancreatic cells [171]. DAMPs are molecules released from stressed or dying cells which act as endogenous danger signals, initiating inflammatory responses and activation of other cell types [172]. The DAMPs that were upregulated in pancreatic cancer-derived exosomes include HMGB1, S100 and histone proteins. To confirm the presence of DAMPs in exosomes isolated from pancreatic cancer-derived exosomes, western blotting for Histone H3 and S100A4 proteins was performed. Figure 2.2 shows the expression of histone H3 in all exosomes, while S100A4 was detected in AsPC-1 and Mia PaCa-2 exosomes but not BxPC-3. The expression levels observed in S100A4 is consistent with the proteomics data, which showed greater expression in MIA PaCa-2 than in AsPC-1, and the absence of S100A4 in BxPC-3 exosomes.

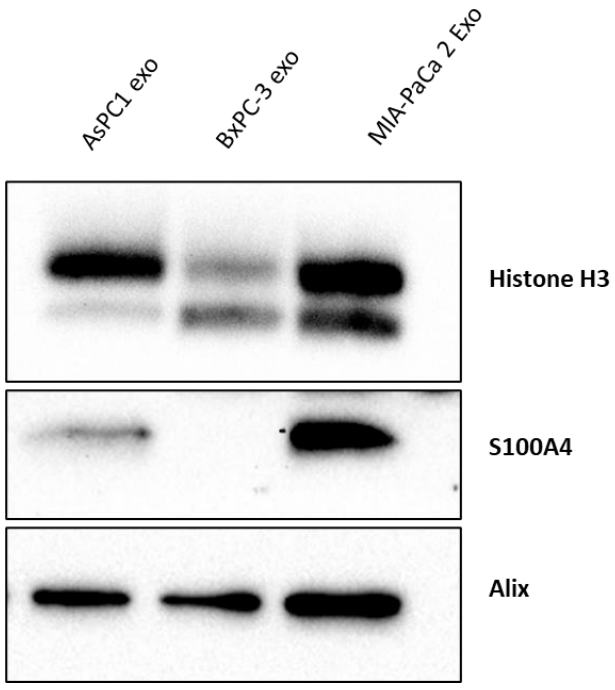


Figure 2.2 Western blot characterisation of DAMP expression in pancreatic cancer-derived exosomes. The exosome lysates (10 µg) were separated on SDS-PAGE and transferred to PVDF membrane. The blot was additionally probed for DAMP molecules, histone H3 and S100A4. Alix was used as housekeeping control. The expression of histone H3 is observed in all exosomes, while S100 A4 is expressed in all but not BxPC-3 exosomes. Representative blot of two independent experiments. Note: The figure shown has been used in a following chapter of this thesis (in Chapter 4) as it was written for research article submission.

iii. Scanning electron microscopy

Exosomes suspended in PBS were fixed with 4% paraformaldehyde overnight. The samples were dehydrated with increasing concentrations of ethanol at 20%, 40%, 60% and 90% ethanol for 10 min each. Samples were left to dry and then processed with platinum coating before imaging on a field emission scanning electron microscopy. Images were taken with assistance from a technical operator at the John de Laeter Centre, Curtin University. Images of exosomes show vesicles with round morphology and within the exosome size range of 30-150 nm (Figure 2.3). Three independent samples were sent for imaging and the clearest images that were obtained and are representative of exosomes are shown in Figure 2.3. Similar images were obtained by a member from co-supervisor Marco Falasca's laboratory using the same exosome isolation procedure and have been published elsewhere [171].

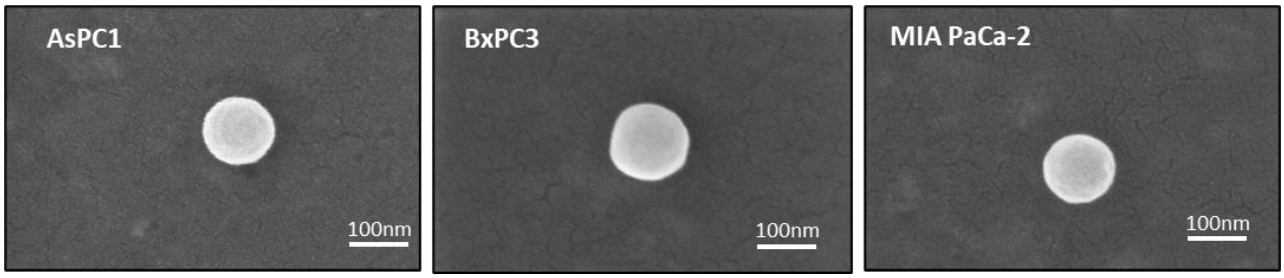


Figure 2.3. Scanning electron microscopy of pancreatic cancer-derived exosomes. Exosomes were fixed with 4% PFA and then dehydrated with ethanol, and platinum coated before imaging on the Neon scanning electron microscope. Three independent samples from each cell line were sent for imaging. Images shown are the clearest that were obtained and give the best representation of exosomes. Scale bar = 100 nm. Note: The figure shown has been used in a following chapter of this thesis (Chapter 4) as it was written for research article submission.

iv. Nanoparticle tracking analysis

Our laboratory, in collaboration with co-supervisor Professor Marco Falasca, routinely conducts Nanoparticle Tracking Analysis (NTA) on the pancreatic cancer-derived exosomes that are isolated within the laboratory. The NTA of these exosomes were conducted by former lab member Katerina Emmanouilidi and the results of the analysis can be found in the supplementary section of their published research article [171]. The reported results of the NTA from the paper is attached below (Figure 2.4), which show the results for the same panel of pancreatic cancer-derived exosomes that have been isolated for experiments used in this thesis i.e., AsPC-1, BxPC-3 and MiaPaCa-2.

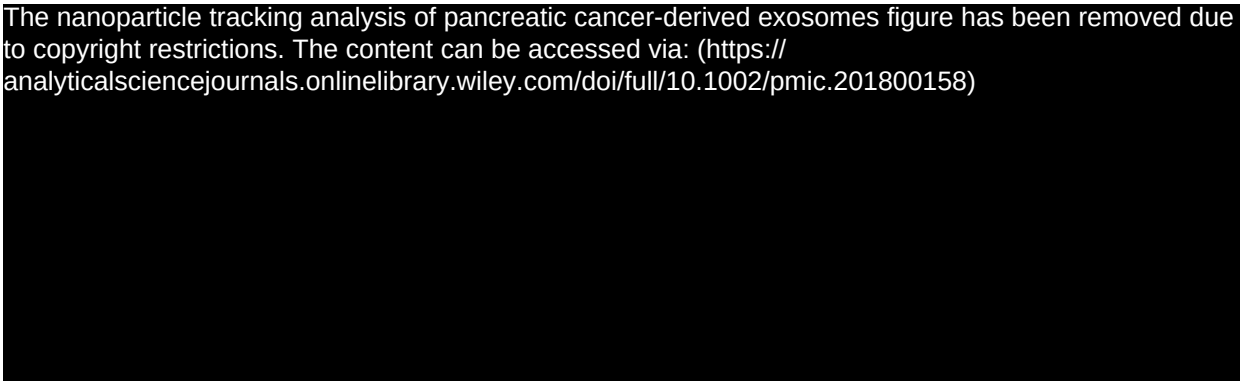


Figure 2.4 Nanoparticle tracking analysis of pancreatic cancer-derived exosomes. NTA characterisation of exosomes isolated from three pancreatic cancer cell lines AsPC-1, BxPC-3 and MIA PaCa-2, published by co-supervisor Marco Falasca's lab [171].

Summary

The choice to use differential ultracentrifugation for exosome isolation for experiments in this thesis was largely based on it being the current gold-standard method, and the capacity to process large volumes of sample at a time. However, there are a few disadvantages with this method such as low purity and low yield, the need for an ultracentrifugation (a costly instrumentation), and a time-consuming process [173]. In our laboratory, it took an average of around 6 hours from the collection of conditioned media to obtaining a final exosome-enriched sample.

One limitation is the low purity that has been reported for exosome isolation with differential ultracentrifugation. Exosomes derived from cancer may be contaminated with protein and immune complexes as they share the same biophysical parameters as exosomes [174]. To negate this issue, it was suggested that following with subsequent density gradient ultracentrifugation would not result in interference with immune complexes, as they have a greater density (> 1.20 g/mL) compared to exosomes (1.13-1.19 g/mL) [174]. Indeed, a study showed that exosomes isolated from gradient ultracentrifugation resulted in greater purity, while exosomes isolated from differential ultracentrifugation were contaminated with a residual matrix composed of biomolecules [164]. The use of density gradient ultracentrifugation requires a complex procedure which would not have been feasible to use in this study. Currently, it is difficult to achieve both high yield and purity with exosome isolation methods. New methods of isolation are emerging such as microfluidic chips which aim to improve yield and purity, and decrease isolation time [161].

Chapter 3

The effect of pancreatic cancer-derived exosomes on neutrophils and implications for cancer-associated thrombosis

3.0 Contribution to review publication:

A brief section on the effects of cancer- and neutrophil-derived exosomes in cancer and neutrophil function was written by the author in the following review article:

- Fyfe D, Dye D, **Abdol Razak NB,** Metharom P and Falasca M. *Immune evasion on the nanoscale: Small Extracellular Vesicles in Pancreatic Ductal Adenocarcinoma immunity.* Seminars in Cancer Biology. 2023. Review.

This chapter will elaborate on cancer-derived exosomes and their effects on neutrophils in cancer, and how it may potentially contribute to cancer-associated thrombosis.

3.1 Introduction

Neutrophils are the most abundant immune cell population in human blood and are regarded as the frontline defenders in response to invading pathogens. They are rapidly recruited to sites of infection and employ vast antimicrobial effector mechanisms, including phagocytosis, production of reactive oxygen species and degranulation [175]. In 2004, a new mechanism was reported by Brinkmann and colleagues, by which neutrophils decondense their chromatin to expel a web-like matrix containing DNA, histones, and antimicrobial proteins [39]. These structures were termed neutrophil extracellular traps (NETs) and were described as a novel mechanism that directly entraps pathogens and provides extracellular killing of pathogens. In recent years, the role of neutrophils has gone far beyond the well-appreciated role of host defence. Accumulating studies are recognising the diversity of neutrophil function and their roles in the pathogenesis of chronic diseases, including autoimmune disorders and cancer [176, 177].

Classically, neutrophils were thought to be a homogenous population with a primary function in innate immune response, fulfilling a specific set of functions [178]. This classical view was assumed mainly owing to their short lifespan (estimated half-life of 6-8 hr) [179] and limited machinery for protein synthesis that restricts phenotypic changes [180]. As a result, the function of neutrophils was not investigated as deeply as other myeloid cells in earlier years. However, more recent and accumulating evidence has revealed neutrophil heterogeneity in various pathologies, altering the old paradigm that neutrophils are a homogenous cell population that only participate in innate immune defence. Now it is recognised that neutrophils are transcriptionally active cells [181], capable of responding to signals, producing cytokines and inflammatory factors that regulate inflammation and the immune system [182-184]. Consequently, the functions of neutrophils now go beyond the basic killing of pathogens, contributing to immune responses through communication with other immune cells, and the initiation or shaping of the immune response of chronic inflammatory diseases (extensively reviewed in [175]).

In particular, the role of neutrophils in cancer has become increasingly apparent in the past decade. With a short half-life, it was not expected that neutrophils would contribute to chronic diseases, although some evidence suggests that cytokines secreted from tumour cells such as granulocyte colony-stimulating factor (G-CSF), interleukin-6 or tumour necrosis factor can prolong the lifespan of neutrophils [185, 186]. In addition, several observations have led to the assumed role of neutrophils in cancer, such as the accumulation of neutrophils in the peripheral blood of cancer patients [183], and an elevated neutrophil-to-lymphocyte ratio is also (NLR) as a predictive marker of poor clinical prognosis in cancer patients [181]. The NLR as an indicator of poor prognosis has been reported in

many tumour types such as breast cancer, metastatic melanoma, pancreatic and prostate cancer [182-184]. Furthermore, a high count of neutrophils was also shown to correlate with an increased risk of cancer-related thrombosis [187].

3.1.1 Tumour-associated neutrophils

Immune cells including neutrophils infiltrate solid tumours and form the tumour microenvironment (TME) along with other cellular and non-cellular extracellular matrix components. While macrophages are recognised to be the major component of the TME infiltrate [188], neutrophils have emerged as players and mounting evidence is suggesting that the role of tumour-associated neutrophils (TANs) may have been underestimated [189]. Many of the studies of neutrophils in cancer were from circulating neutrophils from mouse models or cancer patients, thus studies of tumour-associated neutrophils were limited [190]. The results of various studies suggest TANs have the potential to have both anti-tumour and pro-tumour properties [190], with more studies giving evidence for a pro-tumour role of TANs, especially during the progression of cancer [191]. This pro-tumoral profile can be favoured by cancer themselves, via secretion of cytokines such as interleukin-35 and transforming growth factor-β (TGF-β) [143, 192] or indirectly via tumour-derived exosomes [144].

A high NLR may likely reflect high tumour-infiltrating neutrophils. One study in pancreatic cancer patients observed that a high NLR showed a trend with a higher tumour-infiltrating neutrophils (TANs) [193]. However due to the high level of interpatient variability, statistical significance could not be reached. A few studies have revealed a correlation between TANs with poor prognosis, including in melanoma, head and neck cancer and hepatocellular carcinoma [194-196]. In addition, high densities of tumour-infiltrating neutrophils have been shown to correlate with more advanced-stage diseases of cancer in patients with glioma and gastric cancer [197]. Pancreatic tumours were more likely to be infiltrated by neutrophils if they were the aggressive type with known poor prognosis [198]. While some cancers show consistent correlation with poor prognosis, it is still a matter of debate in some cancers such as colorectal and lung cancer, as these cancers did not show a correlation between TANs with poor prognosis [199, 200].

In 2005, Fridlender and colleagues proposed that neutrophils are capable of the N1 and N2 phenotypes similar to the M1 anti-tumour and M2 pro-tumour phenotypes which is well established in macrophages [143]. Following this, the first attempt to polarise neutrophils to N1 and N2 phenotypes *in vitro* was made [201]. Ohms and colleagues used a cocktail of factors to polarise neutrophils to either N1 or N2 phenotypes with the use of a pan-caspase inhibitor to inhibit spontaneous apoptosis and increase their lifespan. While the authors were able to polarise

neutrophils to different phenotypes, the study had a limitation as the use of a caspase inhibitor meant any increase in neutrophil lifespan due to the stimulation cocktails could not be determined. It is possible that neutrophils' lifespan can be prolonged in cancer, as one study showed the half-life of neutrophils in cancer patients increased to 17 hours [202]. N1 neutrophils have shown direct tumour cytotoxicity by reactive oxygen species production, activation of CD8+ T cells and antibody-dependent cellular cytotoxicity [143, 203, 204]. On the other hand, N2 neutrophils facilitate cancer progression by the reconstruction of extracellular matrix, inducing angiogenesis and increased pro-tumourigenic cytokine production [143, 205]. Despite these studies, the role of N1 and N2 neutrophils in cancer has not been as extensively studied as tumour-associated macrophages. Furthermore, most of the studies have been conducted in mice models and these distinct phenotypes in humans are yet to be clearly established.

The relationship between TANs and their role in cancer-associated thrombosis has not been studied thus far. It has been reported that TANs in the tumour microenvironment can release NETs [204, 206] which can have consequences for CAT given the role of NETs in thrombosis. The differences in NET production from N1 and N2 neutrophils, and whether N1 or N2 neutrophils have a greater propensity to release NET is not known. NET release from TANs is likely to have great implications on thrombosis due to several NET components capable of affecting the activation of coagulation and host cells that have roles in thrombosis. For example, DNA can activate the coagulation cascade [207], histones and cathepsin G can activate platelets [208-210], and myeloperoxidase can promote endothelial dysfunction [211].

3.1.2 NETs in cancer

There have been many reports of NETs being observed in mouse models of cancer and cancer patients [212]. Despite this, the specific cancer-derived mediator/s and signalling mechanism for NET formation in cancer are unknown. It appears that NETs can have variable roles in cancer, by either promoting or inhibiting tumour progression. On the one hand, NETs have been shown to promote apoptosis of cancer cells and improve survival in cancer patients [213, 214]. On the other, an increasing number of studies are inclining towards a pro-tumour role of NETs in various cancers by promoting tumour proliferation, metastasis and tumour angiogenesis [215-219]. NETs can promote tumour progression (both by tumour proliferation and growth) by different mechanisms. NETs isolated from chronic lymphocytic leukemia patient neutrophils were shown to induce activation markers and inhibition of apoptosis in leukemic B cells [220]. Tumour proliferation and migration in diffuse large B-cell lymphoma *in vitro* was stimulated by NETs through activation of NFκB and STAT3/p38 pathways [217]. *In vivo* studies showed that the treatment of tumour-bearing mice with DNAse (to disassemble NETs) resulted in reduced NETs in tumour tissues and also significant

decrease in tumour growth [217]. NETs also increased cancer cell proliferation by stimulating pathways to increase mitochondrial biogenesis via neutrophil elastase [221]. HMGB-1 on NETs was found to mediate colorectal cancer cell proliferation and migration *in vitro* [222]. Interestingly, one study has shown that NETs participate in the awakening of dormant lung cancer cells and inducing their proliferation and metastatic growth in a mouse model injected with dormant mouse cancer cells, after sustained lung inflammation from exposure to LPS [223]. Migration of tumour cells has also been shown to be enhanced by NET induction of endothelial-mesenchymal transition markers in breast and pancreatic cancer cells *in vitro* [224, 225]. The role of NETs in metastasis has also been reported *in vivo*. In a mouse study, NETs were shown to mediate metastasis through NET-bound DNA and interaction with the transmembrane CCDC25 protein on breast cancer cells [216]. The treatment of DNAse (to target NETs) in tumour-bearing mice resulted in reduced metastatic seeding and suppression of lung metastasis [226]**.** The role of NETs in angiogenesis has also been described where isolated NETs were able to induce angiogenesis in human pulmonary artery endothelial cells *in vitro* [218].

With the accumulating evidence, the role of NETs in cancer may have been greatly overlooked as not only can it contribute to cancer-associated thrombosis, but also tumour progression. Thus, it is of great interest to identify specific mediators and the signalling pathways involved, as understanding these and its therapeutic targeting may have the potential to improve outcomes in cancer by impeding the progression of cancer and reducing the risk of thrombosis in cancer patients.

3.1.3 NET formation signalling pathways

With the increasing evidence of NETs in cancer and their roles in both cancer progression and thrombosis, it is pivotal to uncover the signalling pathways of NET formation in cancer. So far studies that have attempted to delineate NET signalling pathways are from *in vitro* stimulation of neutrophils. Various stimuli have been confirmed to release NETs from neutrophils such as PMA, lipopolysaccharide, cytokines and proteins [227]. PMA is the most efficient inducer of NET release and the most frequently used NET stimulus [228]. PMA penetrates through the membrane and activates protein kinase C (PKC) [229, 230] which promotes assembly and activation of NADPH oxidase [231]. PMA-induced NET formation has been shown to be dependent on reactive oxygen species (ROS) generated by NADPH oxidase [232]. In addition, neutrophils from mice when stimulated with PMA, generated ROS and mice deficient with NADPH oxidase failed to produce ROS and subsequent NET release [233]. This finding was also supported in humans, as patients with chronic granulomatous disease which have defective NADPH oxidase in neutrophils, are unable to produce ROS and NET upon stimulation with PMA [234]. Hakkim and colleagues shed light on NET signalling by PMA, showing that the Raf-MEK-ERK pathway is involved which is downstream of PKC

activation and upstream of NADPH oxidase [235]. Another study reported the involvement of p38 MAPK activation downstream of ROS in PMA-induced NET from human neutrophils [232]. Other studies have also reported that Akt [236], Syk and PI3K [237, 238] is required for NET formation induced by PMA.

PMA is a widely used stimulator of NET generation, however it is not a physiological stimulus. Lipopolysaccharide (LPS), the main component of the gram-negative cell wall, is a more biologically relevant stimulus that has been shown to induce NETs. Like PMA, studies have shown that LPS-induced NET is ROS-dependent. Khan et al. reported that LPS-activated c-Jun N-terminal kinase (JNK) which is upstream of NADPH oxidase and signals through toll-like receptor 4 (TLR4) [239]. JNK and TLR4 activation were both required for NET release in LPS-mediated ROS generation and NET release in neutrophils.

High mobility group box 1 protein (HMGB1) is a multi-functional protein and damage-associated molecular pattern (DAMP) molecule which has been shown to induce NETs. HMGB1 is secreted by immune cells or passively released by dying or injured cells which triggers a range of DAMP-induced inflammatory responses. Studies have shown that HMGB1 can bind to different receptors to induce cell activation, including receptors for advanced glycation end products (RAGE) and toll-like receptors TLR2, TLR4 and TLR9 [240]. Tadie et al. reported HMGB1-induced NET release which was dependent on TLR4 activation [241]. This finding was confirmed by Zhou et al. who reported that p38 MAPK and Erk signalling activated downstream of TLR4 in HMGB-1-mediated NETs [242]. Notably, HMGB-1 is relevant to the study of pancreatic cancer as it has been reported to have dual roles in pancreatic cancer where it can function as a tumour suppressor, or be exploited for metastasis in pancreatic cancer [243].

3.1.4 The effect of cancer-derived exosomes on neutrophils and implications for thrombosis

As mentioned in the review which readers are directed to above, neutrophils display plasticity in cancer within the TME, and a limited number of studies have started to recognise the roles of cancer-derived exosomes in the communication and influence on neutrophils in the tumour microenvironment. One group found that exosomes isolated from gastric cancer cells were able to induce polarisation of human neutrophils to an N2 phenotype *in vitro,* promoting gastric cell migration, inducing autophagy and NF-κB activation [144]. Melanoma-derived EVs induced neutrophils to a pro-tumour phenotype with decreased tumour cell cytotoxicity [244]. To date, no other studies have reported the *in vitro* polarisation of neutrophils by cancer-derived EVs or exosomes. Few studies have begun to shed light on the role of cancer-derived exosomes on immune modulation and tumour progression through neutrophils. For example, EVs from gastric cancer cells

induced PD-L1 expression on neutrophils, leading to suppressed T cell immunity *in vitro* [245]. An *in vivo* study in mice showed pancreatic cancer-derived EVs promoted recruitment of bone marrow-derived cells (including neutrophils) which provided a favourable niche for liver metastasis [122]. Studies thus far are lacking, however there is little evidence to suggest that cancer-derived exosomes can influence neutrophils to exert pro-tumour effects and immunosuppression.

As studies are beginning to investigate the effect of cancer-derived exosomes on neutrophils and its effect on immune modulation and tumour progression, one area of study that is yet to be investigated is tumour-derived exosomes and their roles in promoting cancer-associated thrombosis. Many studies have now reported the roles of NETs in thrombosis, through promoting platelet activation and endothelial dysfunction. To date, one study has reported tumour-derived exosomes induced NETs in neutrophils from mice that were primed with granulocyte colony-stimulating factor (G-CSF) [145]. Remarkably, the infusion of tumour-derived exosomes into mice treated with G-CSF accelerated thrombus formation to a level comparable to that seen in tumour-bearing mice. This was the first study to propose a link between tumour-derived exosomes, neutrophils, and cancer-associated thrombosis. While earlier studies have reported that tumour-derived exosomes can polarise neutrophils to the N2 phenotype or pro-tumour phenotype, it remains largely unknown whether NET release is favoured in the N2 phenotype. Evidence appears to be conflicting with some studies reporting NETs are increased in N1 or anti-tumour neutrophils [204, 214], while others report unprompted NET formation in N2 neutrophils [246].

Given the role of NETs in thrombosis, it is possible that cancer-derived exosome-induced NET may play important roles in cancer-associated thrombosis. This could be facilitated by the vast array of proteins present on the NET platform which are capable of recruiting platelets as well as activation of platelets and coagulation. Some examples of the mechanisms by which NET components can induce thrombus formation include: DNA activating the coagulation cascade [207], histones and neutrophil elastase recruiting platelets to facilitate thrombus formation [247] and MPO facilitating endothelial dysfunction [248]. **This part of the thesis aims to confirm the finding that tumour-derived exosomes can induce NETs which was previously observed in neutrophils from mice that were treated with G-CSF. This study will focus on pancreatic cancer-derived exosomes and aims to investigate its ability to directly induce NET formation in human neutrophils *in vitro* and attempt to delineate the signalling pathways involved. Due to the difficulties experienced with working with primary human neutrophils, there was a considerable amount of optimisation which will also be presented below.**

3.2 Results & Optimisation

We have previously shown that neutrophils are activated and release NETs when stimulated with AsPC-1 pancreatic cancer cells and AsPC1 cell-derived conditioned media [249]. To further delineate the pancreatic cancer-derived factor mediating NET release, the conditioned media was separated into its lipid and protein fractions. The protein, but not lipid, fraction stimulated NET release to the same intensity and rapidly within 30 minutes, similar to which conditioned media induced. As proteins can be packaged in exosomes and have been shown to induce signalling in other cell types, it was hypothesised that exosomes derived from pancreatic cancer cells have NET-inducing capacity.

3.2.1 Testing panel of pancreatic cancer cell lines for NET release

To further demonstrate the NET-inducing capacity of pancreatic cancer cells and determine whether the result observed was not specific to the AsPC-1 cell line, another two cell lines were included in the panel. Two cell lines that exhibit different characteristics to AsPC-1 were chosen, which also allowed the examination of any potential differences that may be attributed to the individual cell line characteristics. AsPC-1 cell line was derived from nude mice xenografts initiated with cells from ascites of a patient with adenocarcinoma of the head of the pancreas [250]. Further study had established the metastatic potential of AsPC-1 when inoculated in nude mice [251]. BxPC3 and MIA-PaCa-2 were chosen to be included as primary pancreatic cancer cell lines. BxPC3 was obtained from adenocarcinoma of the head of the body of the pancreas [252], and no evidence of metastasis was found in the patient. MIA PaCa-2 was derived from a patient with pancreas adenocarcinoma, with tumour mass found in the body and tail of the pancreas. [253]. Thus, these three pancreatic cancer cell lines (images in Figure 3.1) will allow examination of both metastatic and primary pancreatic cancer cell lines. The cell culture conditions of these cell lines are described in Chapter 2.

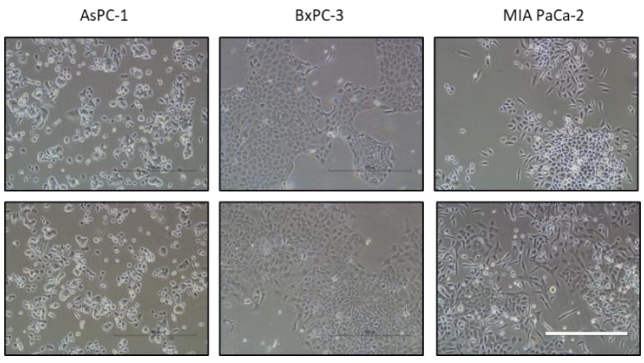


Figure 3.1. Brightfield images of pancreatic cancer cell lines in cell culture. From left to right: AsPC-1, BxPC-2 and MIA PaCa-2. Scale bar = 500 µm.

3.2.2 Isolation of neutrophils and NET quantification assay

Neutrophils were isolated from the blood of healthy human volunteers using Polymorphprep (Axis Shield, Norway), with slight changes to the manufacturer's protocol. Blood was drawn with a 21-gauge needle into a syringe containing EDTA (final 2 mM). The EDTA-anticoagulated blood was layered over an equal volume of Polymorphprep in a 15 ml conical tube and centrifuged at 500 x g for 40 min. The separated neutrophil fraction was collected and washed with cold Hank's buffered salt solution (without magnesium and calcium) and centrifuged at 400 x g for 10 min. Neutrophils were washed twice. The pellet was resuspended in X-VIVO 15 media (Lonza, Switzerland) to a concentration of 5 x 10^6/ml. The purity of neutrophils was routinely >95% as assessed with haematology analyser (Mindray, BC-VET2800).

Before the isolation of neutrophils, pancreatic cancer cells were grown to 90% confluence in 6 well plates. When it was time to harvest cells and incubate with neutrophils, the pancreatic cancer cells were lifted with Accutase (Sigma) to ensure surface proteins were intact as they may potentially be involved in NET release and mediate interactions with neutrophils.

For the NET quantification assay, neutrophils were seeded at 5 x 10^4 cells per well in a 96-well black clear-bottom plate. The neutrophils were allowed to adhere for 15 min in an incubator at 37°C and 5% CO_2. After the adhesion of neutrophils, pancreatic cancer cells AsPC-1, BxPC3 and MIA PaCa-2 were seeded at 5 x 10^4 and 10 x 10^4 cells per well. To detect NET, extracellular DNA was stained with cell-impermeable Sytox Green (5 µM; ThermoFisher, USA) for 5 min at room temperature. NET quantification was measured at 30 min and 3 hr by measurement of fluorescence at 523 nm emission (488 nm laser excitation) on a microplate reader. A well-scan of 100 points per well (10 x 10-point matrix) was measured to sufficiently collect data of NET release across the surface of the well. This was chosen as opposed to a single-point reading which would not accurately reflect NET release due to its non-homogenous nature.

BxPC3 and MIA PaCA-2 cells were incubated with neutrophils at a seeding density of 5 x 10^4 and 10 x 10^4 cells. A seeding density of 5 x 10^4 neutrophils had been determined to be the optimal number for robust NET release with positive control phorbol myristate acetate (PMA). Thus, the ratio of neutrophils to cancer cells was 1:1 or 1:2. Pancreatic cancer cells induced a trend of increased NETs for MIA PaCa-2 compared to controls at 30 min (NET fold change: 2.2 ± 1.7 and 1.3 ± 0.5 with 5 x 10^4 and 10 x 10^4 cells, respectively; ns, n=3) while BxPC-3 cells did not induce NETs (Figure 3.2). In the contrary, BxPC-3 cells appear to result in reduced NET fluorescence reading compared to non-stimulated control (NET fold change: 0.37 ± 0.07 and 0.84 ± 0.3 with 5 x 10^4 and 10 x 10^4 cells, respectively; ns, n=3). Extracellular DNA is known to exist on the surface on BxPC3 and MIA PaCa-2

cells [254] and was observed in our hands (Figure 3.3). Due to the high background of extracellular DNA in MIA-PaCa-2 and BxPC3 cells, they were used as background controls and subtracted from the measurements in the wells containing both neutrophils and pancreatic cancer cells, before calculating for increased NET.

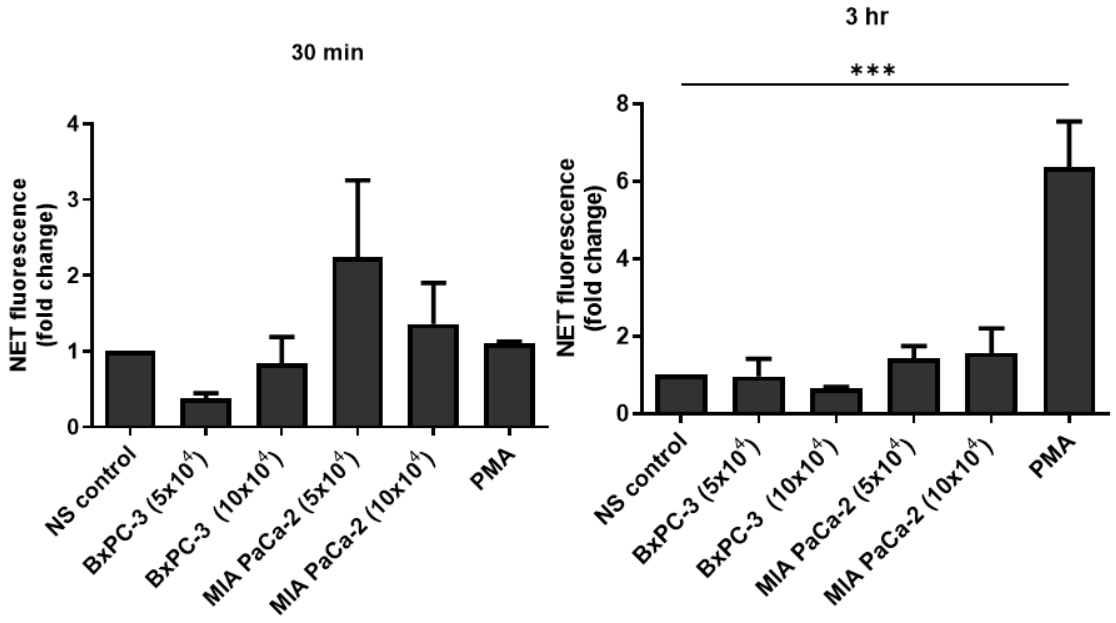


Figure 3.2. Quantification of NET release from neutrophil in the presence of pancreatic cancer cell lines BxPC3 and MIA PaCa-2. Human neutrophils (5 x 10^4) were seeded in a 96-well plate and were allowed to adhere for 30 min. Confluent BxPC3 or MiaPaCa-2 were lifted with Accutase and added to neutrophils at either 5 x 10^4 or 10 x 10^4 cell density. After 30 min incubation, SYTOX Green (5 µM) was added to stain for extracellular DNA. Fluorescence was measured on a plate reader at 504/528 (excitation/emission) after 30 min and 3 hr incubation at 37°C and 5% CO2. NS control refers to neutrophils with PBS as vehicle control. Results represent fold change in NET fluorescence relative to NS control. Data expressed as mean ± SEM. Data was analysed using one-way ANOVA with post-hoc Bonferroni's multiple comparisons test; *** $P < 0.001$, n = 3.

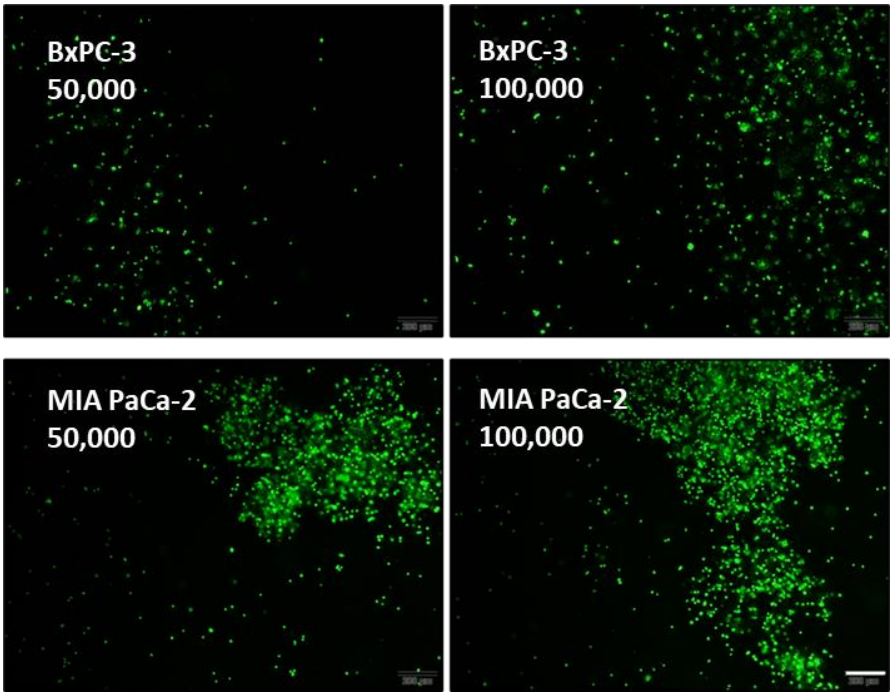


Figure 3.3. Extracellular DNA on the surface of BxPC-3 and MIA PaCa-2 cells. Cells were seeded alone (without neutrophils) in wells for background control and stained with cell-impermeable Sytox Green (5 µM). Cells showed they carry extracellular DNA on their surface. Top: BxPC3 at 50,000 and 100,000 cell seeding density. Bottom: MIA PaCa-2 cells at 50,000 and 100,000 cell seeding density. Scale bar = 200 µm. Images representative of two independent experiments.

3.2.3 Comparison of NET response from neutrophils isolated from syringe and vacutainer blood draw

One step that was vital for subsequent experiments with neutrophils was to ensure neutrophils were not slightly activated during the isolation process, as it may lead to spontaneous NET formation. Vacutainers are widely used to collect blood for the isolation of neutrophils. However due to previous experience working with neutrophils it was evident that neutrophils are highly sensitive cells and are prone to activation. To avoid activation from shear stress, phlebotomy was performed with a 21-gauge needle and blood was collected into a syringe containing EDTA. An experiment that compared the capacity of NET formation from neutrophils isolated from either a vacutainer or syringe showed 30% less NET formation with neutrophils isolated from blood that was drawn using a vacutainer (Figure 3.4). On the other hand, neutrophils isolated from blood that was drawn using a syringe containing EDTA had a much greater response of NET release. The amount of spontaneous NETs observed in the non-stimulated neutrophils was also greater in the neutrophils isolated from vacutainer blood collection. This result is likely due to the shear stress that neutrophils are subject to when drawn through a vacutainer, which may have resulted in the pre-activation of neutrophils

hence they are not fully rested and unable to elicit a full activation response when stimulated. Given the result of this comparison, which would need further repeats to confirm the diminished NET response, a precaution was taken for all blood collections, and blood for the following experiments was collected in a syringe. djjhiiit t

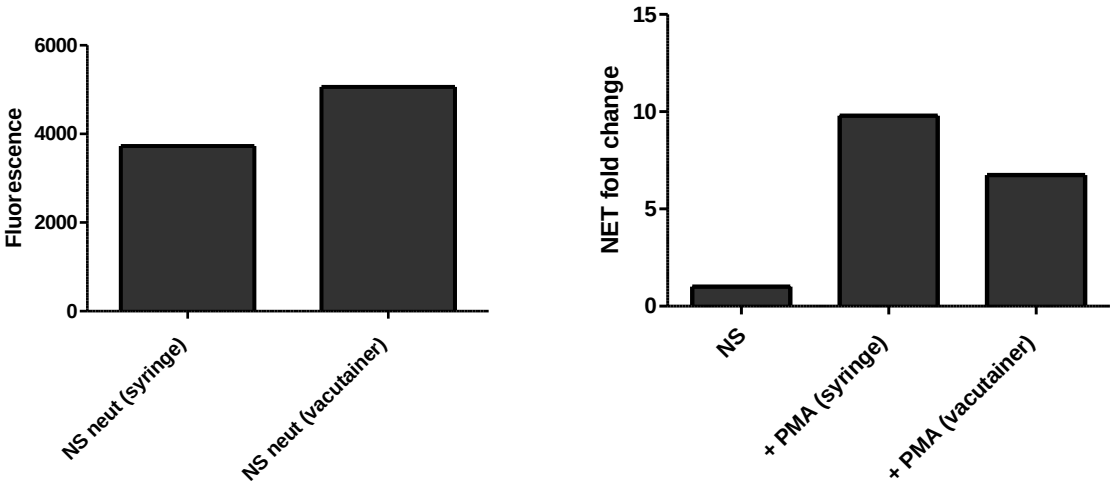


Figure 3.4. Comparison of NET released from neutrophils isolated from different methods of blood collection. a) Background or spontaneous NET was higher in neutrophils isolated from blood collected in a vacutainer. B) Neutrophils were isolated from blood collected in vacutainer and syringe with EDTA anticoagulant. Quantification of NET release from PMA-stimulated neutrophils after 3 hr. PMA is a robust NET activator and served as positive control. NET release was more in neutrophils isolated from blood collected in a syringe. Results are from one independent experiment.

3.2.4 Pancreatic cancer-derived exosomes stimulate NET release

The other two pancreatic cancer cell lines, BxPC3 and MIA PaCa-2, did not induce significant NET release as did AsPC-1 as shown in a previous study. However, it would be interesting to see the impact of direct cancer-derived factors from the cell lines in the form of exosomes on NET release. Furthermore, it will eliminate the interference of extracellular DNA from BxPC3 and MIA PaCa-2 cells that was contributing to the high background in the NET quantification assay.

Exosomes were isolated from pancreatic cancer cells AsPC-1, BxPC3 and MIA-PaCa-2 as described in Chapter 2 (Part 2: Isolation and Characterisation Exosomes). Exosomes from human non-malignant epithelial pancreatic duct cells (hTERT and HPDE) were also used to serve as normal and non-cancer control. Protein concentration of lysed exosomes were first quantified using the Micro BCA Protein Assay Kit (Thermo Fisher) in order to normalise the amount of exosomes being used to stimulate neutrophils. Exosomes were lysed in RIPA buffer and briefly sonicated to release proteins. The sample was centrifuged and the resulting supernatant was used to quantify protein. The equivalent

of one microgram of protein in exosomes was added to neutrophils to assess the NET-inducing capacity. Pancreatic cancer-derived exosomes were able to stimulate NETs by 30 min (Figure 3.5), showing the same rapid rate of NET release observed in the parent pancreatic cancer cells. Interestingly, the capacity of exosomes to induce NET was much greater than the cells, with NET fold change of up to 6-fold for AsPC-1 exosomes (**** $P < 0.0001$, n=4). There was also a significant induction of NETs by BxPC-3 exosomes (NET fold change: 2.77 ± 0.3; * $P < 0.05$, n =4,) and a trend of increased NETs by MIA PaCa-2 exosomes (NET fold change: 1.97 ± 0.3; ns, n=4). Additionally, the use of exosomes allowed the quantification of NET without the interference of any extracellular DNA, which may have obstructed previous quantification with cell lines. The non-cancer control exosomes derived from hTERT and HPDE did not elicit NET, suggesting that the NETs formed are an effect specific to cancer-derived products.

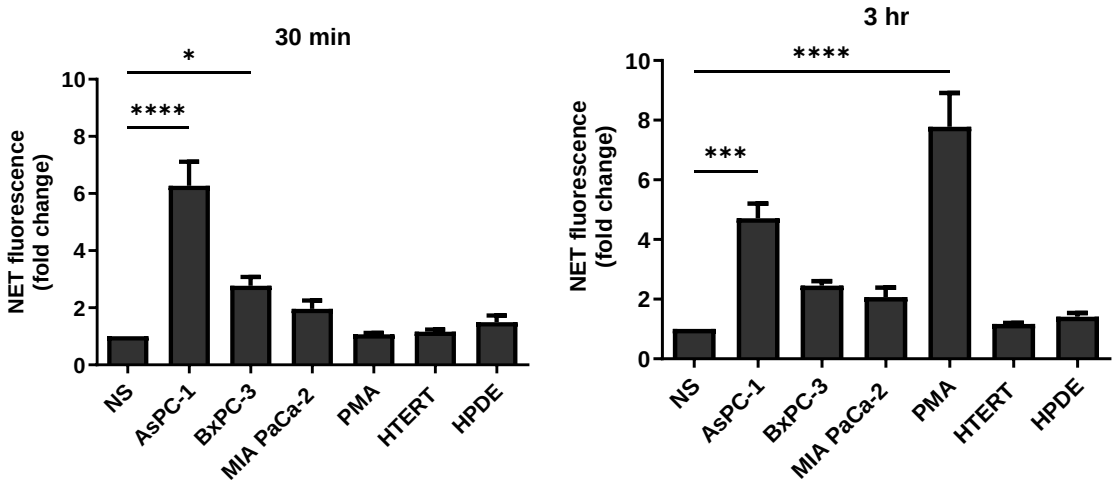


Figure 3.5. Quantification of NET after stimulation with pancreatic cancer cell-derived exosomes. Neutrophils (5 x 10^4) were stimulated with 1 µg exosomes isolated from normal cell lines (HTERT and HPDE) and pancreatic cancer cell lines (AsPC1, BxPC3 and MiaPaCa-2) and incubated for 3 hours. PMA served as positive control. NETs were quantified as extracellular DNA release stained with SYTOX Green (5 µM). Pancreatic cancer-derived exosomes induced NET release by 30 min (n = 4). Data expressed as mean ± SEM. One-way ANOVA followed by post-hoc Bonferroni's multiple comparison test was used to determine significance between means; * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

Note: The figure shown has been used in a following chapter of this thesis (in Chapter 4) as it was written for research article submission.

Attempts to image NET release by exosomes are shown in Figure 3.6. Neutrophils were coated on poly-L-lysine coated glass slides and then stimulated with one microgram of exosomes per coated section. The cells were incubated with exosomes for 30 min, fixed and then stained with SYTOX Green (5 µM). It was observed in neutrophils treated with AsPC1 exosomes the fine structure and

thin strands of NETs, while BxPC-3 and MIA PaCA-2 exosomes were imaged as 'diffuse' NETs as shown by the diffuse nuclei staining which is also indicative of NET release as a result of neutrophil nuclear envelope breakdown. On the other hand, neutrophils that were treated with control exosomes (isolated from fibroblast and HPDE cells) maintained their nuclear morphology, with an absence of diffuse or strands of NETs. The following attempts made to image NET were unsuccessful as there was evidence of NET release in the non-stimulated control. This could be attributed to some activation of the neutrophils during isolation and any excess poly-L-lysine coating solution that was not thoroughly washed away.

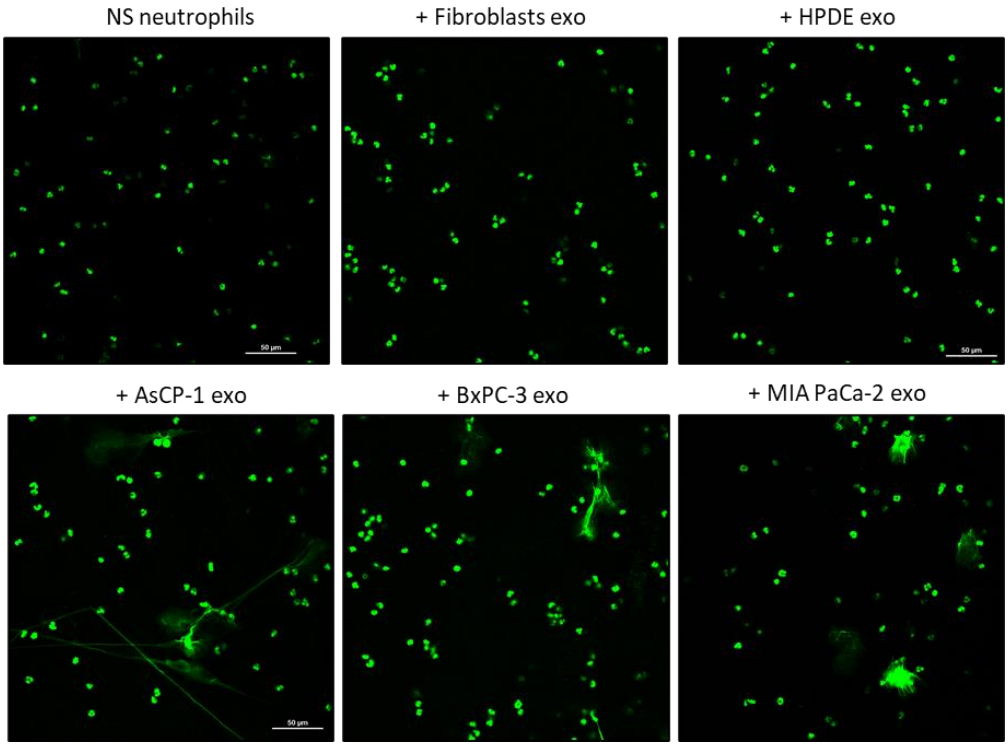


Figure 3.6. Confocal images of NET induced by pancreatic cancer cell-derived exosomes. Neutrophils were allowed to attach on a small section of poly-l-lysine treated glass slides and then treated with 1 µg of exosomes for 30 min. After incubation, cells were fixed with 4% PFA and then stained with SYTOX Green to stain for extracellular DNA. Images were taken on Nikon confocal microscope. Scale bar = 50 µm. Images from one independent experiment.

3.2.5 Delineate signalling pathway of NET release by pancreatic cancer-derived exosomes

The ability of cancer-derived exosomes to induce NETs has been reported in a previous study [145]. This study also demonstrated the role of cancer-derived exosomes and neutrophils as players in the establishment of cancer-associated thrombosis *in vivo.* With the increasing evidence of the role of

NETs in cancer-related complications such as metastasis and compromised organ function, it is important to identify the signalling pathways mediating NET release.

To date, studies have attempted to delineate the signalling pathways in NET release. Some studies have provided evidence of the role of DAMPs as mediators of NET release. HMGB1 has been shown to induce NET release through Toll-like receptor 4 [241, 242]. HMGB1 is known to support cancer immunity in the early stages of cancer development. In collaboration with co-supervisor Prof Marco Falasca's lab, proteomics analysis obtained from the exosomes isolated from pancreatic cancer cells (results published in [171]) revealed that DAMPs such as HMGB1 and S100A4 are highly upregulated in pancreatic cancer-derived exosomes in comparison to control exosomes from non-cancerous cell lines (hTERT and HPNE). Thus, it is hypothesised that DAMP molecules from pancreatic cancer-derived exosomes mediate NET release. As mentioned earlier, HMGB1-mediated NET release was shown to signal through TLR4 with activation of downstream signalling molecules p38 and Erk. Hence, this study attempted to investigate the signalling of pancreatic cancer exosome-mediated NET through the upregulation of p38 and Erk.

3.2.6 Preliminary signalling with pancreatic cancer cell-derived conditioned media

Due to the time-consuming preparation and valuable samples of exosomes, the signalling of pancreatic cancer cell-induced NET was first investigated with the conditioned media (CM) of pancreatic cancer cells. This also allowed optimising the conditions for neutrophil activation and preparation of samples for western blotting. Due to the notoriously difficult nature of working with neutrophils it was not feasible to use exosomes on the first attempt without optimising the best experimental conditions. The results would give an idea of the signalling pathways activated and which to further assess in neutrophils stimulated with cancer-derived exosomes. As hypothesised, the signalling molecules activated downstream of TLR4 receptors were investigated, along with the activation pathways through commonly used NET stimuli (PMA and LPS) including Erk, Syk, PI3K and Akt.

The stimulation of neutrophils before preparation of lysate for western blot was performed in suspension in X-VIVO 15 media. Due to the excessive amounts of proteases present within neutrophils, a potent protease inhibitor diisopropyl fluorophosphate (DFP) was used. Pre-treatment of neutrophils after isolation of neutrophils has been used to inhibit the activity of proteases upon lysis of neutrophils. Neutrophils were pre-treated with DFP for 15 min on ice and then washed twice in ice-cold HBSS without calcium. Neutrophils (1×10^7/ml) were aliquoted in an Eppendorf tube and spun down. The pellets were either resuspended in X-VIVO 15 (for non-stimulated), X-VIVO 15 media with PMA (25 nM; for positive control) or AsPC-1 CM. The preparation of the conditioned medium

can be found in the methods of a previously published paper from our laboratory [249]. After incubation for 5 min at 37°C, the neutrophils were lysed in hot 6X Laemmlli buffer with protease and phosphatase inhibitor cocktail (Cell Signalling) to stop the reaction. Samples were heated at 95°C and sonicated. The samples were loaded (equivalent to 0.25 x 10^6 neutrophils) on SDS-PAGE gel and analysed by western blotting.

The first blot showed phosphorylation of signalling molecules Akt, Syk (Tyr352), Syk (Tyr525/526) and PLC-gamma2 (Tyr579) upon stimulation with AsPC-1 conditioned media (Figure 3.7, n=1). PMA stimulation also showed activation of Erk and Syk, consistent with the literature. However, in our hands, there was no evidence of AKT activation with PMA stimulation.

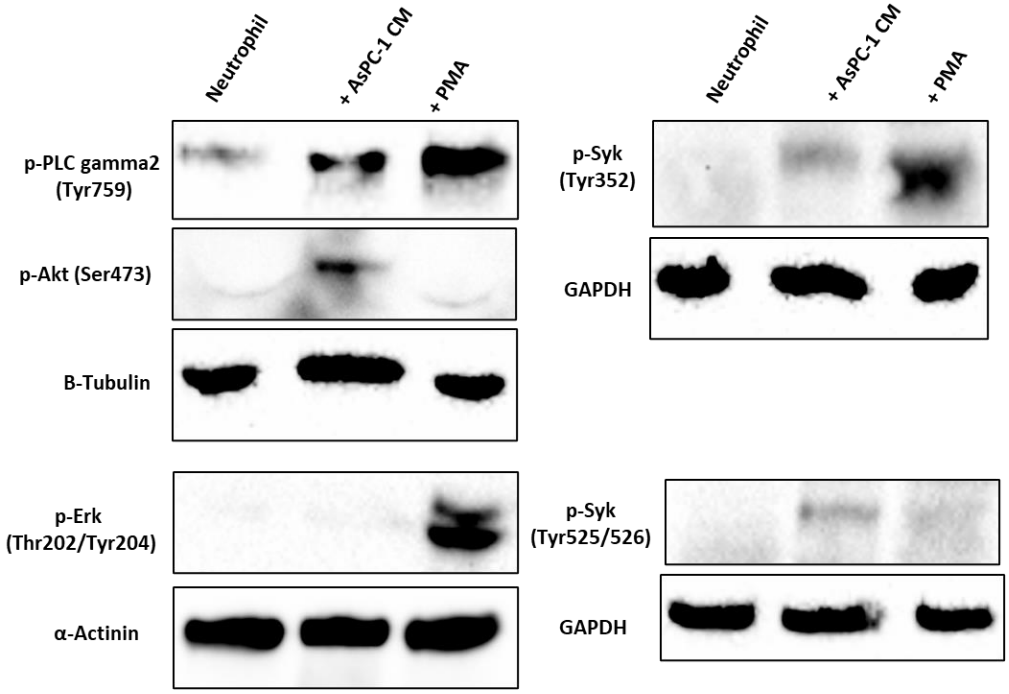


Figure 3.7. Western blots for signalling of neutrophils stimulated with AsPC-1 derived conditioned media. Neutrophils were treated with DFP (5 µm) for 15 min on ice and then washed 2 times at 400 x g for 10 min in HBSS without calcium. Neutrophils 2 x 10^6 (200 µl of 1 x 10^7/ml in Eppendorf tube) were resuspended in V-VIVO 15, PMA or AsPC-1 derived conditioned media (CM) and incubated for 5 min at 37°c before adding hot 6X Laemmli buffer with protease and phosphatase inhibitors (Cell Signaling) to stop the reaction. Samples were heated for 5 min at 95°c and then sonicated. 30 µl of sample (0.25 x 10^6 neutrophils) were separated on SDS-PAGE. The proteins were transferred to the PVDF membrane and probed for signaling proteins. CM = conditioned media. Result from one independent experiment.

Unfortunately, attempts to replicate these results were unsuccessful. Despite following the same conditions and protocol, the blots had poor resolution and loading controls of housekeeping. In some experiments, there were no housekeeping proteins present. This was an indication of poor

samples and the uneven loading controls is possibly due to the proteins being subjected to degradation as a result of the neutrophil's own cargo of proteases which are released upon lysis. In an experiment when housekeeping was detected, similar results were seen for P-Erk and P-Akt, with P-Erk activation in PMA-stimulated and P-Akt activation in CM-stimulated (Figure 3.8, n=1). However, results show that P-Syk is heavily present in non-stimulated neutrophils likely indicating pre-activated neutrophils despite unstimulated conditions.

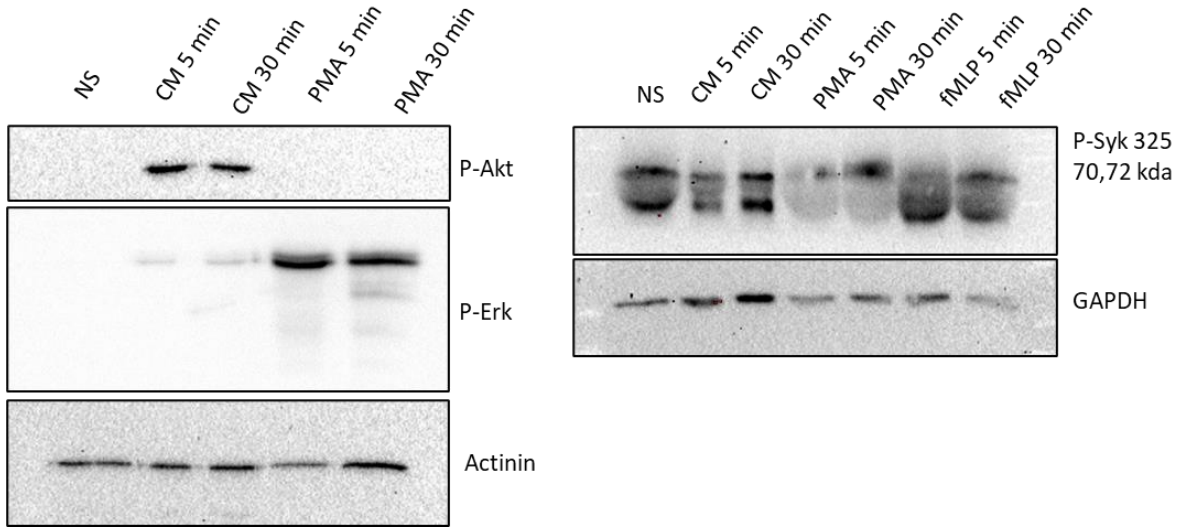


Figure 3.8. Attempts to replicate results from the first western blot of neutrophils stimulated with AsPC-1 conditioned media. Western blots show attempts where housekeeping was detected. Left and right blots are from two different donors. Evidence of poor blot resolution and poor housekeeping with uneven loading. Each blot is from one independent experiment.

3.2.7 Attempt to optimise quality of neutrophil samples for western blotting

Due to the difficulty and inconsistencies with western blotting, it was determined that it was likely due to poor neutrophil lysates, thus sample preparation needed optimisation. DFP is a potent protease inhibitor, however it did not seem to be effective in completely inhibiting protease activity as there was evidence of protein degradation shown from weak housekeeping protein signals. It has also been reported that DFP can reduce oxidative burst in neutrophils [255]. As the production of reactive oxygen species (ROS) is necessary for the induction of PMA-stimulated NETs, the effect of DFP on NET and ROS generation was investigated.

Neutrophils that were pre-treated with DFP showed significantly reduced propensity and inability for NET release compared to stimulated neutrophils without DFP treatment (NET fold change: 1.01 ± 0.1 vs 2.03 ± 0.4; *$P < 0.05$, n=4) (Figure 3.9). This is in line with the requirement of ROS for NET release in PMA-stimulated NET. Indeed, measurement of ROS generation using H2DCFDA dye on a plate reader showed that ROS in PMA-stimulated neutrophils was reduced in DFP-treated neutrophils

(ROS fold change: 1.54 ± 0.04 vs 3.01 ± 0.3; ** P < 0.01, n=3) (Figure 3.10). There was also an increase in spontaneous NET release in resting DFP-treated neutrophils compared to neutrophils without DFP treatment (NET fluorescence: 558 ± 158 vs 327 ± 208; ns, n=4) (Figure 3.11). These results suggests that DFP treatment of neutrophils results in slightly activated neutrophils, which reduces the ROS release capacity and subsequent ROS-dependent NET release in PMA-stimulated neutrophils. Thus, the use of DFP in neutrophil functional assays should be avoided, particularly where ROS is known to be important in mediating the functional response.

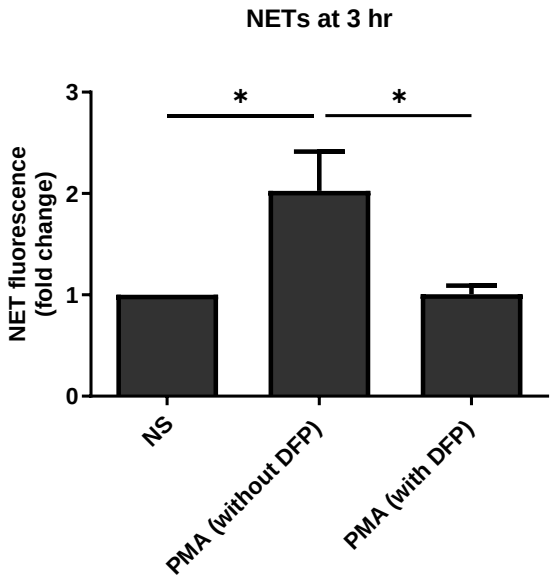


Figure 3.9. DFP treatment in neutrophils inhibits NET generation with PMA stimulation. Quantification of NET using Sytox Green after stimulation with PMA in DFP-treated neutrophils. PMA stimulated neutrophils induced NET which was significantly reduced with DFP pre-treatment in neutrophils. One-way ANOVA followed by post-test Bonferroni's multiple comparison test was used to determine significance between means. Data presented as mean ± SEM; P < 0.05, n=4.

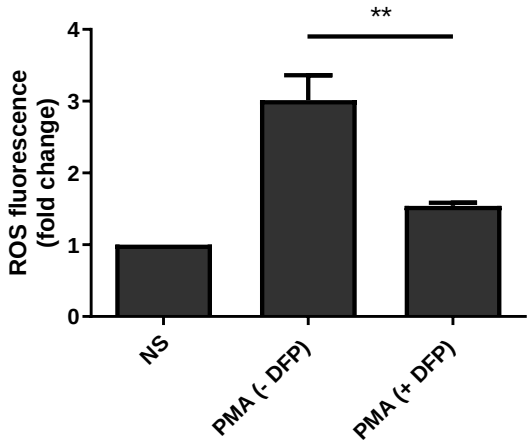


Figure 3.10. DFP treatment in neutrophils reduces ROS generation after PMA stimulation. Quantification of ROS generation in neutrophils using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescent dye after 3 hr stimulation with PMA. ROS was generated in untreated neutrophils and reduced with DFP treatment. One-way ANOVA followed by post-test Bonferroni's multiple comparison test was used to determine significance between means. Data presented as mean ± SEM, **P < 0.01, n=3.

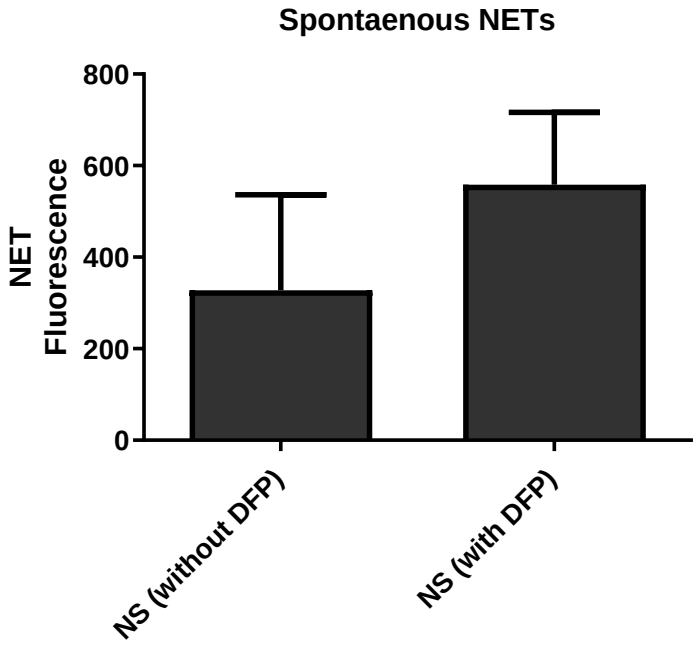


Figure 3.11. DFP treatment in neutrophils results in spontaneous NETs in resting neutrophils. Quantification of NET generation in neutrophils using Sytox Green. Comparison of neutrophils with and without DFP treatment and its effect on NET release in resting neutrophils. Trend of increased NET release from resting neutrophils with DFP treatment. Data presented as mean ± SEM. No significance between means as determined with unpaired t test was. n=4.

3.2.8 Overview of the attempt to optimise the western blotting of pancreatic cancer-derived exosome-stimulated neutrophils

Many factors and reagents were trialled to optimise sample preparation and improve western blotting results. These included lysis buffers, protease inhibitors, addition of beta-mercaptoethanol (BME) to the culture media, and different stimulation conditions (suspension vs adherent cells). Different lysis buffers used included Laemmli, HEPES (with NP40) and RIPA lysis buffers. A variety of protease inhibitors was used, including phenylmethylsulfonyl fluoride, sodium orthovanadate and protease and phosphatase inhibitor cocktails. The use of BME was introduced to keep the neutrophils more rested, and there was evidence of less protein degradation in housekeeping protein in the trial. A comparison between neutrophils with and without BME in the cell culture media showed better NET production in response to PMA, and less spontaneous NET in the unstimulated neutrophils. Thus, it was chosen to include the use of BME in neutrophil cell culture media in subsequent experiments. Further optimising prompted to try different cell stimulation conditions. Neutrophils were first stimulated in suspension; however, it was difficult to obtain pellets of the stimulated cells as activated neutrophils would form a smear along the wall of the pellet. To negate this issue, neutrophils were stimulated in 6-well plates. The results of each trialling method are presented in the following section.

Attempt to improve the inhibition of protease activity within neutrophils

The absence of housekeeping proteins in several attempts for signalling in neutrophils stimulated with AsPC-1 conditioned media led to the trial of a different lysis buffer with a cocktail of potent protease inhibitors. Neutrophils are known to be loaded with proteases and without sufficient inhibition, it is likely they will breakdown signalling proteins of interest upon cell lysis. A protocol was adapted from Bussmeyer et al. [256] which included a washing step of neutrophils with phosphatase inhibitors after stimulation. The cells were washed in ice-cold PBS containing 25 mM NaF and 0.5 mM Na3VO4. The pellet was then resuspended in lysis buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% [vol/vol] Nonidet P-40, 20% [vol/vol] glycerol, 1 mM dithiothreitol) with phosphatase inhibitors sodium fluoride (50 mM NaF) and sodium orthovanadate (1 mM Na3VO4) and 2X Roche complete ULTRA protease inhibitor cocktail. After 15 min on ice, cell debris was spun down at 12,000 x g for 20 min at 4°C. Lysates were stored at -80c until western blot was performed.

Using this protocol and lysis buffer, western blots with good housekeeping proteins were obtained (Figure 3.12). From these blots, it appears that activation of Erk is not involved in AsPC-1 stimulated neutrophils. Upon the reintroduction of stimulation with exosomes, there was no evidence of Akt or

Erk activation (Figure 3.13), and attempts to identify other signalling proteins which may be activated in exosome-stimulated neutrophils appeared to need optimisation as it was absent in positive control (data not shown).

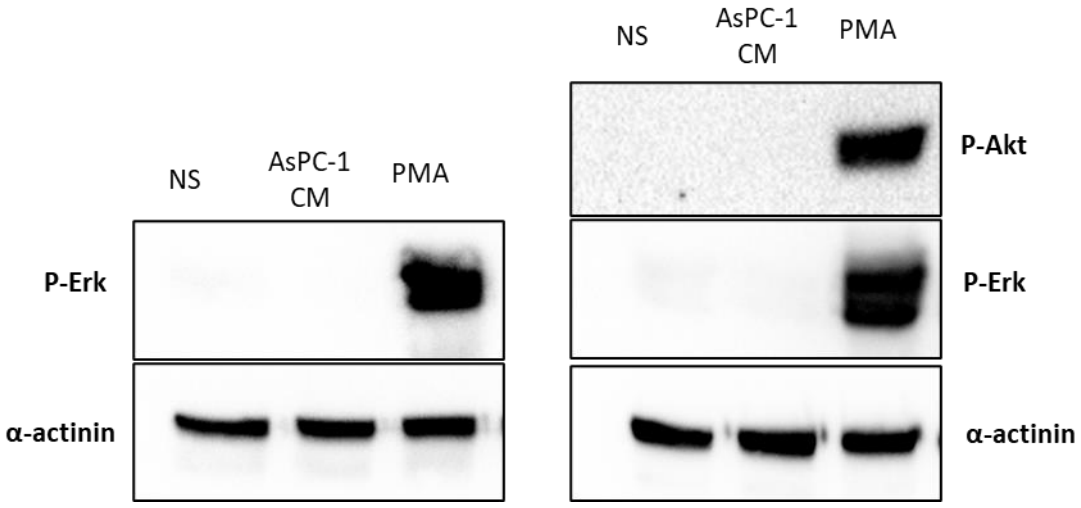


Figure 3.12. Western blot trials with HEPES NP-40 lysis buffer. After stimulation for 5 min, neutrophils were washed with ice-cold PBS including phosphatase inhibitors. The pellet was resuspended and lysed in HEPES-NP40 lysis buffer. Lysate equivalent to 0.5 x 10^6 neutrophils were analysed by western blot. Blots shown are from two independent experiments.

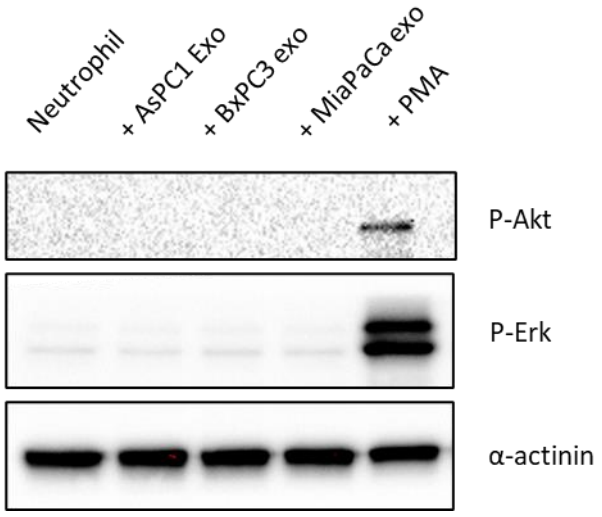


Figure 3.13. Western blot of neutrophils stimulated with exosomes isolated from three pancreatic cancer cell lines. Neutrophils were treated with exosomes for 3 min and lysates were prepared using HEPES-NP40 lysis buffer. Samples were run on an SDS-PAGE gel and the blot was probed for signalling proteins p-Akt and p-Erk, and α-actinin was used for housekeeping. PMA was used as positive control. Result from one independent experiment.

Shift to identify activation in phospho-tyrosine signalling

With the increasing difficulty in showing evidence for the upregulation of specific signalling proteins by western blotting. The decision was made to shift to western blotting for phospho-tyrosine activation which would show broad activation and indicate signalling of proteins through phosphorylation at the tyrosine residue. This led to another trial of lysis buffers with a protocol adapted from a paper published by Rollet et al. that showed western blotting for tyrosine phosphorylation [257]. The authors used Laemmli buffer supplemented with cocktail of protease and phosphatase inhibitors (62.5mM Tris-HCL pH 6.8, 4% SDS, 5% beta-mercaptoethanol, 8.5% glycerol, 2.5 mM sodium orthovanadate, 10 mM paranitrophenylphosphate (PPI), 12 ug/ml leupeptin, 12 ug/ml aprotinin, 1.25 mM PMSF and 0.05% bromophenol blue). Another measure that was taken to keep neutrophils more rested, was the addition of beta-mercaptoethanol (BME) to the culture media of neutrophils with the aim to reduce oxidative stress on the cells. The addition of BME seemed to improve protein lysate sample quality as there was less degradation of beta-actin observed (Figure 3.14). Thus, it was chosen to include BME in the cell culture for future experiments.

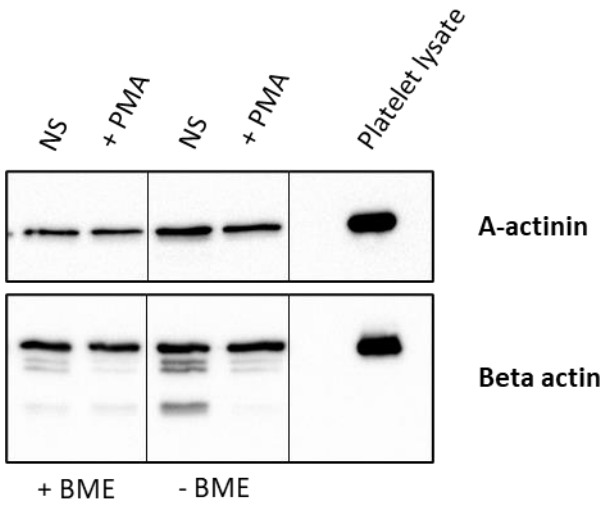


Figure 3.14. The addition of BME in the culture media of neutrophils and its effect on housekeeping proteins. Equal amount of neutrophils (0.4 x 10^6) were loaded in all lanes. NS= non-stimulated neutrophils. The presence of BME in the cell culture media appeared to improve the signals for beta-actin housekeeping as indicated by the evidence of less protein degradation observed in the lanes. Platelet lysate was used as a positive control for the housekeeping proteins which showed strong signals for both α-actin and beta-actin. Blot from one independent experiment.

Effect of using different blocking buffers in the phospho-tyrosine western blots

While optimising for tyrosine phosphorylation blots it became apparent that the recommended blocking buffer was not ideal. Cell Signalling Technology recommended the use of 5% skim milk for blocking for the anti-phosphotyrosine antibody (P-Tyr-1000). However, milk contains casein proteins, a phosphoprotein that can bind to the anti-phosphotyrosine antibody being used, resulting in non-specific binding and high background. A switch to using 5% bovine serum album (BSA) in Tris-buffered saline (TBS-T) saw a drastic change in phospho-tyrosine blots with less background noise (Figure 3.15).

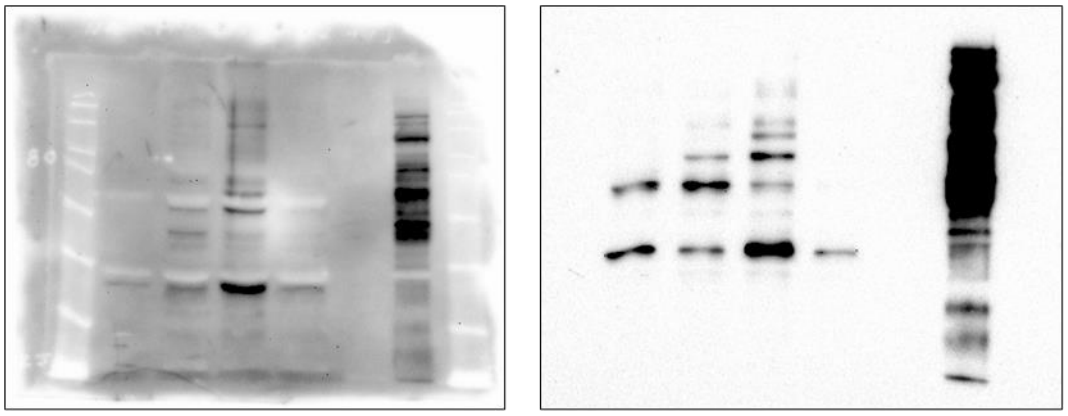


Figure 3.15. Comparison of milk and BSA blocking of blots that were probed for phospho-tyrosine. Left: Blot blocked with 5% milk in TBS-T. Right: Blot blocked with 3% BSA in TBS-T. Samples were lysed with Laemmli buffer lysis containing cocktail of protease and phosphatase inhibitors. Blot from one independent experiment.

Impact of storage on samples and subsequent western blotting experiments

Another issue that became apparent was the evidence of protein degradation from storage of the neutrophil lysates at -80°C. Samples were stored at -80°C until western blots were performed. In the process of optimising conditions for the anti-phosphotyrosine antibody, which involved increasing the concentration of the secondary antibody to 1:50,000 dilution, there was a great reduction in signals as evidenced by the lack of protein bands (Figure 3.16). Despite, the increase in secondary antibody the signals did not appear to be stronger and the exposure time had to be extended again to 30 min to accumulate signals. Thus, it was important that neutrophil lysates were not kept in storage for long periods of time and that western blots were performed as soon as possible, preferably on the same day of sample preparation or the next day at the very latest.

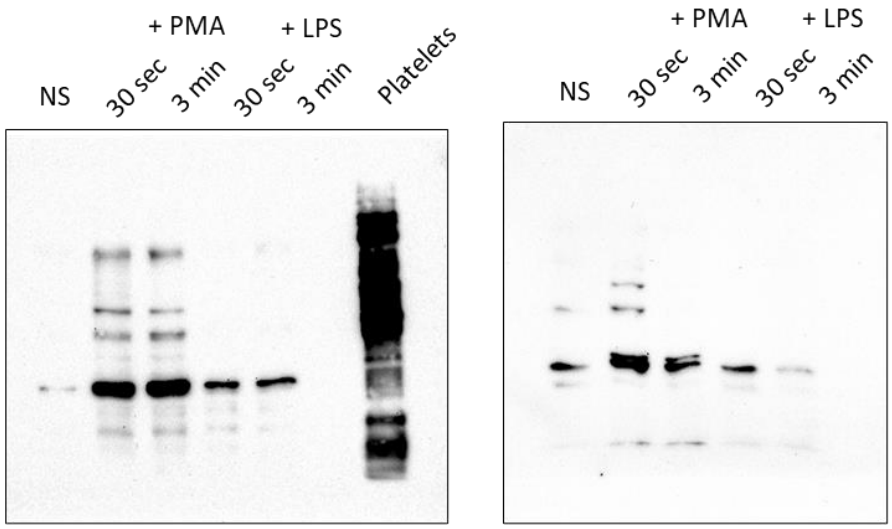


Figure 3.16. Effect of neutrophil lysate sample storage on western blotting. Comparison of blots with the same neutrophil lysate sample. **Left:** Blot was run immediately after sample preparation with 1:100,000 secondary antibody dilution. **Right:** Blot was run with the same samples but after storage in -80°c for three days. The secondary antibody was adjusted to be stronger at 1:50,000 dilution. For both blots, a dilution of 1:2,000 for primary antibody, and exposure time of 30 min was used. Blots are from one independent experiment.

Effect of different stimulation conditions

To continue optimisation of phospho-proteins, a change to the stimulation protocol was trialled. According to a paper published by Arndt et al. [258] the stimulation of non-suspended, and not suspended neutrophils by LPS yielded activation through Syk and PI3K pathways. This report was intriguing and led to investigate the changes that would be observed in our hands if neutrophils were stimulated in non-suspension. Neutrophils were seeded at a concentration of $2x10^6$/ml in a 6-well plate and allowed to adhere for 10 minutes before treatment with agonist and preparation of lysate. The phosphotyrosine profile improved and showed a better phosphorylation profile in PMA-stimulated compared to non-stimulated (Figure 3.17a). P-Syk was also present in PMA-stimulated neutrophils, but not in LPS-stimulated neutrophils (Figure 3.17b).

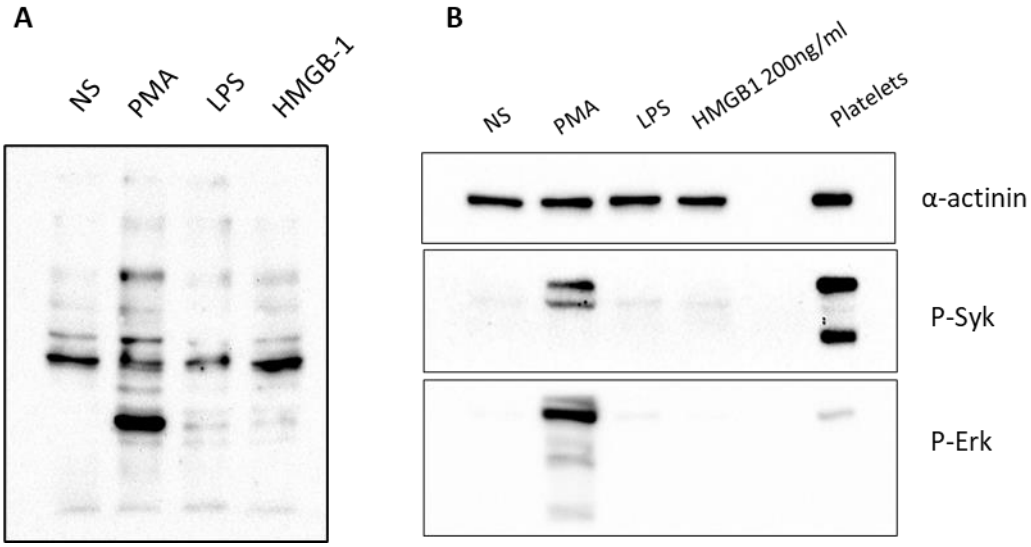


Figure 3.17. The effect of stimulation of adherent neutrophils in 6-well plates before lysis. A) Blot probed for phosphotyrosine. PMA and LPS served as positive controls. Recombinant HMGB-1 was included as an agonist and used at 200 ng/ml. B) Blots for signalling proteins p-Erk and P-Syk. α-actinin was used a positive. Platelets (activate lysate) was used as positive control. Blots from one independent experiment.

After many attempts and trials, it became increasingly apparent that the pursuit of identifying the signalling pathways induced by the pancreatic cancer-derived exosome was no longer feasible. Many months of optimising were undertaken and a wise decision had to be made to stop and switch to another angle to avoid using up more time. The focus in neutrophils was shifted and led to the investigation in other cells known to be important in thrombosis i.e. platelet and endothelial cells, which are in the chapters that follow.

3.3 Discussion

Pancreatic cancer-derived exosomes induce NETs

This study was able to demonstrate the ability of exosomes isolated from pancreatic cancer-derived cell lines AsPC-1, BxPC-3 and MIA PaCa-2 to stimulate NET release from neutrophils. AsPC-1-derived exosomes showed the greatest and significant induction of NETs, compared to exosomes derived from BxPC-3 or MIA PaCa-2. AsPC-1 induced NET was around 6-fold compared to non-stimulated, while NETs induced by BxPC-3 and MIA PaCa-2 exosomes were almost 3-fold less. This variability in NET response may be associated with the different characteristics of the cell lines. AsPC-1 is derived from mouse xenograft tumours initiated by ascites of a patient with metastatic pancreatic cancer. On the other hand, BxPC-3 and MIA PaCa-2 cell lines are derived from primary tumours, with BxPC-3

known to not be metastatic. Both exosomes from these cell lines showed a trend of increased NET of about 2-fold. Thus, the increased potency for AsPC-1 exosomes to induce NET may be associated with its metastatic characteristic. This could support the reports that demonstrate NETs as a player in facilitating metastasis [215, 216] whereby metastatic cancers have a greater propensity to induce neutrophils to release NETs and serve as a platform to support metastasis. One study found that tumour derived-EVs were responsible for NET formation in lymph nodes which also promoted metastasis at this site [259].

Another factor that is important to note is the time in which NETs were released. Exosomes induced a rapid production of NETs (within 30 min), while PMA induced NET after 3 hours. The slow process of NET formation by PMA is in line with the classical mechanism of NET release which was first detailed in 2007 [234]. Using a series of imaging experiments, neutrophils stimulated with PMA were shown to undergo significant morphological changes over the course of 3 hours, which were dependent on ROS generated by NADPH oxidase. The other mechanism of NET release is the rapid and ROS-independent process. This was first shown in neutrophils stimulated by *Staphylococcus aureus* [260], and later by other mediators such as activated platelets and *Candida albicans* [261, 262]. It was not investigated in this study whether exosome-induced NET is dependent on ROS. However, it was established in an earlier study from our laboratory that AsPC-1 conditioned medium induced NET independently of ROS [249]. It is likely that NETs induced by exosomes which are derived from cancer cell conditioned media, follow the same mechanism seen by cancer CM-induced NET as a similar response with time to induce NET was observed, however further studies will be needed to confirm this.

At the time when these results were obtained, it was the first report known to show direct induction of NET by cancer-derived exosomes. Another study in 2017 by Leal and colleagues showed that bone marrow cells from mice treated with GCS-F released NETs after stimulation with 4T1 cancer-derived exosomes *ex vivo*. NETs were confirmed as they were released from Ly6G+ positive cells (indicating neutrophils) and were positive for citrullinated histone staining, a marker for NET formation. Thus, this study demonstrated the release of NET from mice neutrophils after treatment with GCS-F, a factor known to induce neutrophilia and predispose NET release in a mouse model of cancer [40]. A recent study has shown that pre-injection of tumour-derived exosomes into a mouse model before the inoculation of cancer cells led to increased NET formation and metastasis. The important role of EVs was demonstrated with the knockdown of Rab27a, essential for EV formation, resulting in less EV secretion and subsequent NET formation. The result from this study shows the direct ability of pancreatic cancer-derived exosomes to stimulate neutrophils *in vitro* and the generation of NETs. This has implications for the studies that have shown NET facilitating metastasis, as it identifies a

potential direct mediator of NET generation in the cancer microenvironment and inhibiting such pathway of exosome-induced NET can impede other significant pathological processes which are mediated by NET.

NET formation signalling pathways

After establishing that pancreatic cancer-derived exosomes directly stimulate neutrophils to induce NET, the next investigation was to identify the specific mediators and signalling pathways involved. This would be pivotal as the mediator/s and signalling pathways identified could provide potential therapeutic targets for the inhibition of NET release in cancer. It was hypothesised that exosome-derived DAMPs may be involved in mediating NET release, as HMGB-1 has been shown to induce NET [241, 242], and is a molecule that is upregulated in pancreatic cancer-derived exosomes compared to normal exosomes as determined by proteomics analysis [171]. Another protein that was shown to be upregulated in pancreatic cancer-derived exosomes is S100A4. So far, there have been no reports of S100A4 inducing NETs directly. However, S100A9 has been shown to induce NET both in mice and human neutrophils [263, 264]. S100 proteins are calcium-binding proteins that can signal through TLR4 and RAGE receptors [265]. Interestingly, RAGE ligands were found to condition neutrophils for NET formation as its inhibition with the RAGE antagonist FPS-ZM1 for 3 hours before stimulation with tumour necrosis factor-α (TNF-α) prevented NET formation [264]. Whether or not pancreatic cancer-derived exosomes induce the same conditioning effect via S100A4 will need further investigation, as this study only investigated direct stimulation which showed rapid NET response by 30 minutes. It would be interesting to see if the same conditioning effect is observed before stimulation with factors that are highly present within the tumour microenvironment, and known to play roles in tumour growth, such as TNF-α or TGF-β.

The attempts to identify NET signalling pathways by pancreatic cancer-derived exosomes were unsuccessful due to several problems encountered. Despite this, according to the earlier blots performed with pancreatic cancer CM stimulation (Figure 3.7 and 3.8), it appears that Akt activation is involved in the signalling of CM-induced NET. This is in line with the evidence that Akt is involved in the generation of NETs in PMA-stimulated neutrophils [236] and also in ROS-independent NET release [266]. On the other hand, it appears that Erk is not involved in CM-induced NET. Erk has been shown to be involved in HMGB-1 induced NET in neutrophils by one group [242], however it was reported that Erk is not essential in ROS-independent NETosis [266] Thus, the preliminary results suggest that CM-induced NET signalling is in line with ROS-independent NET signalling.

In addition to signalling, the method of uptake by which the exosomes would present their mediating factor to neutrophils is another area of study. The mechanisms through which exosomes

are taken up by cells have been briefly outlined in the previous chapter. Since only few studies have reported the ability of cancer-derived exosomes to induce NET, there is still a need for further investigation on how these effects are mediated in terms of exosome uptake. Whether cancer-derived exosomes mediate receptor-ligand interactions, are or internalised before eliciting a cellular response are yet to be investigated.

Working with the sensitivity of neutrophils

There were several issues encountered which proved neutrophils are difficult and very sensitive cells to work with, demonstrating their fragility and ease to activation. Examples include the difference observed between blood collection in vacutainers and syringes. The collection of blood with a syringe is a slow and controlled method that would enforce less shear stress when compared to being under the influence of a vacuum created in a vacutainer. Shear stress is known to activate neutrophils and enhance its adhesion to blood vessel walls, enabling their transmigration [267]. In addition to shear stress, disturbances to blood flow and other turbulence can also cause activation of neutrophils. Thus, it is plausible that the neutrophils isolated from blood collected with a vacutainer were likely to be slightly activated, which not only led to increased NET release after stimulation with PMA, but also increased spontaneous NET which refers to the release of NET from unstimulated neutrophils. The comparison between syringe and vacutainer blood collection was only performed once and more repeats would be needed to confirm this effect. Nevertheless, a precaution was taken to collect blood with a syringe to avoid any potential diminishing effect on neutrophil activation from a vacutainer collection. Another example was observed in an experiment for optimisation of western blots where neutrophils that were incubated at 37°C for stimulation appeared to have an activated and clumpy appearance in suspension. Out of curiosity, neutrophils that were sitting on the bench at room temperature for the same amount of time were taken and analysed to compare for differences in activation of signalling proteins. It was evident that the non-stimulated samples on 37°C had induced activation of Erk, while it was only slightly present in samples prepared from neutrophils on the bench (Appendix 3.1). Thus, it was evident that small adjustments such as the stimulation at 37°C or obtaining blood via vacutainer can cause slight activation This would greatly impact downstream results as it means that the non-stimulated were pre-activated from the isolation process and would be more prone to activation even in unstimulated conditions.

Western blotting optimisation

The attempts to optimise neutrophil samples and conditions for western blotting presented in this chapter were summarised and do not reflect all the attempts made. Two studies with protocols analysing signalling in proteins were referenced earlier in the optimisations section [256, 257]. From

these studies, the methods, particularly the lysate sample preparation was adapted. Despite following these, the results obtained were not optimal or failed to replicate the signalling which was observed in preliminary results. One factor which was different is the cell culture media used, where RPMI medium was used in the referenced studies. In our earlier neutrophil studies comparing NET response to PMA treatment in different medium (HBSS, RPMI and X-VIVO 15) it was shown that X-VIVO 15 media resulted in the strongest response and kept the neutrophils well rested with low levels of spontaneous NETs in untreated neutrophils (data not shown). Thus, all experiments with neutrophils were chosen to be in X-VIVO 15 media. It is not known if the media used would have had a downstream effect on the ability to obtain signalling in neutrophils and experiments would be required to determine any effect or improvement with using other media. In addition, there is much less literature available on human neutrophils and signalling by western blot compared to human peripheral blood mononuclear cells which may further highlight the difficulty of the analysis in neutrophils. Several papers also lack the detail needed (such as the ingredients of lysis buffer used) to be able to replicate the signalling in their studies.

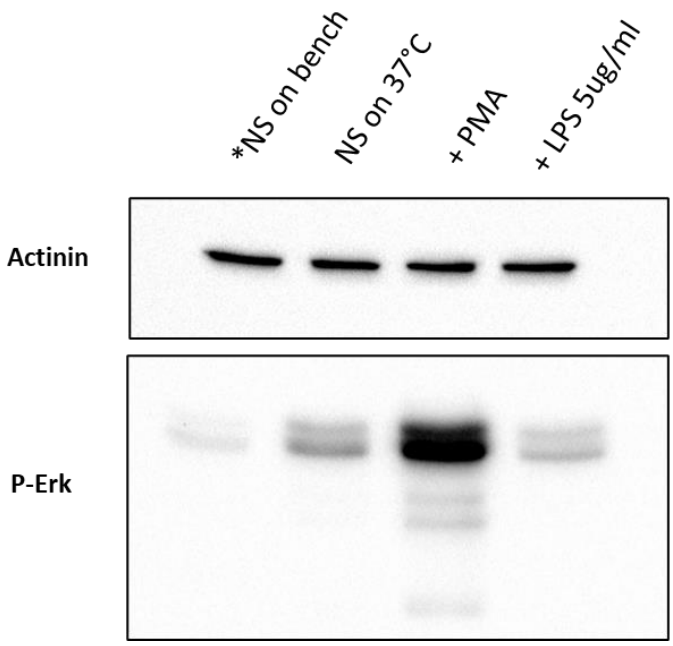
With a further look into the literature, it became more apparent that it could be difficult to identify activation of just one signalling protein. For example, to identify activation of p38 in LPS-stimulated neutrophils, it was reported that neutrophil stimulation needed the addition of plasma as it carries the LPS binding protein which LPS-mediated activation of p38 is dependent on [268]. It was also shown in this chapter that p-Syk was only detectable in PMA-stimulated when stimulated in non-suspended conditions. Another example was observed when the lysis buffer was changed to RIPA buffer for phospho-tyrosine blots which resulted in better resolution blots and activation profile with positive control (data not shown). Unfortunately, further attempts with inclusion of exosomes resulted in issues with the western blotting protocol (poor transfer) and evidence of activation in the non-stimulated neutrophils. Thus, it is evident that the detection of proteins can be very specific to the signalling protein of interest, adding to the difficulty and complexity of western blotting in neutrophils. In addition, the issue discovered with sample degradation after storage meant that it was best for western blots to be run on the same day of sample preparation. The isolation of neutrophils from whole blood takes up to 3 hours, in combination with the time to stimulate cells, prepare lysates, and run a western blot, is an additional 8 hours. It was therefore not feasible to continue and optimise conditions for each antibody of signalling proteins that were thought to be involved in NET induction.

Future studies

Due to the time restraints and difficulties in working with neutrophils and their sensitive nature, a number of experiments were not able to be pursued. The author hoped to investigate the ability of pancreatic cancer-derived exosomes to drive neutrophil polarisation to an N1 or N2 phenotype. This would be a significant observation with implications for pancreatic cancer-derived exosomes as mediators for the N1 and N2 phenotype observed in cancer. It is important to note that so far, there has only been one study so far that reported the polarisation of neutrophils to the N2 phenotype by tumour-derived exosomes in 2018 [144]. This was followed by another relatively recent study in 2022 which showed melanoma-derived exosomes could promote neutrophils with a pro-tumour phenotype [244]. Given the increasing evidence and the roles of the pro-tumour phenotype in cancer, it would be essential to identify the specific mediators, which could largely be mediated by cancer-derived exosomes. It is surprising that no other studies have reported or yet confirmed these findings, however this could be attributed to the difficulty with working with neutrophils and inability for other groups to replicate these findings.

If conditions were able to be optimised in this study, it would be desirable to uncover the specific pathways involved in pancreatic cancer exosome-mediated NETs. Studies would include inhibition studies with receptors, potentially TLR4 or RAGE, and signalling pathways Erk, Akt and Syk to determine is these are necessary for NET generation. The studies would include the recombinant HMGB-1 and S100A4 proteins as stimulants to compare the NET and signalling response and the effect upon its inhibition. These would be pivotal to understanding the NET signalling pathways involved in pancreatic cancer. It would also be of interest to investigate the method of uptake of exosomes by exosomes, whether by internalisation or receptor-mediated processes. While many experiments were not able to be carried out in this part of the study, the result that pancreatic cancer-derived exosomes, particularly from a metastatic cell line AsPC-1, was able to directly stimulate neutrophils to generate NETs is a novel finding, and given the important role of NETs in cancer it has implications in both cancer progression and thrombosis in cancer.

Appendix 3.1



A western blot from an attempt to optimise sample preparation conditions for western blotting. Neutrophils were left untreated or stimulated with PMA or LPS at 37°C degrees for 5 min, then lysed with HEPES-NP40 lysis buffer for western blotting. Neutrophils that were resting for 5 min on the bench at room temperature were also taken and lysed for analysis on the difference of activation in P-Erk. It is evident that the incubation at 37°C alone (Lane 2) activated Erk when compared to non-stimulated neutrophils that were at room temperature on the bench (Lane 1).

Chapter 4

Pancreatic cancer-derived exosomes do not directly activate but prime platelets for a prothrombotic effect via neutrophils

4.1 Introduction

Cancer patients are at greater risk of developing thromboembolic complications compared to non-cancer patients, leading to increased mortality, morbidity and poorer prognosis [269, 270] . Depending on the type of cancer there is a 4- to 7-fold greater risk in patients with cancer compared to those without cancer [271, 272]. Patients with pancreatic, brain and lung cancer are classified as high risk and the highest incidence of venous thromboembolism occurs in these patients [273]. The first report that described a link between thrombosis and malignancy was in 1865 by Armand Trousseau. However, despite the long-established association of cancer and thrombosis, the mechanisms are not completely understood.

The study of extracellular vesicles has attracted great interest in thrombotic disease, particularly in cancer-associated thrombosis, due to the common occurrence of thrombotic complications in malignancy. Cancer cells express tissue factor and release tissue factor-positive microparticles [274-276]. Microparticles (MP), also referred to as microvesicles (MV), are a larger subtype of extracellular vesicles that are formed by membrane shedding. It has been shown that the presence of tissue factor (TF), the primary initiator of the coagulation cascade, on cancer-derived microparticles contributes to thrombosis in cancer patient [274, 277]. TF-positive cancer-derived MPs have been shown to carry procoagulant properties both *in vitro* and *in vivo* [37, 274, 277]. The analysis of plasma from cancer patients have shown increased levels Furthermore, the high activity of TF on microparticles has also been associated with thrombotic events in cancer [23, 25]. However, reports are conflicting as some studies have reported that TF activity of MPs did not correlate with cancer-associated thrombosis [24, 278]. Interestingly, Bang et al. reported that although high levels of cancer-derived extracellular vesicles in plasma of cancer patients were associated with increased thrombosis, there was no association with TF-positive extracellular vesicles with thrombosis [279].

In addition to direct activation of coagulation, extracellular vesicles may initiate prothrombotic effects through the activation of platelets and neutrophils, which both have been shown to be vital in propagating thrombosis [34, 36]. MVs isolated from pancreatic cancer cells were shown to enhance thrombosis in mice through the activation of platelets which was dependent on TF [36]. This study also showed TF-dependent *in vitro* activation of platelets. However, Gomes and colleagues showed evidence for TF-independent activation of washed platelets by breast cancer-derived extracellular vesicles [280]. Thus, the mechanism of cancer-derived MV activation of platelets is not clear and evidence for the role of TF are conflicting. Neutrophils can release neutrophil extracellular traps (NETs) which have been shown to be important in the development of thrombi in deep vein thrombosis [209, 281] and proposed to be important in cancer-associated

thrombosis [187]. Thus far one group has shown that cancer-derived extracellular vesicles are capable of inducing NETs [145]

Much of the studies investigating cancer-derived extracellular vesicles in cancer-associated thrombosis have focused on microparticles. Exosomes are a smaller subtype of extracellular vesicles that are generated from multivesicular bodies. So far very few studies have investigated the role of cancer-derived exosomes in thrombosis, thus their role may be underestimated. Due to the high risk of thrombosis in pancreatic cancer patients, studies will be carried out using pancreatic cancer-derived exosomes (PDEx). **This study aims to investigate PDEx and their prothrombotic effects by examining its ability to promote coagulation, activate platelets, and stimulate NET release via platelets.**

4.2 Materials and Methods

Cell culture and maintenance

Exosomes were isolated from pancreatic cancer cell lines AsPC-1, BxPC-3 and MIA PaCa-2. AsPC-1 and BxPC-3 were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum. MIA PaCa-2 was cultured in DMEM media (Sigma-Aldrich) supplemented with 10% foetal bovine serum. All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Exosome isolation

Exosomes were isolated from the conditioned medium of pancreatic cancer cell lines AsPC-1, BxPC-3 and MIAPaCa-2 cells. Approximately 5 x 10^6 cells were seeded in one T175 cm^2 culture flask (three flasks for each biological replicate). Cells were seeded in their respective medium used for maintenance. When the cells reached 60-70% confluence, the medium was switched to contain exosome-depleted FBS to prevent cross-contamination of exosomes. After 72hr the conditioned medium was pooled (approximately 45 ml) and processed through differential centrifugation to remove cells and cell debris at 480 x g for 5 min and 2,000 x g for 10 min, respectively. The conditioned media was filtered through a 0.22 µM filter. Exosomes were pelleted at 100,000 x g for 2 hr and washed with PBS before a final centrifugation at 100,000 x g for 2 hr. Exosomes were resuspended in filtered PBS and stored at -80°c. The protein concentration of lysed exosomes was quantified using Micro BCA assay kit (Sigma Aldrich), which was used to normalise exosomes used in the experiments.

Western blotting

For exosomal lysates, exosomes were lysed in RIPA buffer (NaCl 150 mm, Nonidet P-40 1%, Sodium deoxycolate 0.5%, EDTA 2.5 mm, SDS 0.1%, Tris-HCl 25 mm) and heat treated at 95°C for 10 min. Lysates were electrophoresed on a 10% sodium dodecyl sulphate–polyacrylamide (SDS-PAGE) gel at 200 V and transferred to a 0.22 µm polyvinylidene difluoride membrane. Membranes were blocked for 1 hr at room temperature with 5% milk in TBST. Primary antibodies were probed overnight at 4 °C in 3% BSA in TBST. All antibodies were purchased from Cell Signaling Technology and were used at a dilution of 1:1000. Membranes were washed three times in TBST then incubated with secondary antibody for 3 hr at room temperature. Bands were visualized with ChemiDoc MP Imaging System (BioRad).

For platelet samples, platelets which have been incubating exosomes (50 µg/ml) for 4 hr at 37°c were centrifuged at 1,000 x g for 7 min and then lysed with RIPA buffer supplemented with protease inhibitor cocktail (Cell Signalling Technology). Western blot analysis was performed as described above.

Scanning Electron Microscopy

Exosomes that were resuspended in PBS were mixed with equal volumes of 4% paraformaldehyde (Sigma) on a glass coverslip and left to fix overnight at room temperature. Fixed samples were dehydrated with increasing concentrations of ethanol (10, 20, 40 and 90%) for 10 min each. The coverslips were mounted onto aluminium stubs with carbon tape. Imaging was performed by the Microscopy and Microanalysis Facility, John de Laeter Centre, Faculty of Science and Engineering, Curtin University, Bentley, WA.

Coagulation Assay

A plate-reader based kinetic assay to screen for procoagulant activity in human plasma was adapted from Still and colleagues [282]. To prepare microvesicle-depleted plasma, citrated blood (0.32% sodium citrate) was collected from healthy donors and centrifuged at 1550 x g for 20 min to obtain cell-free plasma. To further remove endogenous extracellular vesicles the plasma was spun at 18,000 x g for 1 hr at room temperature. Microvesicle-depleted plasma was stored at -80°c until ready to use. For the clotting assay, exosomes with or without inhibitor were added to wells of 96-well plate, in addition to calcium chloride (final 10 mM). Assays were performed at room temperature. Citrated plasma was added to each well to initiate coagulation. The measurement of coagulation was performed on a plate reader immediately after the addition of citrated plasma. A reduction in light transmittance occurs as a result of fibrin generated, which is monitored as increased absorbance in a clear 96-well plate. Absorbance was measured at a wavelength of 565 nm

on an EnSight Multimode microplate reader (PerkinElmer). Absorbance measurements were made over a time course (every 30 sec for 1 hour) and a kinetics curve was generated by the plate reader instrument which was used for analysis.

Preparation of washed human platelet

Blood was collected from healthy volunteers into a syringe containing acid-citrate-dextrose (1:7, v/v) with informed consent from the Curtin University Human Research Ethics Committee (approval number HR54/2014). Blood was centrifuged at 150 x g for 20 min. The platelet-rich plasma was centrifuged at 800 x g for 10 min in the presence of PGE2 (1 µM) to pellet platelets and washed three times with CGS buffer (14.7 mM trisodium citrate, 33.33 mM glucose and 123.2 mM NaCl, pH 7) in the presence of prostaglandin E2. Platelets were resuspended to 1 x 10^9/ml in 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_2PO_4, pH 7.4).

Washed platelet aggregation

Washed platelets (3 x 10^8/ml) in Tyrode's HEPES buffer supplemented with 1.8 mM calcium chloride were incubated with exosomes (10 µg/ml and 50 µg/ml) at 37°c. Platelet aggregation was monitored under stirring conditions (1200 RPM) in a light transmission aggregometre (Model 700 Aggregometre, Chrono-log Corporation) for at least 30 min. In some conditions, 1% of autologous plasma was included in the reaction.

Platelet-dense granule secretion assay

Platelet secretion was determined by ATP release using the luciferin-luciferase reagent (Chrono-Lume, Chrono-Log, USA). Briefly, 90 µl of platelets (1 x 10^8/ml) in Tyrode's HEPES buffer supplemented with calcium was added to 10 µl of exosomes (10 µg/ml final). Platelets were incubated on a shaker at 37°c for 10 min before adding 5 µl of Chrono-Lume reagent. Luminescence was measured on multiplate reader.

CD73 activity assay

CD73 activity was evaluated from the levels of inorganic phosphate levels resulting from the hydrolysis of exogenous AMP. Exosomes were first diluted 1:10 in assay buffer. Samples were diluted 1:1 with the inhibitor (AB680; 2 nM) or buffer and incubated for 1 hr at 37°C. AMP (100 µM) or buffer was added to the samples and incubated for 1 hr at 37°C. Phosphate concentrations were measured using Malachite Green detection kit following manufacturer's instructions.

CD73 ELISA

An ELISA was performed on pancreatic cancer-derived exosomes using a CD73 ELISA kit from Abcam (ab254513). Exosome samples were diluted 1:10 and 100 µl of sample was added to the microplate before following the protocol according to manufacturer's instructions.

Neutrophil isolation

Neutrophils were isolated from human blood using PolymorphPrep (Axis-Shield, Norway), with minor changes to the manufacturer's protocol. Blood was collected with EDTA (2 mM) as an anticoagulant. The blood was layered over PolymorphPrep 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).

Priming of platelets with exosomes

Washed platelets (1 x 10^8/ml) were incubated with exosomes (50 µg/ml) for 4 hr at 37°c. The platelets, referred to as exosome-primed platelets, were centrifuged at 800 x g for 5 min and then washed and resuspended in Tyrode's HEPES to a concentration of 1 x 10^9/ml. A final concentration of 1 x 10^8/ml of primed or non-treated platelets were added to neutrophils for quantification of NET release.

NET quantification assay

Neutrophils (5 × 10^4) were seeded in 96-well clear-bottom plate and allowed to adhere for 15 min at 37 °C 5% CO_2 before addition of exosomes or exosome-primed platelets (1 × 10^8/mL). Cell stimulation cocktail (Thermofisher) and lipopolysaccharide (Thermofisher) were used as positive control treatments. 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 an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). A second reading was obtained at 3 hr. Treatments were performed in replicates of four. The amount of NET released was determined by calculating fluorescence fold change from unstimulated control.

4.3 Results

Characterisation of exosomes isolated from pancreatic AsPC-1, BxPC-3 and MIA PaCa-2

Exosomes were isolated from the conditioned media of cultured pancreatic cancer cell lines AsPC-1, BxPC-3 and MIA PaCa-2 using differential ultracentrifugation. To confirm the enrichment of exosomes, western blotting was performed to verify the presence of exosome markers CD63 and Alix, and the absence of GM130, a Golgi membrane protein that should be absent in exosomes. Alix is one of the well-established markers of exosomes [283]. Western blotting confirmed that CD63 and Alix were present in exosome lysates, while GM130 was not (Figure 4.1a & b). Scanning electron microscopy was performed on exosomes to evaluate size range and morphology. Enriched exosomes were found to be within the exosome size range of 30-100 nm with a round morphology (Figure 4.1c). Furthermore, given the roles of tissue factor in coagulation, western blot of tissue factor on exosomes was also performed to assess the expression in the three pancreatic cancer cell-derived exosomes. Tissue factor was detected in AsPC-1 and BxPC-3-derived exosomes, while absent in MIAPaCa-2-derived exosomes (Figure 4.2b). The expression of TF in exosomes reflects the expression in the pancreatic cancer cell lines, as flow cytometry analysis of the pancreatic cancer cells also showed expression of TF in AsPC-1 and BxPC-3, but not MIA PaCa-2 cells (Figure 4.2a). The mean fluorescence intensity (MFI) of tissue factor compared to isotype control was analysed. Both AsPC-1 (MFI of anti-TF: 10508 ± 5933 vs isotype: 380 ± 200) and BxPC-3 (MFI of anti-TF: 14615 ± 7304 vs isotype: 220 ± 113) had a trend increase in MFI of anti-tissue factor compared to isotype control (Figure 4c, $n \geq 3$). Mia PaCa-2 on the other hand, showed similar MFI values between anti-TF (MFI: 410 ± 239) and isotype control (MFI: 403 ± 235; $n = 4$) indicating an absence of TF expression as expected. There is a large variation between tests, which may be due to the use of different flow cytometers.

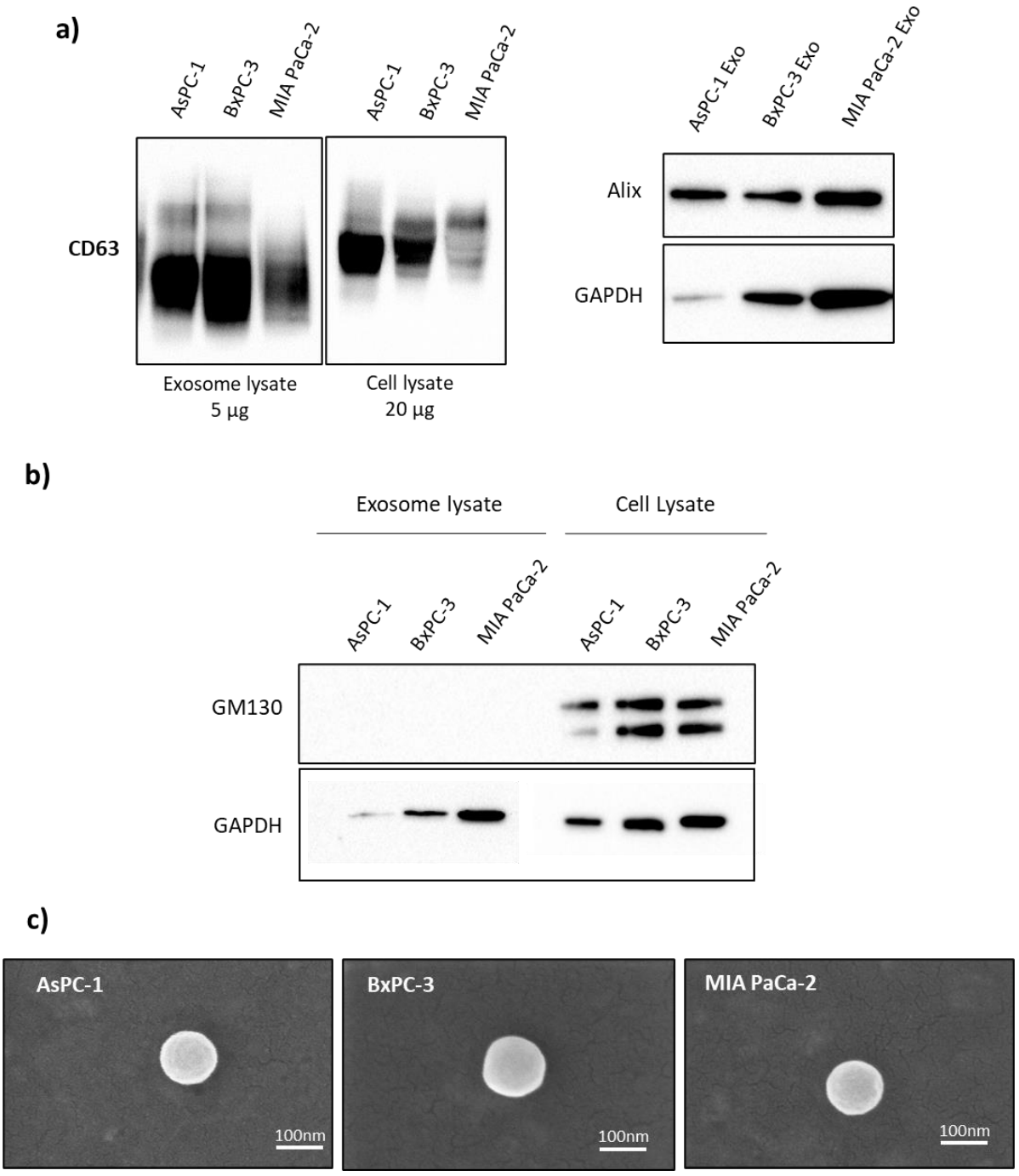


Figure 4.1. Characterisation of pancreatic cancer-derived exosomes (PDEx) isolated from AsPC-1, BxPC-3 and MIA PaCa-2 pancreatic cancer cell lines. a) Presence of exosomal markers CD63 and Alix in exosomes. CD63 is enriched in exosome lysates (5 µg loading) when compared to cell lysates with greater protein loading (20 µg). Results representative of two independent experiments. B) Absence of Golgi marker GM130 in exosome lysates, while present in cell lysates. C) Scanning electron microscopy images showing the expected morphology and size of exosomes. Three independent samples from each cell line were sent for imaging. Images shown are the clearest that were obtained and give the best representation of exosomes.

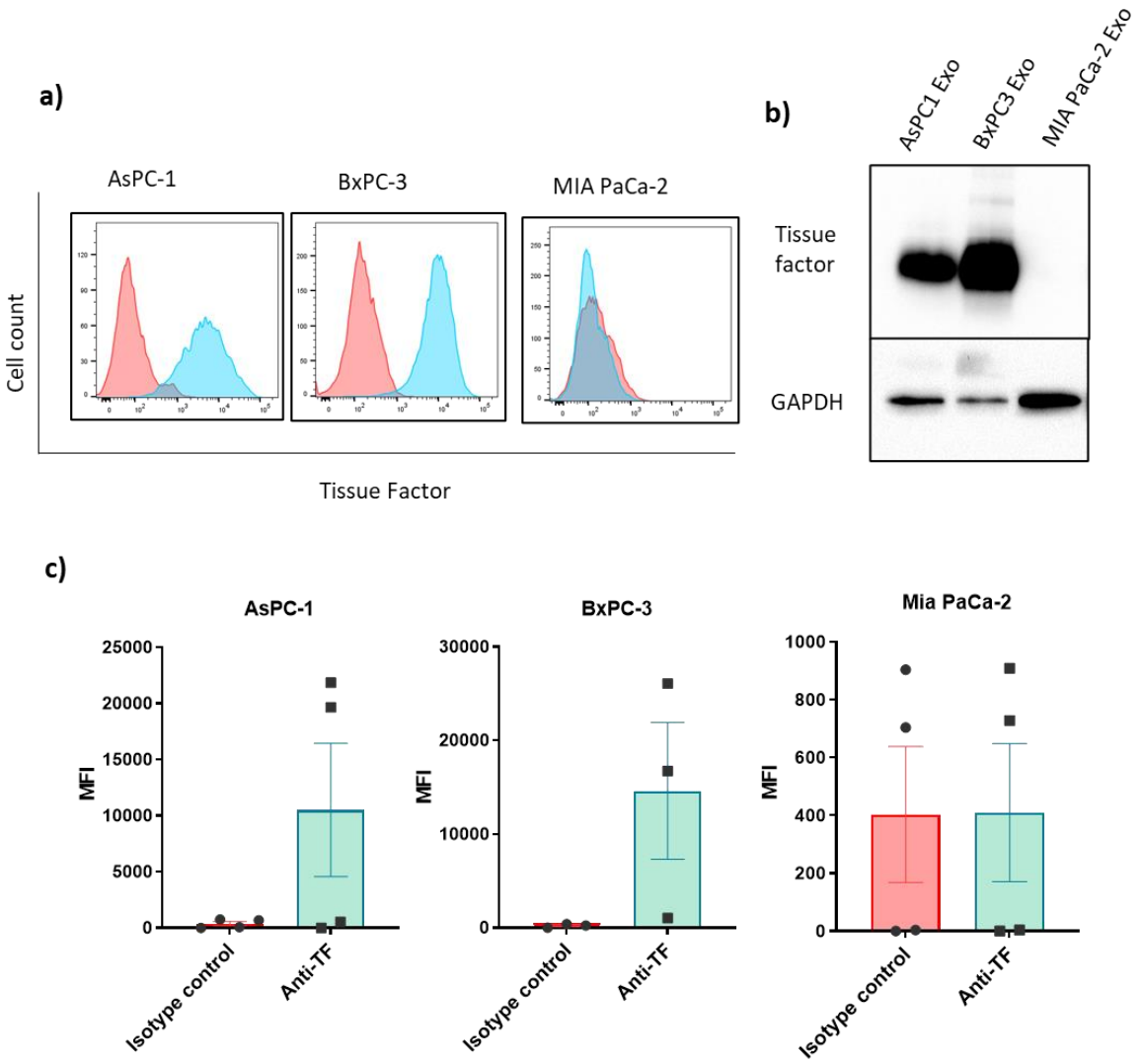


Figure 4.2. Tissue factor expression in pancreatic cancer-derived exosomes and the parent cell lines. a) Flow cytometry analysis of tissue factor surface expression on pancreatic cancer cell lines AsPC-1, BxPC-3 and MIA PaCa-2. Histogram representative of three independent experiments. b) Western blot analysis of exosomes isolated from three pancreatic cancer cell lines. 5 µg of exosome lysate was loaded and separated on SDS-PAGE gel. Tissue factor is present in AsPC-1 and BxPC-3 but not MIA PaCa-2 exosomes. Blot representative of two independent experiments. c) Column graphs of mean fluorescence intensity (MFI) of the isotype control and anti-tissue factor (anti-TF) antibody for each cell line. Data expressed as mean ± SEM. No significant difference between means as determined by unpaired t-test ($n \geq 3$).

Pancreatic cancer-derived exosomes promote coagulation

Several studies have shown that TF-positive microvesicles possess procoagulant properties *in vivo*, through correlation with elevated blood coagulation markers and thrombotic events [284]. To determine the ability of pancreatic cancer-derived exosomes to directly promote coagulation *in*

vitro, fibrin generation in plasma was measured using a 96-well spectrophotometric measurement-based assay. Exosomes (10 μg/ml) were incubated with microvesicle (MV)-depleted plasma in the presence of 10 mM calcium to facilitate re-calcification of the citrated plasma. MV-depleted plasma was used to ensure there was no contamination of endogenous blood-derived MVs. Figure 4.3 shows the capacity for all exosomes to initiate coagulation at a rate that occurs faster than the control (plasma with calcium chloride) suggesting exosomes are directly potentiating coagulation. This potentiation is demonstrated by the increased absorbance in AsPC-1 (0.189 ± 0.01; ** $P < 0.01$, n=6), BxPC-3 (0.211 ± 0.02; *** $P < 0.001$, n=6) and MIA PaCa-2 (0.151 ± 0.05; ns, n=6) exosomes, compared to the control reaction (0.058 ± 0.01; n=6) (Figure 4.3b, n≥3). AsPC-1 and BxPC-3 exosomes showed almost immediate initiation of coagulation, which started within minutes, while the control reaction initiated coagulation after 10 minutes (Figure 4.3a). Figure 4.3c shows the lag time for the initiation of clotting as depicted by the start of an increase in absorbance in the kinetic assay. All exosomes had initiated clotting with significantly less lag time (AsPC1: 80 ± 33 sec; **** $P < 0.001$; BxPC3: 80 ± 23 sec, **** $P < 0.001$; MIA PaCa-2: 520 ± 57 sec, ** $P < 0.01$, n=6) compared to control (785 ±75 sec, n=6) (Figure 4.3b). However, MIA PaCa-2 appears to have a longer lag time compared to AsPC-1 and BxPC3 exosomes. These differences in coagulation profiles between exosomes may be due to the presence of tissue factor on AsPC-1 and BxPC-3 but not MIA-PaCa-2. Thrombin (0.25 U/ml) served as positive control which resulted in almost immediate initiation of coagulation (60 ± 30 sec, **** $P < 0.001$, n=6).

To investigate the role of thrombin in exosome-mediated coagulation, hirudin (a direct thrombin inhibitor) was added to the plasma and exosome reactions. There was a clear inhibition of exosome-mediated coagulation with the addition of 1 U/ml thrombin in all reactions with exosomes (Figure 4.3b, n=3) as all reactions were similar to the calcium chloride control, indicating a thrombin-dependent mechanism of coagulation mediated by pancreatic cancer-derived exosomes.

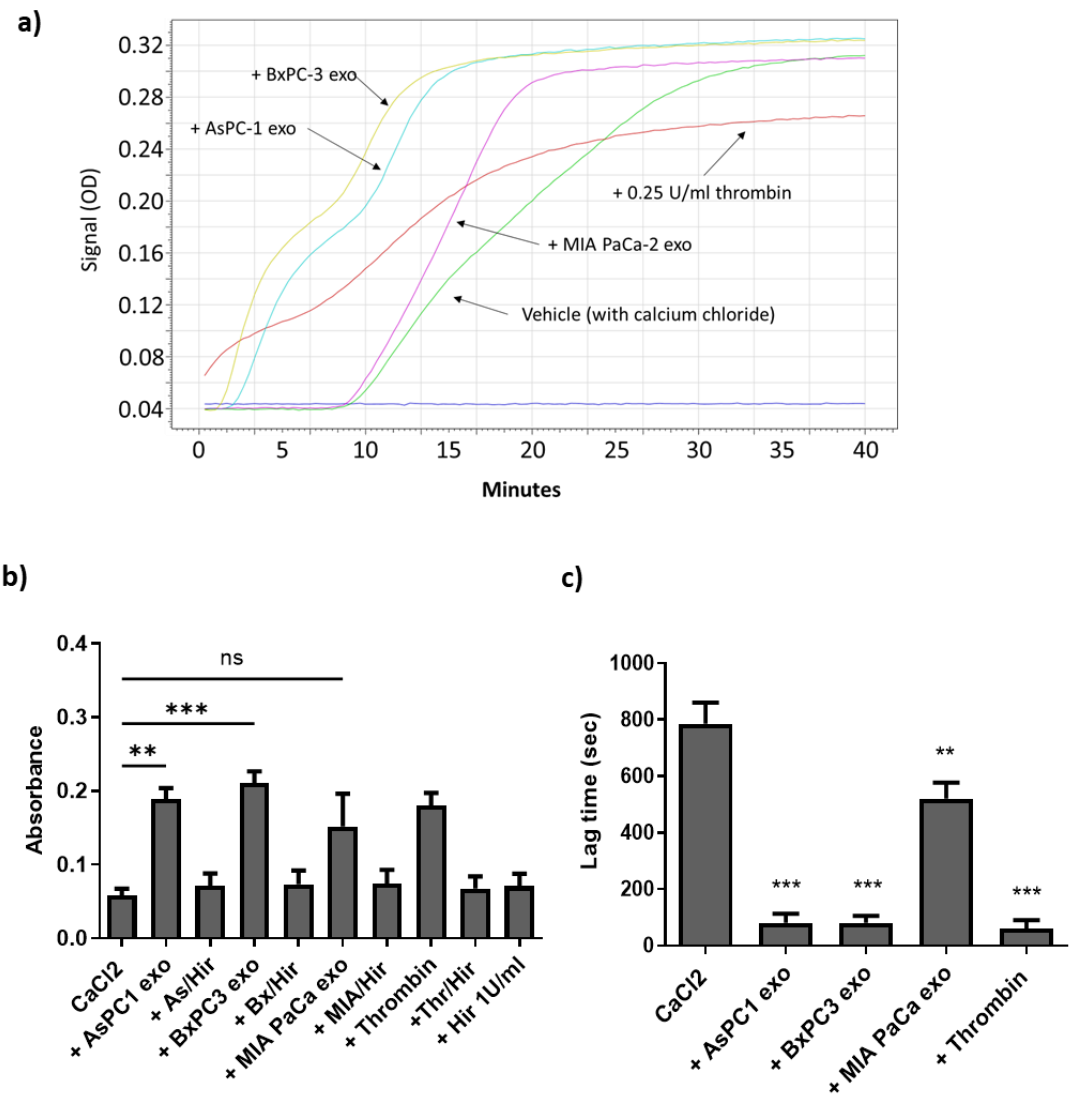


Figure 4.3 PDEx potentiate coagulation. a) Exosomes (10 µg/ml) were added to human microvesicle-depleted plasma in the presence of 10 mM calcium chloride to initiate coagulation. Thrombin served as positive control. Vehicle refers to PBS vehicle for exosomes which included calcium chloride. 10 mM calcium chloride was included in all reactions to initiate clotting. The curve depicts the increase in absorbance (or decreased light transmittance) as a result of fibrinous clot formation. Graph representative of six independent experiments. b) Thrombin-dependent mechanism of exosome-induced coagulation. Effect of direct thrombin inhibitor, hirudin (1 U/ml) on clotting induced by exosomes. The absorbance in each treatment when clotting has initiated in the control reaction (calcium chloride alone) is shown (n ≥ 3). c) Lag time of coagulation with exosomes which is defined by the time (seconds) at which clotting is initiated (n=6). Data analysis was performed with one-way ANOVA followed by post-test Bonferroni's multiple comparison test. Data expressed as mean ± SEM.

Effect of PDEx on platelet aggregation

As PDEx were shown to facilitate coagulation, we wanted to further investigate the pro-coagulant effect of exosomes through platelets as they are known to play a major role in coagulation. To date, there have been few studies reporting the ability of cancer-derived MVs to induce platelet aggregation in either the presence or absence of plasma. Gomes et al. were the first to report extracellular vesicles (mostly in the exosome size range) isolated from breast cancer cells, induced platelet aggregation in the absence of plasma, indicating a TF-independent mechanism. On the other hand, earlier studies have shown cancer-derived MVs (isolated from pancreatic cancer) were able to aggregate platelets only in the presence of plasma, suggesting a TF-dependent process. These studies used a concentration of exosomes ranging from 0.5 to 50 μg/ml.

We assessed exosome-induced platelet aggregation using 10 and 50 μg/ml of exosomes in the absence or presence of plasma. Exosomes were incubated with platelets for at least 30 minutes. While there was no aggregation observed with exosomes in the absence of plasma (Supplemental Data Figure 4.1 – found in the Appendices at the end of this chapter) there was variability observed among donors with the addition of 1% plasma in the reaction with 10 μg/ml of exosomes (Figure 4.4a). AsPC-1 and BxPC-3 exosomes at 10 μg/ml caused aggregation in 2 out of 4 donors. With a higher concentration of exosomes (50 μg/ml), there was a more consistent aggregation of platelets with AsPC-1 and BxPC-3, where aggregation was seen in 3 out of 4 donors (Figure 4.4b). In contrast, there was consistently no aggregation with MIA PaCa-2 exosomes at both low and high concentrations in the presence of plasma. Any aggregation observed with exosomes was induced within 5 to 15 min of incubation. Examples of the aggregation traces obtained when there was aggregation with 10 and 50 μg/ml exosomes are shown in Figure 4.5. Overall, there was a greater lag time with 10 μg/ml exosomes (Figure 4.5a) compared to 50 μg/ml exosomes (Figure 4.5b). In addition, the aggregation induced by exosomes appeared to be a dose-dependent response as platelets with 50 μg/ml exosomes aggregated faster (Fig 4.5b) and with greater aggregation percentage (AsPC1: 68 ± 18 %; BxPC-3: 70 ± 17 %, n=4) when compared with 10 μg/ml exosomes (AsPC-1: 43 ± 34%; BxPC-3: 53 ± 33, n=4) (Figure 4.4a & b). These data suggest there is a threshold of 50 μg/ml for exosomes to have a consistent effect on the aggregation of platelets. Overall, any platelet aggregation that was observed is suggested to be TF-dependent due to exosome-induced platelet aggregation occurring only in the presence of plasma, and not in the TF-negative MIAPaCa-2 exosomes.

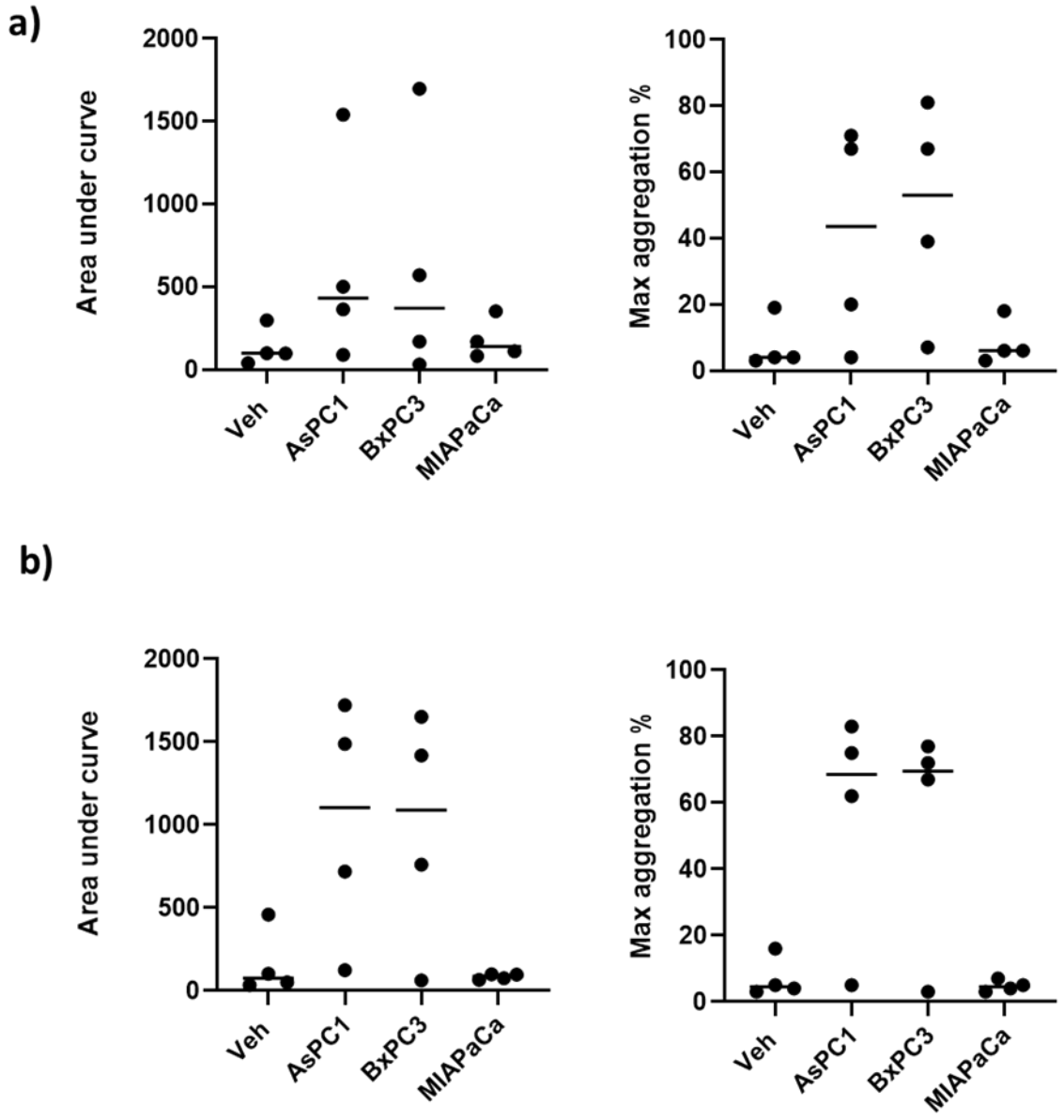
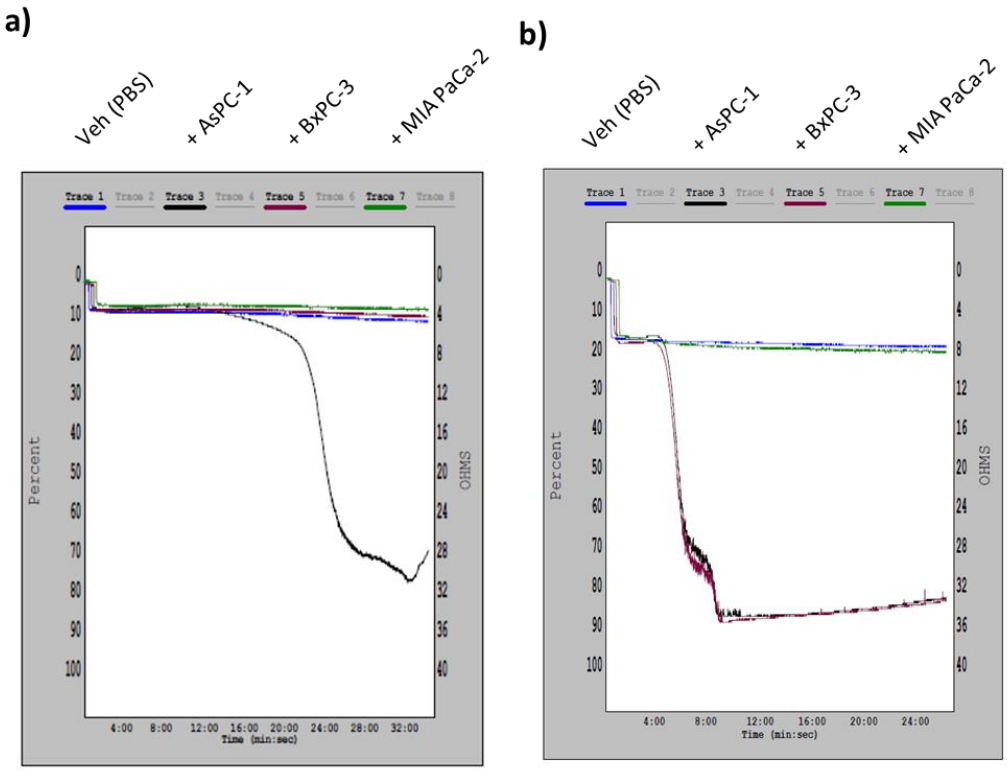


Figure 4.4. Effect of exosomes on washed platelet aggregation in the presence of plasma. Effect of 10 µg/ml exosomes on the area under the curve and amplitude on washed platelet aggregation in the presence of 1% plasma in A) 10 µg/ml exosomes and B) 50 µg/ml exosome (n = 4). There was a greater consistency of platelet aggregation with 50 µg/ml of exosomes while aggregation with 10 µg/ml exosomes was inconsistent with AsPC-1 and BxPC-3 exosomes. MIA PaCa-2 exosomes did not induce aggregation at either 10 µg/ml and 50 µg/ml exosome concentrations.



10 µg/ml exosomes

50 µg/ml exosome

Figure 4.5 Effect of exosomes on washed platelet aggregation trace in the presence of 1% plasma. Example trace of washed platelet aggregation curves when aggregation was observed with a) 10 µg/ml exosomes and (b) 50 µg/ml exosomes. Washed platelets were incubated with 1% homologous plasma and left to stir at 1200 RPM for 1 hr. In the donors (3 out of 4) which demonstrated aggregation with 50 µg/ml exosomes, there was reduced lag time and greater percentage aggregation compared to 10 µg/ml exosomes.

The effect of PDEx on dense granule secretion (ATP release)

As there was no direct effect of exosomes on platelet aggregation observed, we assessed other modes of activation, as platelet activation may still occur in the absence of platelet aggregation. ATP release was measured using the CHRONOLUME reagent. After a 10 min incubation of exosomes with platelets, there was no significant release of ATP with either 10 µg/ml or 50 µg/ml exosomes (Figure 4.6a). Interestingly, there appeared to be a decrease in ATP release with exosomes, which was greater with 50 µg/ml (ATP fold change vs 1.00 in AsPC1: 0.305 ± 0.2; BxPC3: 0.770 ± 0.05; MIA PaCa-2: 0.500 ± 0.06, ns, n=3) (Figure 4.6a). To determine whether exosomes were responsible for the observed reduced ATP release, the assay was repeated with the addition of collagen to the reaction. Collagen is known to stimulate granule release from platelets. While not very noticeable, the treatment with exosomes showed a trend in reduction of ATP release (ATP fold change with AsPC1: 4.40 ± 1.1, BxPC3: 5.20 ± 1.4, MIA PaCa-2: 4.52 ± 1.3, n=3) when compared to collagen-activated

platelets without the presence of exosomes (ATP fold change: 5.30 ± 1.2) (Figure 4.6b). These data suggest that cancer-derived exosomes may be involved in reducing ATP release from platelet activation, and potentially dampening a complete activation response in platelets.

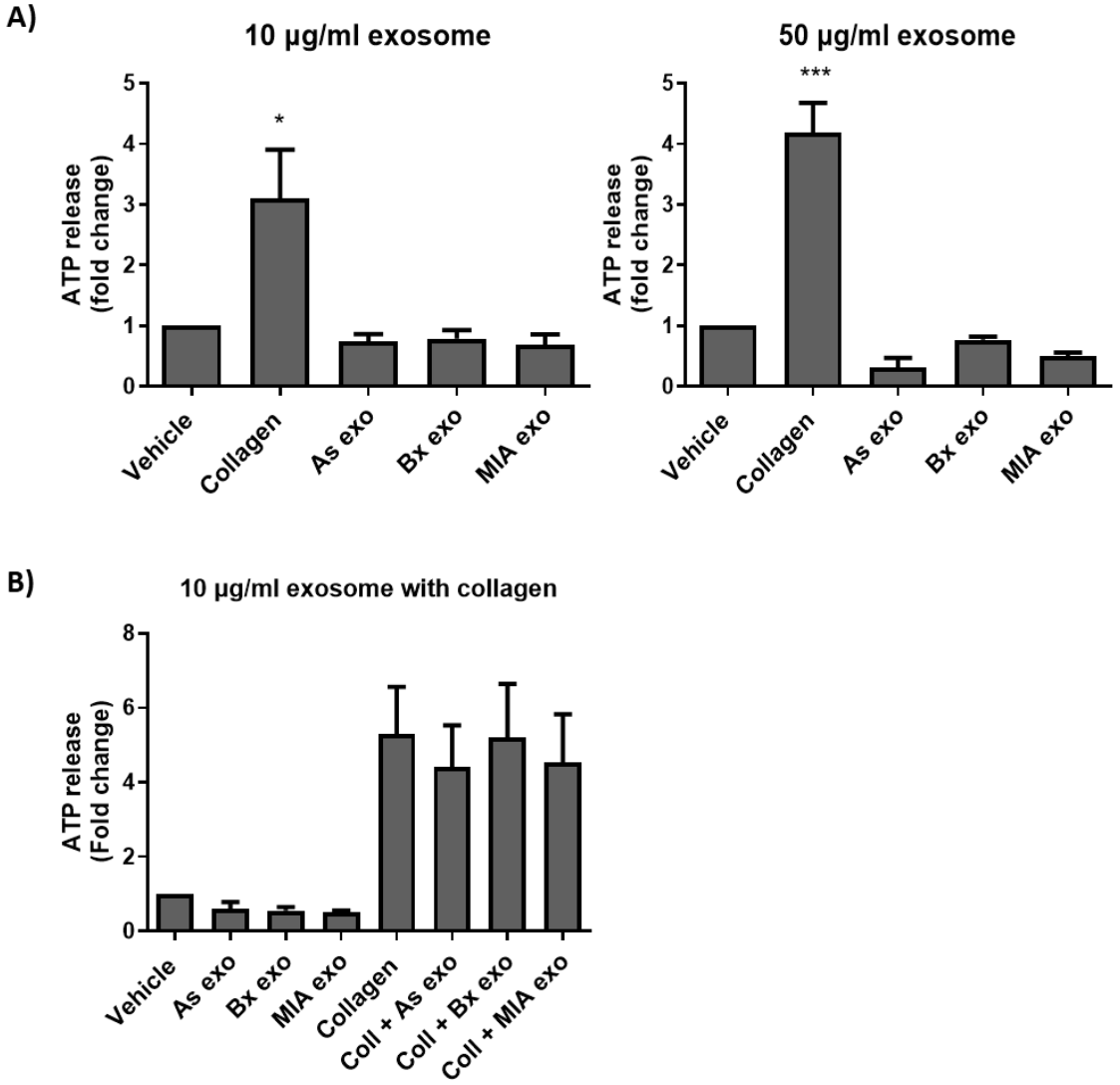


Figure 4.6 Effect of exosomes on dense granule secretion from platelets. A) Exosomes were incubated with both 10 and 50 µg/ml exosomes and assessed for ATP release. Collagen 10 µg/ml served as positive control. n=3. B) The effect of 10 µg/ml of exosomes on ATP release when incubated together with collagen (n=3). Results are expressed as fold change in ATP release relative to vehicle control (PBS). Data presented as mean ± SEM, * $P < 0.05$, *** $P < 0.001$. Data analysis was performed with one-way ANOVA followed by post-hoc Bonferroni's multiple comparison test. There was a trend of reduced ATP release when exosomes were included in the collagen and platelet reactions, suggesting exosomes may be reducing ATP release from collagen-activated platelets.

Evidence of CD73 activity on PDEx

One possible explanation for the observed slight reduction in ATP release from platelets in the presence of exosomes is the activity of ectonucleotidase such as CD73. CD73 is known to convert extracellular ATP to adenosine, which has immunosuppressive effects in the tumour microenvironment [285]. In addition, one study has previously shown that exosomes from diverse cancer cell types express CD73 [286]. Thus, it is probable that any ATP released from platelets may have been cleaved by CD73 expressed on PDEx, as evidenced by a slight reduction of ATP that is also observed in collagen-stimulated platelets in the presence of exosomes.

To determine if the exosomes isolated from pancreatic cancer cells in this study have ATP hydrolytic activity, a Malachite Green assay was performed. CD73 activity was observed in all AsPC-1, BxPC-3 and MIA PaCa-2 exosomes (Figure 4.7a). To confirm that the result was due to CD73 activity, a CD73 inhibitor, AB680 (1 nM), was used to inhibit CD73 activity. In AsPC-1 and BxPC-3 exosomes, ATP hydrolytic activity was inhibited with AB680, with little to no phosphate production detected (Figure 4.7a). In contrast, the phosphate production by MIA PaCa-2 exosomes was increased in the presence of AB680. A test with recombinant CD73 (rCD73) and AB680 confirms that the assay was working as rCD73 resulted in increased phosphate production which was reduced with the addition of AB680 (Figure 4.7a). To confirm that these effects were due to CD73 activity on exosomes, an ELISA for CD73 was performed. Indeed, all exosomes showed presence of CD73 with greater expression in MIA PaCa-2 exosomes (CD73 ng/ml in AsPC1: 27.1 ± 7.0; BxPC3: 23.1 ± 8.4; MIA PaCa-2: 130 ± 1.7) (Figure 4.7b).

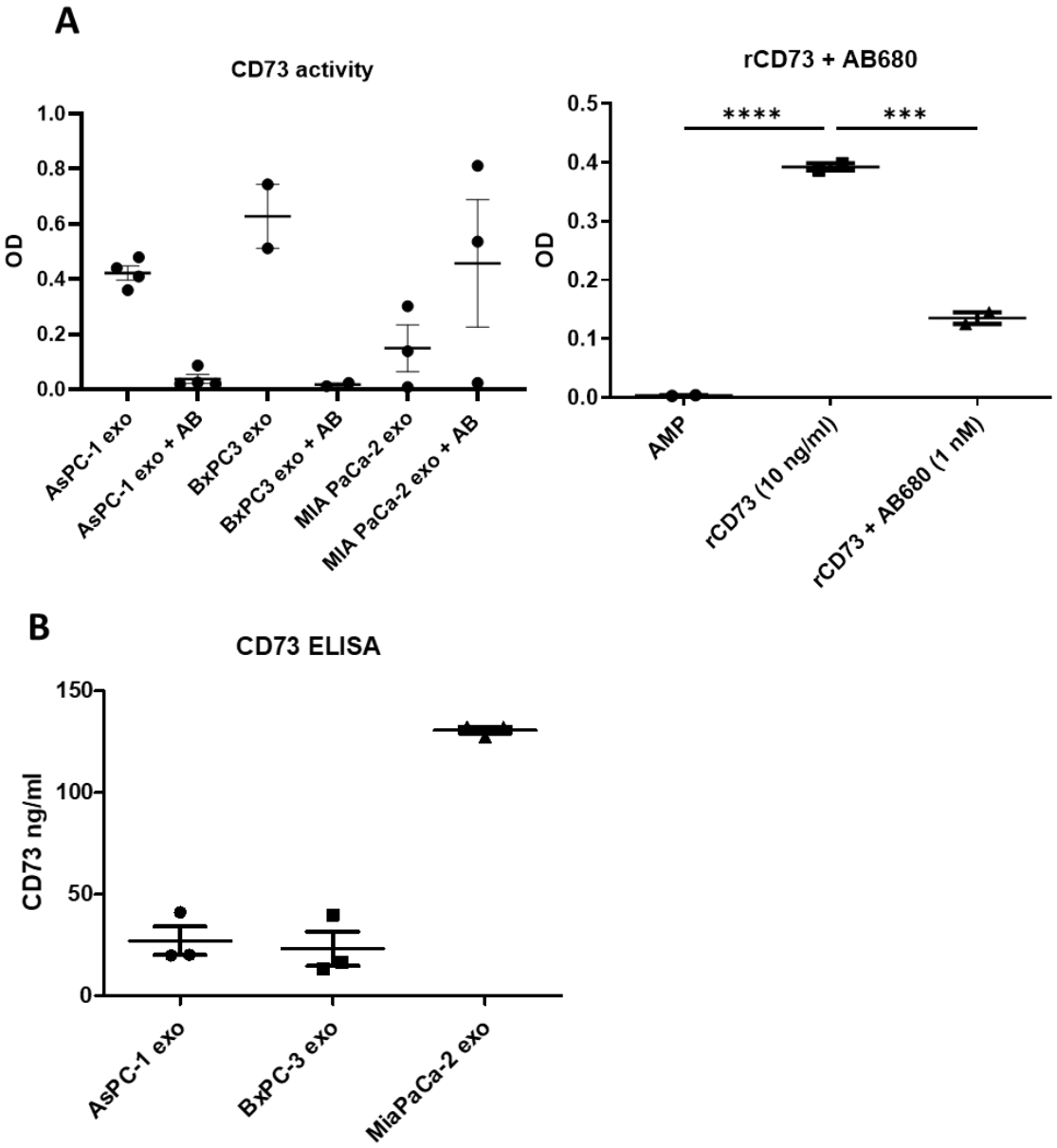


Figure 4.7 Malachite green assay and CD73 ELISA of exosomes*.** a) Right: Exosomes were tested for their ability to produce inorganic phosphate from AMP. AsPC-1 and BxPC-3 exosomes showed hydrolytic activity which was inhibited with the CD73 inhibitor AB680. MIA-PaCa 2 exosomes showed little to no phosphate production and an increase in activity was found with the addition of AB680 inhibitor. Data presented as mean ± SEM. Each dot represents the average of each exosome stock ($n \geq 2$; n refers to the number of different exosome stock). Right: Positive control test with recombinant CD73 (rCD73) and CD73 inhibitor AB680 using AMP as the substrate. Recombinant CD73 resulted in increased absorbance indicating production of inorganic phosphate, which was inhibited with the CD73 inhibitor AB680. Data were analysed using one-way ANOVA with post-hoc Bonferroni's multiple comparison test (* $P < 0.0001$, *** $P < 0.001$; $n = 2$). b) Quantification of CD73 in exosomes using CD73 ELISA. Data presented as mean ± SEM ($n = 3$; n refers to the number of different exosome stock).

Uptake or association of PDEx with platelets

As exosomes do not seem to elicit robust platelet activation responses as assessed by platelet aggregation and ATP release, or P-selectin and platelet spreading (Supplemental Data Figure 4.2 and 4.3), we hypothesised that exosomes may play a role in priming platelets to induce activation of other cell types which have roles in thrombosis. It has been shown that ingestion of extracellular vesicles can facilitate intracellular signalling. To explore this idea, we investigated the ability of platelets to ingest or become associated with exosomes. Platelets were incubated with exosomes (50 μg/ml) for 4 hr then centrifuged at 800 x g. Centrifugation at this speed will spin down platelets and any associated exosomes, but not exosomes that have not been ingested or associated with platelets via adhesion. The platelet pellets were lysed then run on SDS-PAGE gel and probed for histone H3. Histone proteins are known to not exist in resting platelets as it is a DNA-associating protein and are therefore absent in the anuclues platelet. Western blot confirmed the absence of Histone H3 in untreated platelets (Figure 4.8b). The incubation of exosomes with platelets for 4 hr resulted in the presence of histone H3 in the platelet lysates, indicating an association of exosomes with platelets (Figure 4.8b, n=3). Variation in the levels of histone H3 in platelets after incubation with exosomes is evident, suggesting different levels of association of exosomes to platelets which may be cell line dependent. We also confirmed the presence of histone H3 in exosome lysates (Figure 4.8a, n=3), indicating that the histone H3 observed in platelets is derived from exosomes. These results suggest that exosomes have become associated with exosomes after prolonged incubation, however it cannot be certain whether the exosomes have been ingested into platelets, or were associated with platelets by adhesion.

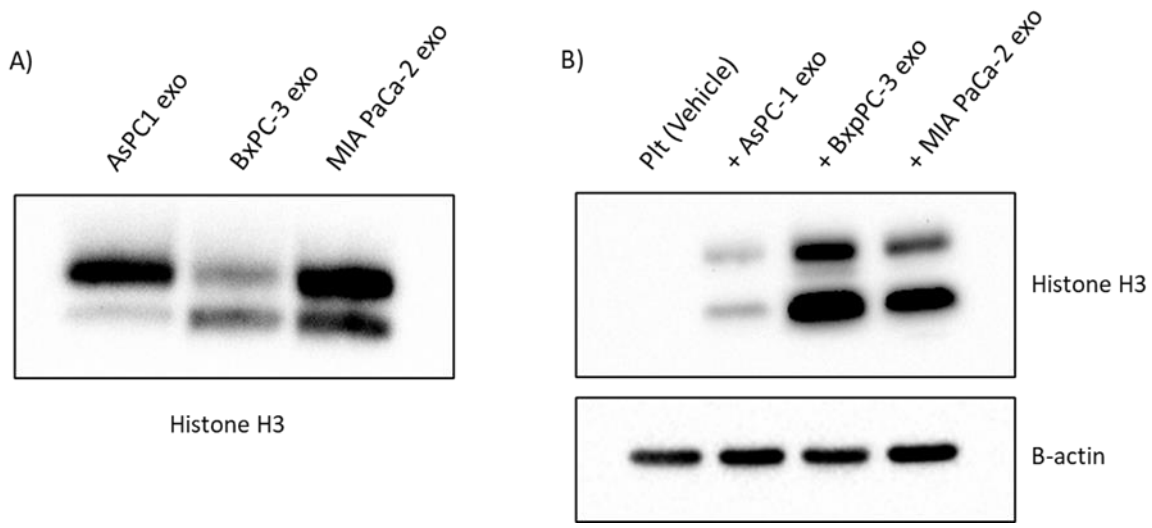


Figure 4.8 Exosomes are associated with platelets after a 4 hr incubation. A) Western blotting on exosome lysates (10 µg loading) confirms the presence of histone H3 in all exosomes. B) Platelets which have been incubated with exosomes for 4 hr were spun the washed. The platelet pellets were lysed and run on SDS-PAGE to detect histone H3. Histone protein was present in platelets that were incubated with exosomes but not in vehicle control (PBS). Blots are representative of three independent experiments.

PDEx stimulate NET release directly, and indirectly through platelets

With no potent activation of platelets observed with PDEx, it was thought that platelets may be under the influence of exosomes without signs of activation and may elicit their effect or transfer their contents to other cells elsewhere to induce a pro-thrombotic effect. Given the previous report of interactions between platelets and neutrophils in propagating thrombosis [155], it was hypothesised that platelets are primed by PDEx and these primed platelets can stimulate NET release. In addition to the link between platelets and neutrophils in thrombosis, an observation from an earlier study from our lab contributed to this hypothesis. We have previously shown that when platelets are pre-incubated with AsPC-1 cells for 4 hours, they cause rapid NET release compared to untreated platelets [249]. It is possible that within the 4 hour pre-incubation, exosomes from AsPC-1 may be transferred to or induce effects on platelets, which are then transferred to neutrophils to induce NET release. Other priming effects on platelets have been suggested via TLR4 receptor where LPS does not induce platelet aggregation, but can cause a potentiation of aggregation with low dose agonist [287, 288]. Furthermore, Clark et al. demonstrated that LPS-activated platelets did not cause platelet activation or aggregation but were able to induce NET formation which was not achieved with LPS or platelets alone [289]. Together, these led to the hypothesis that similarly to the priming

of platelets by LPS, exosomes prime platelets which does not cause direct activation of platelets and can induce activation in neutrophils and NET release.

We first confirmed that exosomes can directly stimulate neutrophils to release NETs. Exosomes were able to induce NET release which occurred within 30 min, as opposed to 3 hr which was seen with PMA, indicating a rapid form of NET release. The greatest induction of NET was by AsPC-1 exosomes (NET fold change relative to control: 6.27 ± 0.7; *** P < 0.001, n=4). BxPC-3 exosomes also induced significant NET release within 30 min with 2.77 ± 0.3-fold change in NET (* P < 0.05, n=4) compared to control MIA PaCa-2 exosomes induced a trend of increased NET release with 1.96 ± 0.25-fold change in NET release. Exosomes from non-cancer cell lines were also included as controls which did not result in induction of NETs.

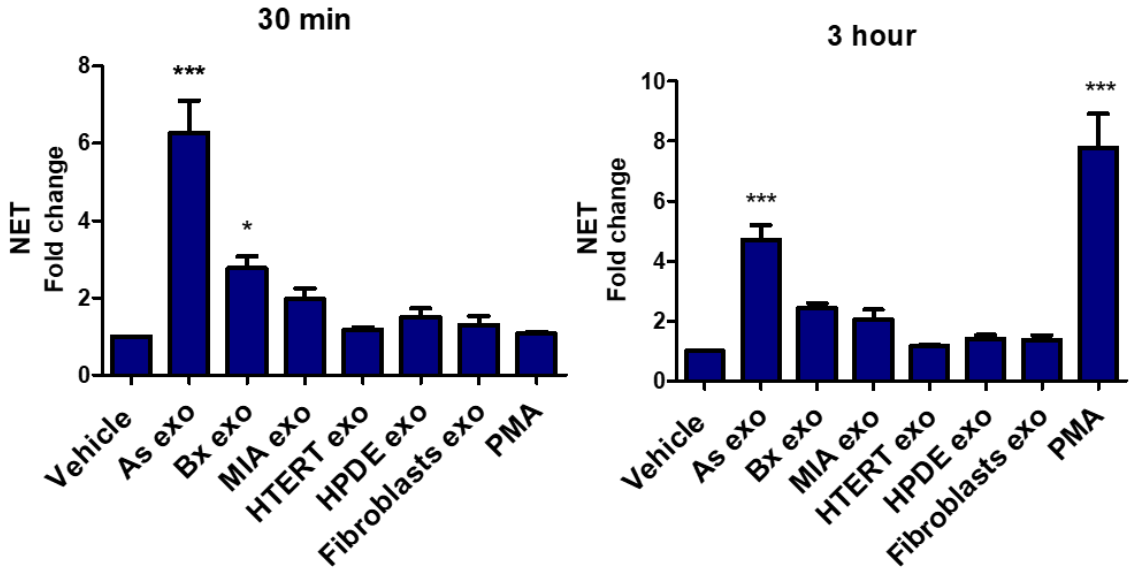


Figure 4.9 Pancreatic cancer-derived exosomes stimulate neutrophils to release NETs. Exosomes (10 µg/ml) were incubated with neutrophils. The release of NETs was quantified using SYTOX Green which binds to extracellular DNA. Fluorescence was measured on a plate reader at 30 min and 3 hr. Data expressed as fold change in NET fluorescence relative to vehicle control. Exosomes from non-cancerous, normal cell lines HTERT, HPDE and fibroblasts were also tested and did not induce NETs. PMA served as positive control for NET at 3 hr. Data analysed with one-way ANOVA followed by Bonferroni post-test. Data presented as mean ± SEM. * P < 0.05, *** P < 0.001, n=4.

To further investigate whether AsPC-1 cells-primed platelet inducing NET was mediated by exosomes, exosomes were pre-incubated with platelets for 4 hours, then washed to remove excess exosomes before incubation with neutrophils. Platelets that were pre-incubated with exosomes, referred to as exosome-primed or PDEx-primed platelets, induced rapid NET release (Figure 4.10). AsPC1-primed platelets induced a trend of increased NET (4.23 ± 1.1-fold change) compared to

control at 30 min. BxPC3- and MIA PaCa-2-primed platelets induced significant NETs with 5.60 ± 1.8 and 5.30 ± 1.8-fold change (* P < 0.05, n=4), respectively. In this experiment, collagen-activated platelets were also included to determine the effect of activated platelets on NET release. There was no induction of NET at either 30 min or 3 hr (Figure 4.10).

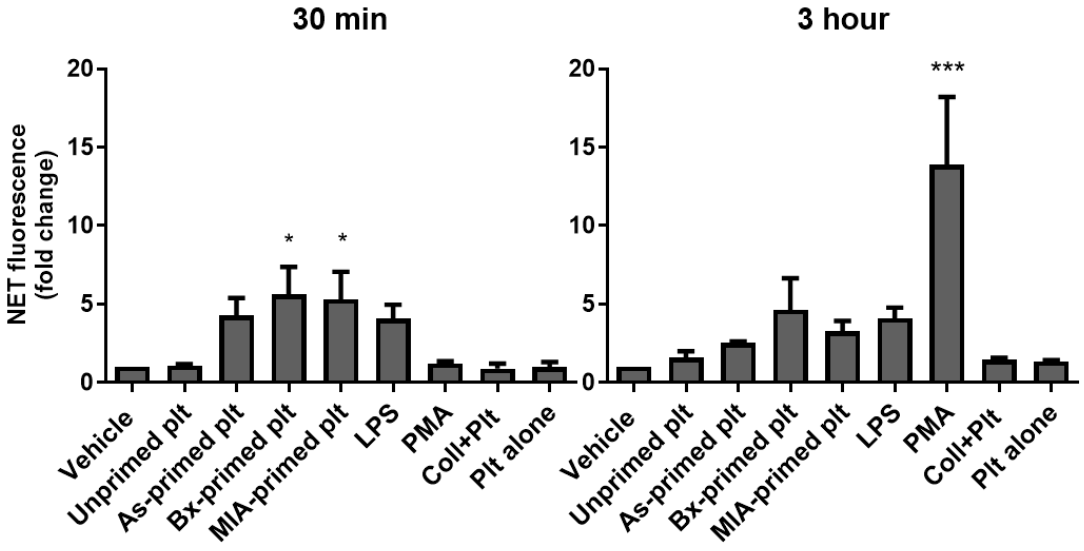


Figure 4.10 Exosomes have a priming effect on platelets which are capable of eliciting NET release. Exosomes (10 µg/ml) were incubated with washed platelets 1 x 10^8/ml for 4 hr to prime platelets. Platelets (plt) were then spun down to remove any exosomes that were not associated to platelets and resuspended in PBS before incubation with neutrophils. NET quantification was assessed by Sytox Green and fluorescence detected using a plate reader. Data expressed as fold change in NET fluorescence relative to vehicle control. Data were analysed with one-way ANOVA followed by Bonferroni post-test. Data presented as mean ± SEM. * P < 0.05, *** P < 0.001; n=4.

4.4 Discussion

This study was able to confirm the procoagulant effect of EVs that has been observed in earlier studies, as well as identifying that PDEx, in our hands do not directly induce potent platelet activation (as assessed by aggregation) in the absence of plasma, rather a dampening effect on platelet may be induced by PDEx. The novel finding in this study is that PDEx were able to prime platelets to stimulate NET release, a phenomenon, which to the author's knowledge, has not yet been described elsewhere. However, as mentioned earlier in this chapter, other reports of platelet priming have been described with LPS via TLR4 on platelets, resulting in NET release. Thus, the results from this study suggest that the same concept of platelet priming can be demonstrated with other cancer-derived agonists.

All of the studies which have indicated a role for TF tumour-derived EVs with a procoagulant effect were described as microparticles (also referred to as microvesicles), the subcategory of EVs with a greater size range and are released by shedding of the plasma membrane. This study demonstrated that exosomes, the subcategory with smaller sized vesicles and exosomal origin, derived from pancreatic cancer cells were similarly capable of inducing coagulation and fibrin generation in plasma. It is interesting to note that there were differences observed in the coagulation kinetic profile, particularly with MIA PaCa-2. This is seen in the increased lag time compared to the other two PDEx (Figure 4.3c) as well as coagulation to a lesser extent as indicated by less absorbance seen once clotting in the control sample (with calcium chloride) was initiated (See Figure 4.3b). This may be attributed to the absence of TF on MIA PaCa-2. As the other two exosomes carry TF, it provides additional exogenous activation of the coagulation cascade, therefore enhancing its response and inducing initiation to occur with significantly less lag time. Apart from these differences, there was a trend to indicate coagulation activation by Mia PaCa-2 exosomes, which was significantly activated by AsPC-1 and BxPC-3 exosomes. Given the well-established effect of tumour-derived EVs in inducing coagulation, the targeting or inhibition of these vesicles may be a potential therapeutic option for preventing the hypercoagulable state in cancer patients which may potentially lead to reduced thrombotic events in cancer patients.

In addition to a pro-coagulant response, another potential pathway for thrombosis in cancer by PDEx is through the activation of platelets. While several studies have shown that the activation of platelets by tumour-derived EVs is dependent on TF, one study was able to show a TF-independent mechanism [280]. This study was unable to confirm their findings, as the aggregation of platelets by PDEx in our hands, was only observed in the presence of plasma. It appears that since this study was reported in 2017, no other lab groups have reported and replicated this response. The author suggests that the direct and TF-independent effect activation of platelets by breast cancer-derived EVs seen by Gomes and colleagues may potentially be due to contamination of traces of thrombin in the platelet preparation. Thrombin being a strong inducer of platelet activation, traces of thrombin from the plasma that remains after platelet isolation may be causing aggregation by breast cancer exosomes reported in their study. On the contrary, this effect reported by Gomes et al. may be specific to breast cancer-derived exosomes, and not applicable for pancreatic cancer-derived exosomes.

As there was no overt direct activation of platelets by PDEx, other more subtle mode of platelet activation was assessed. The effect on platelet dense granule secretion was assessed using ATP secretion assay. Likewise, there was no significant induction of ATP release by PDEx on washed platelets. However, there was evidence to suggest that there may be a dampening effect on the

activation of platelets, implicating a suppressive effect on platelet activation. This was observed when platelets were incubated with collagen, together with the presence of PDEx. Though not significant, there appears to be a slightly reduced ATP release from platelet when incubated together with PDEx, compared to platelets with collagen (Figure 4.6b). This prompted the question of whether PDEx has an active role in cleaving any secreted ATP from activated platelets, which may explain the reduced levels of ATP observed. Intriguingly, upon testing the PDEx for CD73 activity by Malachite Green assay, there was evidence of CD73 activity, which was inhibited accordingly by the CD73 inhibitor, AB680. Furthermore, there were confirmed amounts of CD73 quantified within exosomes, as determined by ELISA. The quantified values ranged between 20-130 ng/ml. There was a distinctively large amount of CD73 quantified in MIA PaCa-2, however it was strange that despite this, there was little CD73 activity observed, and the presence of the AB680 inhibitor increased, rather than decreased, CD73 activity on MIA PaCa-2 exosomes.

The possibility of functional machinery on the surface of PDEx is an interesting finding. Exosomes are well known to mediate intercellular communications through transfer of their contents such as microRNA or proteins. CD37 on PDEx entails another mechanism whereby exosomes can induce effects on immune cells through ATP cleaving and generation of adenosine. The effects of adenosine of the tumour microenvironment are well established (reviewed in [290]). Adenosine functions as an immunosuppressive metabolite, thus suppressing immune cells and facilitating tumour immune evasion and the growth and progression of cancer. Thus, there is potential for CD73 machinery on the surface of PDEx impacting levels of adenosine in the pancreatic tumour microenvironment.

The novel finding from this study is the evidence that PDEx primes platelets, and these primed platelets are able to induce rapid NET release. This is a key finding that has implications for platelets in cancer, where they potentially may be under the effect of exosomes without signs of activation but can elicit certain activation responses with other cells. Platelets in the circulation would be exposed to great amounts of exosomes released from cancer cells. Exosomes may be regulating the activation of platelets as they do not induce direct strong activation but instead induce a priming effect where platelets can travel through the circulation in an inactivated state to the distant site of metastasis or thrombosis. At the site, platelets would then exert their role in the activation of other cells. The potential immunosuppressive effect of platelets by exosomes described earlier may further facilitate the travel of exosome-primed platelets through the circulation. These are some possible scenarios which may occur to explain the significance and role of exosome-primed platelets. In addition, this primed platelet effect by PDEx may serve as an explanation for the unique study showing evidence of tumour-educated platelets in cancer [291]. The alteration of the RNA profile

seen in the tumour-educated platelets may be due to the transfer of exosome contents to platelets in cancer.

As one can expect, it seems to be a highly complex interaction between tumour cells, platelets and immune cells which would not have been able to be delineated by this study. Observational studies within mouse models of cancer looking at exosome-primed platelets and their effect on immune cell interactions, tumour progression and thrombosis would be valuable to help elucidate the role and relevance of pancreatic cancer exosome-primed platelets *in vivo.*

Future studies

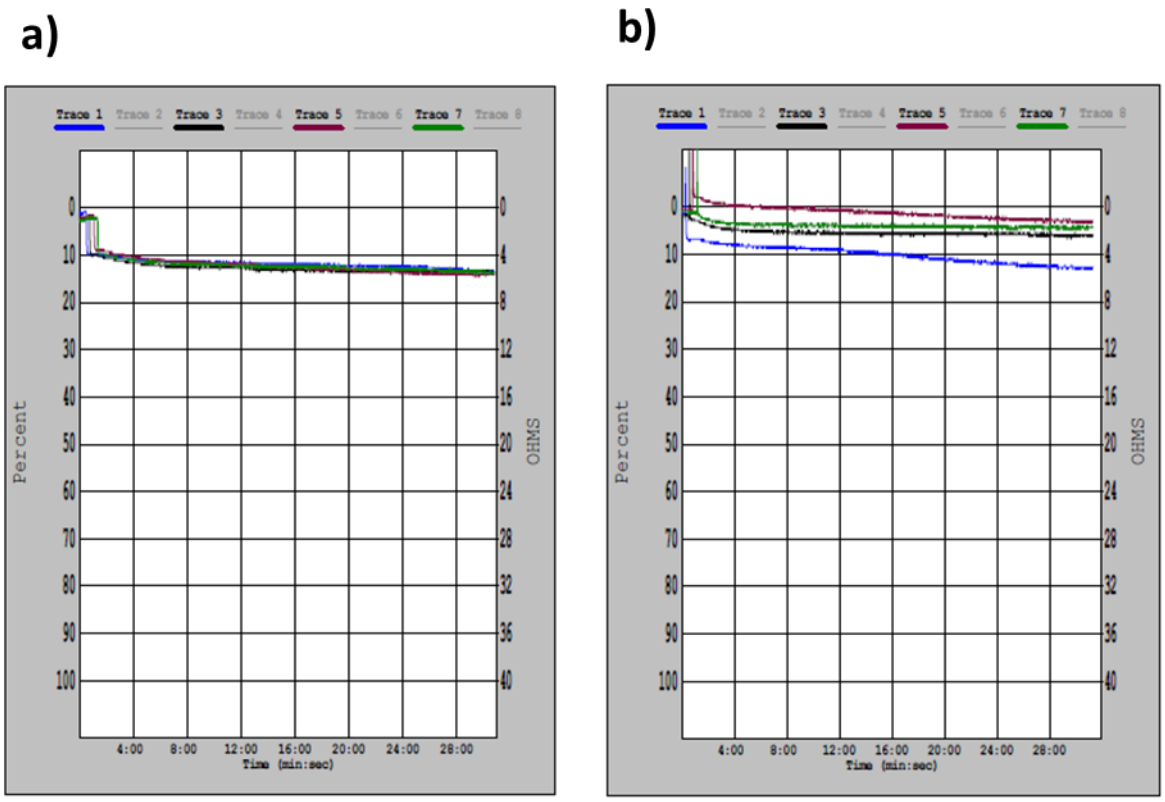
Additional experiments would benefit to confirm some trends observed in this study. For example, the ability of PDEx-derived exosomes to cleave ATP when in the presence of collagen was not a strong trend, and a dose response with PDEx would help to see that more exosomes could result in greater reduction of ATP release, suggesting greater CD73 activity induced by a higher concentration of exosomes.

Another question that arises is how platelets are being primed by exosomes. It was suggested in this study that exosomes are taken up by platelets, as shown by the presence of histone H3 in platelet lysates after the incubation of exosomes. This protein is not normally present in the platelet as it lacks a nucleus. To begin to delineate the mechanism of uptake and potential exosome-derived mediators involved in the priming effect, a few more experiments can be conducted. The first would be to confirm the method of uptake which can be determined by repeating the same uptake blot with the presence of an uptake inhibitor. Another study has shown that pancreatic cancer-derived exosomes were taken up by endothelial cells by the process of clathrin-mediated endocytosis, a process which was inhibited by dynasore, a dynamin inhibitor [292]. Dynamin is a GTPase protein that is essential for membrane fusion upon clathrin-mediated endocytosis [293] Therefore, the same studies can be repeated with the presence of dynasore, and the absence of histone H3 in platelets after incubation with exosomes would suggest exosomes being taken up into platelets via clathrin-mediated and dynamin-dependent endocytosis.

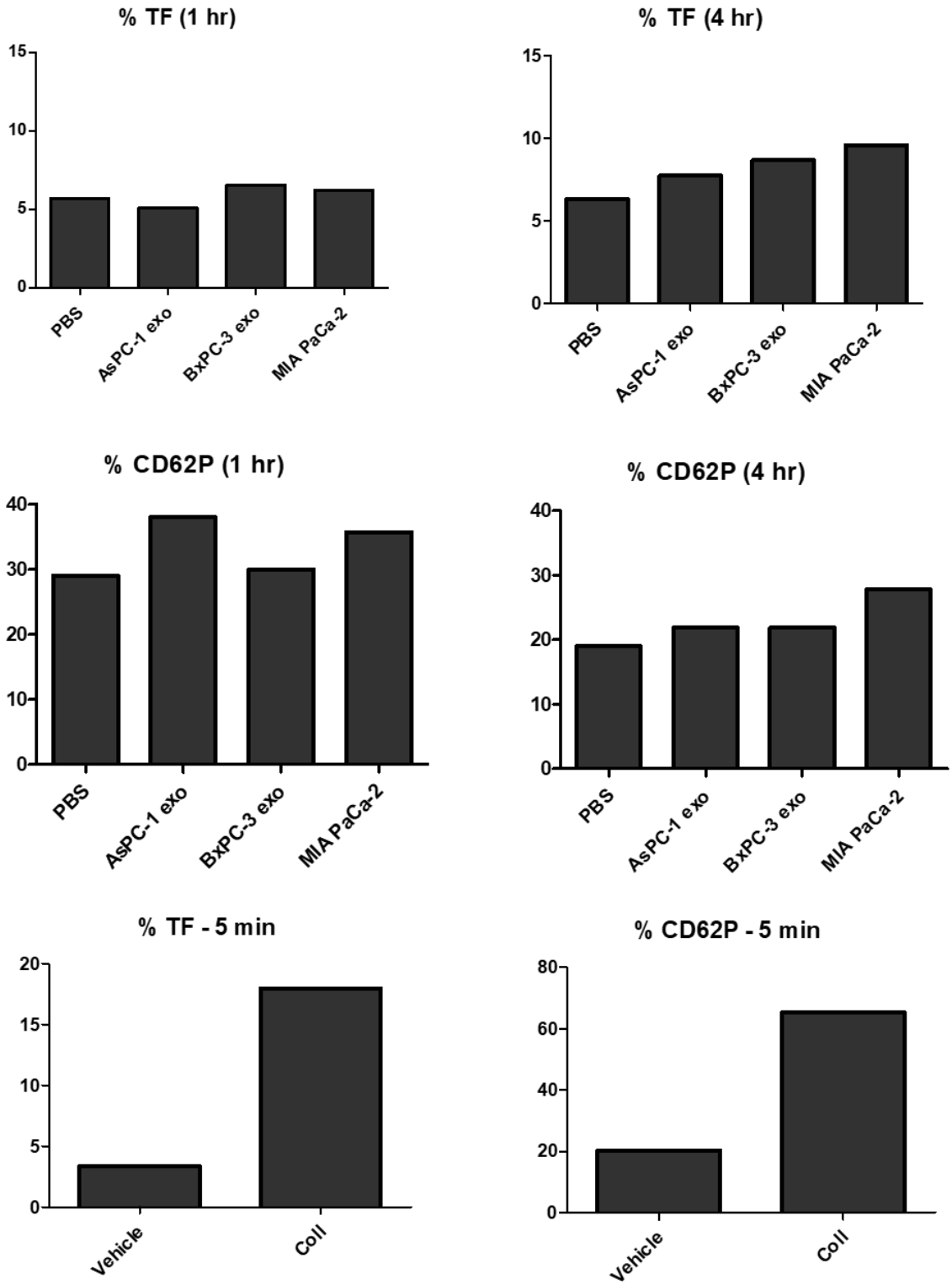
The second would be to perform the same blots with molecules that may likely mediate the priming effect. As mentioned in the previous chapter, DAMP molecules are proteins of interest due to their wide range of effect on immune cells. Thus, the uptake of proteins such as HMGB-1 and S100A4 can be repeated to confirm that other proteins within exosomes also become associated with platelets. Third, would be determining if exosome uptake is an essential step in exosome-primed platelets induced NET formation, which can be investigated by using the dynasore inhibitor.

Appendices for Chapter 4

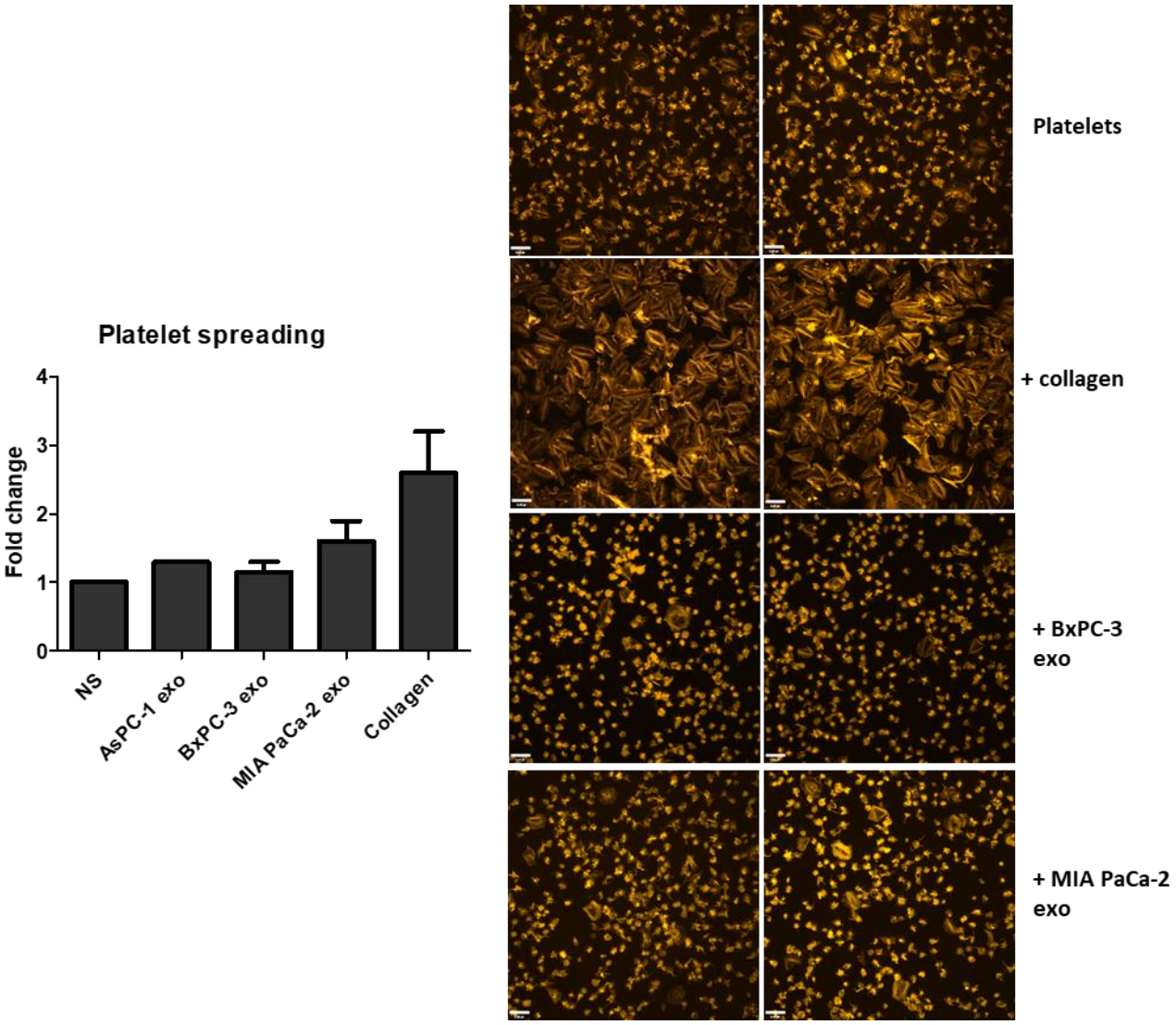
Supplementary Figures for Chapter 4



Supplemental Figure 4.1. Effect of exosomes on washed platelet aggregation in the absence of plasma. A) Effect of 10 µg/ml exosomes on washed platelet aggregation trace without plasma in the reaction (n=4)**. B)** Effect of 50 µg/ml exosome on washed platelet aggregation trace without plasma in the reaction (n=3). Exosomes at both concentrations did not induce platelet aggregation in the absence of plasma.



Supplemental Figure 4.2. Effect of pancreatic cancer-derived exosomes on the surface expression of CD62 (P-selectin), a marker of platelet activation, and tissue factor (TF) expression. Flow cytometry analysis was used to assess the upregulation of markers. Exosomes did not increase activation marker P-selectin or upregulation of surface TF. PBS served as vehicle control for exosomes. Collagen (coll) test was included as positive control which increased TF and CD62P by 5 min. Results representative of one independent experiment.



Supplemental Figure 4.3. Effect of exosomes on platelet spreading a) Surface area quantification of two independent experiments. Collagen served as positive control. Platelets were incubated with exosomes (1 ug) or collages on poly-l-lysine coated glass slides. After incubation platelets were stained with Phalloidin to visualise F-actin filaments to observe changes to platelet structure after stimulation. Collagen induced platelet spreading while minimal changes were observed with exosomes. **b)** Confocal images of platelets after stimulation with collagen and exosomes with F-actin staining. Results from two independent experiments.

Chapter 5

The effect of pancreatic cancer-derived exosomes on endothelial cells

5.1 Introduction

Apart from the roles and activation of neutrophils and platelets in mediating thrombosis in cancer (as described in the previous chapters), another important player is the activation of endothelial cells. For many years since its discovery, the endothelium was viewed as an inert barrier that separated blood from the vessel wall. Over time, evidence emerged to recognise that the endothelial cell lining is an active layer involved in regulating many homeostatic processes such as haemostasis, vasomotor tone, nutrient trafficking, and blood fluidity through the expression and synthesis of anticoagulants [294, 295]. Endothelial cells possess both anticoagulant and procoagulant mechanisms, which can be evoked depending on the specific tissue needs and local stress factors. Under physiological conditions, endothelial cells express anticoagulant and antiplatelet factors to prevent unnecessary fibrin formation and platelet adhesion [296]. On the other hand, in response to endothelial perturbations at sites of inflammation, endothelial cells become activated and transform into a pro-thrombotic phenotype [297].

A normal resting endothelium possesses several anti-coagulant and anti-platelet properties, which together function to achieve a non-thrombogenic surface [298, 299]. These include the expression of heparin-like proteoglycans and thrombomodulin to sequester thrombin, or the secretion of prostacyclin to inhibit platelet activity [298, 299]. In addition, a healthy or 'quiescent' endothelium has the ability to avert the progression of cancer, by inhibiting cancer cell proliferation and invasiveness, leading to the inhibition of tumour growth and metastasis *in vivo* [300, 301]. In cardiovascular disease, the endothelium is considered the main target organ of which damage to results in hypertension and thrombosis [302]. Similarly, the well-known increased risk of thrombosis in cancer may well be a result of the adverse changes to the endothelium that is induced by the cancer.

5.1.1 Mechanisms and markers of endothelial dysfunction

Endothelial dysfunction is broadly defined as endothelial cells exhibiting functional changes that lead to a shift from maintaining homeostasis, towards a proinflammatory response with proliferative and prothrombotic properties and reduced vasodilation [300]. The term 'endothelial dysfunction' is more commonly applied in the context of cardiovascular disease, where endothelial dysfunction is said to precede the development of atherosclerosis. Historically, endothelial dysfunction was restricted to reduced bioavailability of nitric oxide and impaired vasodilation of the large arteries, but it has become apparent that this is an oversimplification of the vast roles and biology of the endothelium [303]. Now, with over 40 years of research, the context in which endothelial dysfunction is studied has changed. It is now recognised that endothelial dysfunction is involved in the pathophysiology of

other diseases other than cardiovascular, such as inflammatory, metabolic and infectious diseases [304, 305]. Endothelial dysfunction encompasses changes to several functions of the endothelium and includes endothelial activation [306]. The activation of endothelial cells in cancer and its relevance to promoting thrombotic interactions will be discussed.

Activation of the endothelium with inflammatory stimuli such as inflammatory cytokines, damage-associated molecular patterns and reactive oxygen species, results in pro-adhesive properties, and the impairment of anti-coagulant and anti-inflammatory properties [307]. The expression of anti-coagulant thrombomodulin is downregulated, and the procoagulant tissue factor is upregulated [308]. Expression of cell adhesion molecules such as P-selectin, vascular cell adhesion molecule-1 (VCAM-1), or intercellular adhesion molecule-1 (ICAM-1) promotes the adhesion of leukocytes and platelets [309, 310]. P-selectin is expressed on activated endothelial cells which can enhance thrombosis by facilitating rolling and adhesion and increasing the recruitment of neutrophils via P-selectin glycoprotein ligand-1 (PSGL-1) [155]. VCAM-1 and ICAM-1 allow for stronger adhesion of neutrophils onto endothelial cells [307]. Endothelial cell activation also drives the expulsion of von Willebrand factor (vWF) from Weibel-Palade bodies (WPBs) which promote platelet adhesion [311].

Increased endothelial permeability is another indicator of endothelial activation. Vascular permeability and the regulation of the contents that pass through the endothelium are important for maintaining homeostasis. Increased permeability in the endothelium can be due to the exposure of inflammatory cytokines [312]. The key features of increased permeability are the disruption of intercellular junctions and gap openings between endothelial cells [312]. An increase in endothelial permeability can be reversed or diminished once the stimulus is removed, however can be sustained in chronic inflammation and cancer.

5.1.2 Endothelial activation in cancer

The secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-8, RANTES, G-CSF and GM-CSF has been shown to be induced by a cocktail of tumour-derived factors containing tumour necrosis factor α (TNF-α) and angiogenic factors [313]. In addition, gene expression analysis of activated endothelial cells by tumour-derived factors indicated a pro-thrombotic phenotype such as increased tissue factor, and decreased expression of thrombomodulin [313]. Gene expression for cell adhesion molecules were also upregulated, including VCAM-1 and ICAM-1, while anti-inflammatory cytokines such as eNOS and VE-cadherin were decreased [313]. The activation of endothelial cells also resulted in increased permeability of dysfunctional EC after stimulation with a cocktail of factors mimicking the tumour microenvironment [313].

Other secreted factors from cancer cells have been shown to directly activate endothelial cells, leading to an increased upregulation in cell adhesion molecules, vWF release or increased endothelial permeability. Endothelial cells of the tumour vasculature have been described to be leaky, due to the loss of polarity between endothelial cells and release of permeability factors such as vascular endothelial growth factor (VEGF) from cancer cells [314-317]. The release of metalloproteinase by tumour cells promoted endothelial cell activation via protease-activated receptor-1 [318]. Upregulation of ICAM-1 was observed by interaction of tumour-derived CD44 with endothelial E-selectin [319]. Several papers have shown the activation of endothelial cells by tumour-derived VEGF. Melanoma cancer-derived VEGF induced WPB exocytosis from endothelial cells [320]. VEGF-A from cancer cell supernatant stimulated VWF fibre generation in endothelial cells [321]. Bauer and colleagues showed tumour-derived VEGF-A mediated stimulation of endothelial cell activation through VWF release, which facilitated platelet activation and tumour progression [322]. Similarly, a recent study showed tumour-derived VEGF mediated vWF release and platelet aggregation [323].

5.1.3 Effect of cancer-derived exosomes on endothelial cells

So far there have been minimal studies on the role of cancer-derived exosomes on endothelial activation, and most studies have focused on the effect on angiogenesis. With the lack of studies with cancer-derived exosomes, studies with endothelial activation by cancer-derived EVs will be included. One study reported that cancer-derived EVs expressing tissue factor activated IL-8 secretion and E-selectin expression in endothelial cells via protease-activated receptor1 [324]. Colorectal cancer-derived exosomes induced increased permeability in endothelial cell [325]. An earlier study found that cancer-derived EVs promoted endothelial cell migration which was thought to be involved in the angiogenic activity of cancer-derived EV [326]. Since then, several studies have reported that cancer-derived EVs and exosomes promote angiogenesis in endothelial cells [327-330]. A recent study showed the release of vWF by small extracellular vesicles in cancer induced activation of endothelial cells by promoting angiogenesis which facilitated metastasis [331].

The activation and dysfunction of endothelial cells may not only activate a pro-inflammatory and pro-thrombotic phenotype but has also been shown to support metastasis and inflammation in cancer [313]. Thus, the effect of tumour-derived exosomes on endothelial cells may be underestimated with a dual role effect in cancer progression and thrombosis. So far, the studies on the effect on cancer-derived exosomes on endothelial cells have focused on the effect of exosomes inducing angiogenesis, thereby promoting tumour growth and metastasis.

This study aimed to assess other activation responses in endothelial cells with the stimulation of pancreatic cancer-derived exosomes (PDEx), including permeability, expression of cell-adhesion molecules and signalling pathways activated. Furthermore, it is hypothesised that PDEx induces the activation of endothelial cells which can promote prothrombotic interactions such as the adhesion and activation of neutrophils, which will also be investigated.

5.2 Materials and methods

5.2.1 Cell culture

Human microvascular endothelial cell-1 (HMEC-1) cells were obtained from American Type Tissue Collection. HMEC-1 cells were grown in MCDB-1 medium supplemented with 10 mM glutamine (Gibco Life Technologies Australia Pty Ltd., Mulgrave, Australia), 10 ng/ml epidermal growth factor (ThermoFisher Scientific, USA), 1 µg/ml hydrocortisone (Stemcell Technologies, Vancouver, BC, Canada) and 10% foetal bovine serum (Bovogen, Victoria, Australia). Cells were maintained in an incubator at 37°C and 5% CO_2 and were grown to 80-90% confluency before subculturing at a ratio of 1:8 to 1:12. Cells were not fed with growth medium between subcultures as it would result in difficult to detach cells at the time of subculture.

5.2.2 Isolation of pancreatic cancer-derived exosomes

Refer to Chapter 2 (Part 2: Isolation and Characterisation of Exosomes)

5.2.3 Western blot

Western blot was performed with cytoplasmic and nuclear extractions prepared from stimulated HMEC-1 cells. Cells were seeded in a 6-well plate until 90% confluent and then serum starved in MCDB-1 medium with 1% foetal bovine serum for 1 hour. Exosomes (10 µg) and LPS (2.5 µg/ml) were added to cells for 30 min. After stimulation, cells were washed with ice-cold phosphate-buffered saline (PBS), scraped and centrifuged at 130 x *g* for 5 min. The pellet was resuspended in 100 µl of hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM DTT, 0.3% Nonidet P-40) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signalling Technology) and incubated on ice for 10 min. The lysed cells were centrifuged at 1,500 x g for 5 min at 4°C and the supernatant was designated at the cytosolic fraction. The resulting nuclear pellet was resuspended in nuclear extraction buffer containing 25% glycerol, 20 mM HEPES, 0.6M KCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA to obtain nuclear proteins. Samples were incubated on ice for 15 min then centrifuged at 14,000 x *g* for 10 min. The supernatant is the nuclear extraction.

Cytosolic and nuclear extractions were aliquoted and stored at -80°C until ready for western blotting. Protein quantifications on the extractions were measured using the Pierce™ 660nm Protein Assay Kit (ThermoFisher Scientific, Waltham, USA).

Rabbit antibodies specific for p-p38 MAPK (Thr180/Tyr182), p-Erk1/2 (Thr202/Tyr204) and p-Akt (Ser473), NF-KB p65 and IKBα were used for analysis. All antibodies were obtained from Cell Signalling Technologies. For western blot analysis, 10 μg of protein was loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel was transferred to a polyvinylidene difluoride (PVDF) blotting membrane with 0.2 μm pore (GE Health Care Life Sciences) at 40 V for 1 hr. The blots were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween (TBS-T) at room temperature for 1 hr. The blots were rinsed three times with TBS-T before incubating with the primary antibodies at 1:1000 dilution in 3% bovine serum albumin. After overnight incubation at 4°C, blots were rinsed with TBS-T and primary antibodies were detected using secondary anti-rabbit horseradish peroxidase-conjugated antibodies at 1:40,000 dilution. Protein signals were developed using the Amersham ECL Prime Western blotting detection reagent (GE Healthcare Lifesciences, USA) and imaged using ChemiDoc imaging system (Bio-Rad, USA).

5.2.4 Flow cytometry analysis

HMEC-1 cells were seeded in a 6-well plate and grown to form monolayer. Once confluent, cells were stimulated in complete media with 10 μg/ml exosomes or LPS 2.5 μg/ml (eBioscience LPS solution 500X; ThermoFisher #00-4976). After 16 hr treatment, cells were washed with cold PBS and lifted with Accutase. Cells were resuspended in 1% BSA and 2mM EDTA at a concentration of 4-5 x 10^6/ml. Cells were stained with Alexa Flour 674-conjugated mouse anti-human ICAM (CD54) antibody or anti-mouse IgG1 isotype, for 15 min in the dark at room temperature. Stained HMEC-1 cells were washed and then fixed with 1% paraformaldehyde before analysis on the flow cytometer (BD LSRFortessa™ cell analyser).

5.2.5 Neutrophil adhesion assay

HMEC-1 cells were seeded in a 6-well plate and left for 2 to 3 days to form a monolayer. Once a monolayer was formed, cells were washed with PBS and stimulated in fresh media. For exosomes, 10 μg/ml was used to treat the endothelial monolayer. Cells were stimulated for 16 hr with exosomes or LPS 2.5 μg/ml as positive control.

Fluorescent labelling of neutrophils

The following day after HMEC-1 monolayer treatment, neutrophils were isolated from a healthy donor (as previously described in Chapter 3). Neutrophils were labelled with 1 µM CellTracker Orange CMRA dye (ThermoFisher Scientific, Waltham, USA). The final washed neutrophil pellet was resuspended in X-VIVO 15 media (Lonzo, Switzerland) containing Orange CRMA dye (1 µM). Neutrophils were left to incubate in the dark for 15 min at room temperature, then centrifuged at 400 x *g* for 10 min. The pellet was resuspended in X-VIVO 15 media at a concentration of 2 x 10^6/ml.

Neutrophil binding assay

After 16 hr stimulation with exosomes, the HMEC-1 monolayer was washed with filtered X-VIVO 15 media with 3% BSA. The labelled neutrophils (2 x 10^6/ml) were added to each well (2 mL per well) and incubated in the dark for 20 min at 37°C and 5% CO_2. After incubation, the supernatant containing unattached neutrophils was removed and HMEC-1 cells were washed four times with PBS. Two ml of X-VIVIO media was added back to the wells and attached neutrophils were imaged using Olympus Inverted IX-51 microscope on the TRITC channel. A minimum of 5 fields of view were captured. The average count from 5 to 10 random fields of view was calculated to determine the number of neutrophils bound to exosome-treated HMEC-1 cells.

5.2.6 Permeability assay of HMEC-1 cells

The *In Vitro* Vascular Permeability Assay (Green) kit (Merck Millipore catalogue #17-10398) was used to image the disruption of endothelial cell barrier integrity after exosome treatment. The protocol followed was according to manufacturer's protocol with use of 12mm round glass coverslips in a 24-well plate. Coverslips were coated with poly-L-Lysine and incubated for 20 min at room temperature, then rinsed twice in PBS. Coverslips were treated with glutaraldehyde for 15 min at room temperature. Biotinylated-gelatin was coated on the coverslips for 10 min at room temperature. Substrates were disinfected with 70% ethanol and incubated at room temperature for 30 min. Ethanol was removed and coverslips were rinsed twice with PBS. Residual free aldehyde was quenched with MCDB131 growth medium and incubated at room temperature for 30 min, protected from light.

HMEC-1 cells that were 70-80% confluent were used and seeded at 76,000 cells per well. Cells were left undisturbed on a horizontal surface for 15 min to encourage even cell distribution. HMEC-1 cells were allowed to grow in the incubator maintained at 37°C and 5% CO_2 for 2-3 days until a monolayer was formed. Once monolayer was achieved, cells were treated with 5 µg exosomes, 5 µg/mL LPS or

80 nM PMA. After 16 hr treatment, cell culture media was removed and cells were stained with fluorescein-streptavidin for 10 min at room temperature, before fixation with 4% formaldehyde in PBS for 30 min. Cells were washed with PBS and then mounted with ProLong Gold mounting media with DAPI (Molecular Probes, Life Technologies) and imaged using PerkinElmer Ultraview Spinning Disk confocal microscope. Images were taken from random fields of view. The percentage area of HMEC-1 permeability as determined by fluorescein-streptavidin staining was analysed using Image J software. The average of percentage areas of five to six images were taken for analysis to compare with non-stimulated HMEC-1.

5.3 Results

5.3.1 Effect of endothelial cell permeability with treatment of PDEx

The effect of PDEx on endothelial HMEC-1 was first examined using an *In Vitro* Vascular permeability imaging assay kit. HMEC-1 cells were treated with PDEx for 16 hours. Little to no changes in the fluorescence of fluorescein-streptavidin, indicating sites of permeability, were observed in HMEC-1 cells stimulated with PDEx for 16 hours compared to non-stimulated HMEC-1 (Figure 5.1). LPS, which was used as positive control, also did not induce a significant increase in permeability. PMA on the other hand, induced a 4-fold change in permeability compared to non-stimulated HMEC-1 (Figure 5.1a).

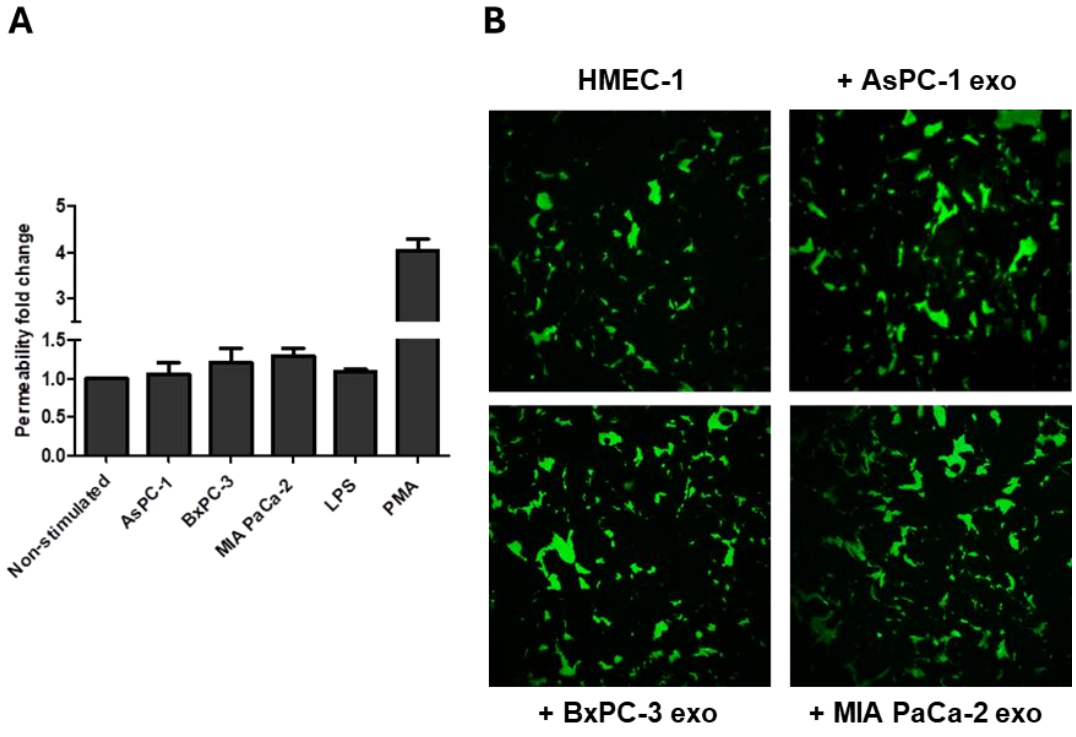


Figure 5.1 *(See legend on next page)*

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Figure 5. 1 The effect of PDEx on HMEC-1 cell permeability. HMEC-1 cells were grown on biotinylated-coated coverslips and left in culture to form a confluent layer. Cells were treated with 5 µg exosomes (1 µg/100 ml) and left to incubate for 16 hours. After treatment, cells were washed and stained with fluorescein-streptavidin for 10 min, then fixed with 4% paraformaldehyde. Cells were mounted and imaged on the Ultraview Spinning Disk confocal microscope. A) Fold change in surface area quantification of fluorescein-streptavidin staining sites of intercellular permeability. PDEx-stimulated HMEC-1 were compared to non-stimulated HMEC-1. Data expressed as mean ± SEM; n = 2. B) Confocal images of cells treated with PDEx showing staining in sites of intercellular permeability. Images are representative of two independent experiments.

5.3.2 Upregulation of ICAM-1 on HMEC-1 cells treated with pancreatic cancer-derived exosomes

The upregulation of ICAM-1 is a marker of endothelial activation and mediates interactions with neutrophils. Endothelial cells were incubated with PDEx for 16 hours and assessed for upregulation of ICAM-1 by flow cytometry. There was an upregulation of ICAM-1 observed with PDEx which was greatest in BxPC-3-derived exosomes (MFI fold change: 25.6 ± 14; non-significant (ns), n = 3) compared to non-stimulated control (Figure 5.2a). MIA PaCa-2 exosomes induced a moderate level of upregulation (11.9 ± 6.7-fold change; ns) while AsPC-1 exosomes induced the least upregulation (5.40 ± 3.2-fold change; ns). LPS (2.5 µg/ml) was used as a positive control and induced a great increase in ICAM-1 upregulation (34.0 ± 11-fold change; ns).

In addition to the upregulation of ICAM-1, an attempt was made to examine upregulation of tissue factor to assess a pro-thrombotic phenotype in endothelial cells. The results are included in the representative dot plot (Figure 5.2b). However, it was later discovered that it may not be likely to see an upregulation in tissue factor. As studies that show tissue factor upregulation on the surface of endothelial cells with LPS stimulation have indicated that it is an event that occurs 4 hours after stimulation, with levels returning to baseline after 6 hours [308]. As the stimulation with exosomes in this experiment surpassed 6 hours, it was decided that the analysis of tissue factor was not included in the subsequent flow cytometry experiments.

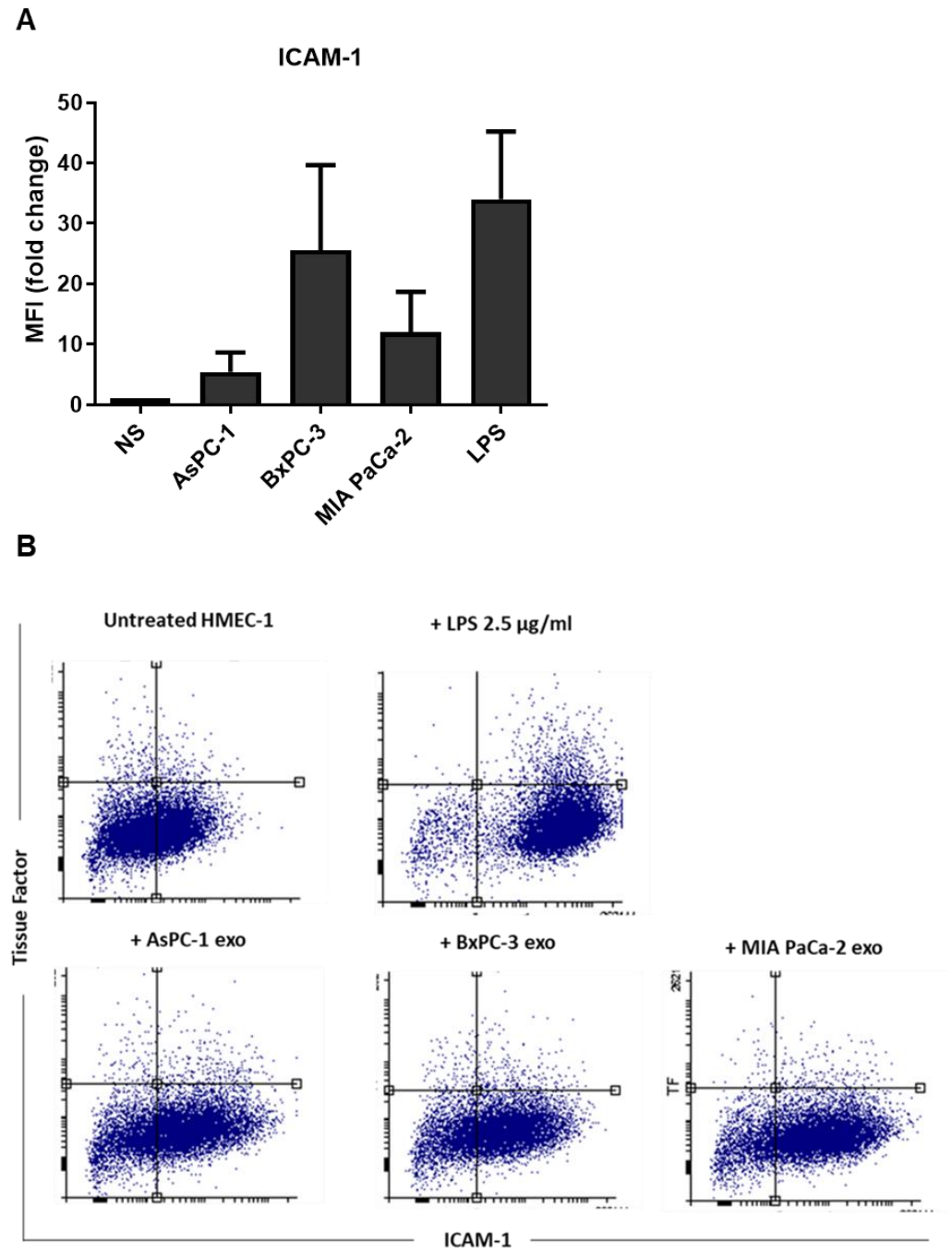

Figure 5. 2 Effect of PDEx on ICAM-1 upregulation of HMEC-1 endothelial cells. HMEC-1 cells were seeded in a 6-well plate and incubated with PDEx for 16 hr and assessed for ICAM-1 using flow cytometry. After 16 hr stimulation, the cells were lifted with accutase, washed and incubated with Alexa Flour 674-conjugated mouse anti-human ICAM (CD54) antibody. Cells were fixed with 1% paraformaldehyde and analysed on BD LSR Fortessa. A) Fold change of ICAM-1 median fluorescence intensity (MFI) in PDEx stimulated relative to non-stimulated (ns) control. Significance between means was analysed using one-way ANOVA followed by post-test Bonferroni's multiple comparisons. Means between groups were non-significant. Data expressed as mean ± SEM; n = 3. B) Representative dot plot of ICAM-1 fluorescence from three independent experiments. This experiment also included the analysis of tissue factor upregulation which showed no difference to untreated HMEC-1.

5.3.3 Increased neutrophil adhesion to HMEC-1 cells treated with PaCa-derived exosomes

Upon seeing a large increase in the upregulation of ICAM-1 in HMEC-1 cells, the impact this would have on neutrophil adhesion was investigated. A neutrophil adhesion assay was performed in a 6-well plate starting with a monolayer of HMEC-1 cells. Once a monolayer was formed, cells were treated with PDEx for 16 hours. The following day, neutrophils were isolated and labelled with CellTracker Orange CMRA dye. After the 16 hour incubation of HMEC-1 with PDEx was complete, the HMEC-1 cells were washed and incubated with the fluorescent-labelled neutrophils for 20 min. HMEC-1 were then thoroughly washed and media replaced with fresh X-VIVO 15 and imaged on the Olympus Inverted IX-51 microscope. The treatment of HMEC-1 with PDEx led to an increase in neutrophil binding (fold change with AsPC-1: 8.03 ± 3.8; BxPC-3: 4.33 ± 1.1; MIA PaCa-2: 3.23 ± 0.4; ns, n = 3) compared to non-stimulated control (Figure 5.3a). Figure 5.3b shows representative images of neutrophil adhesion to PDEx-stimulated HMEC-1.

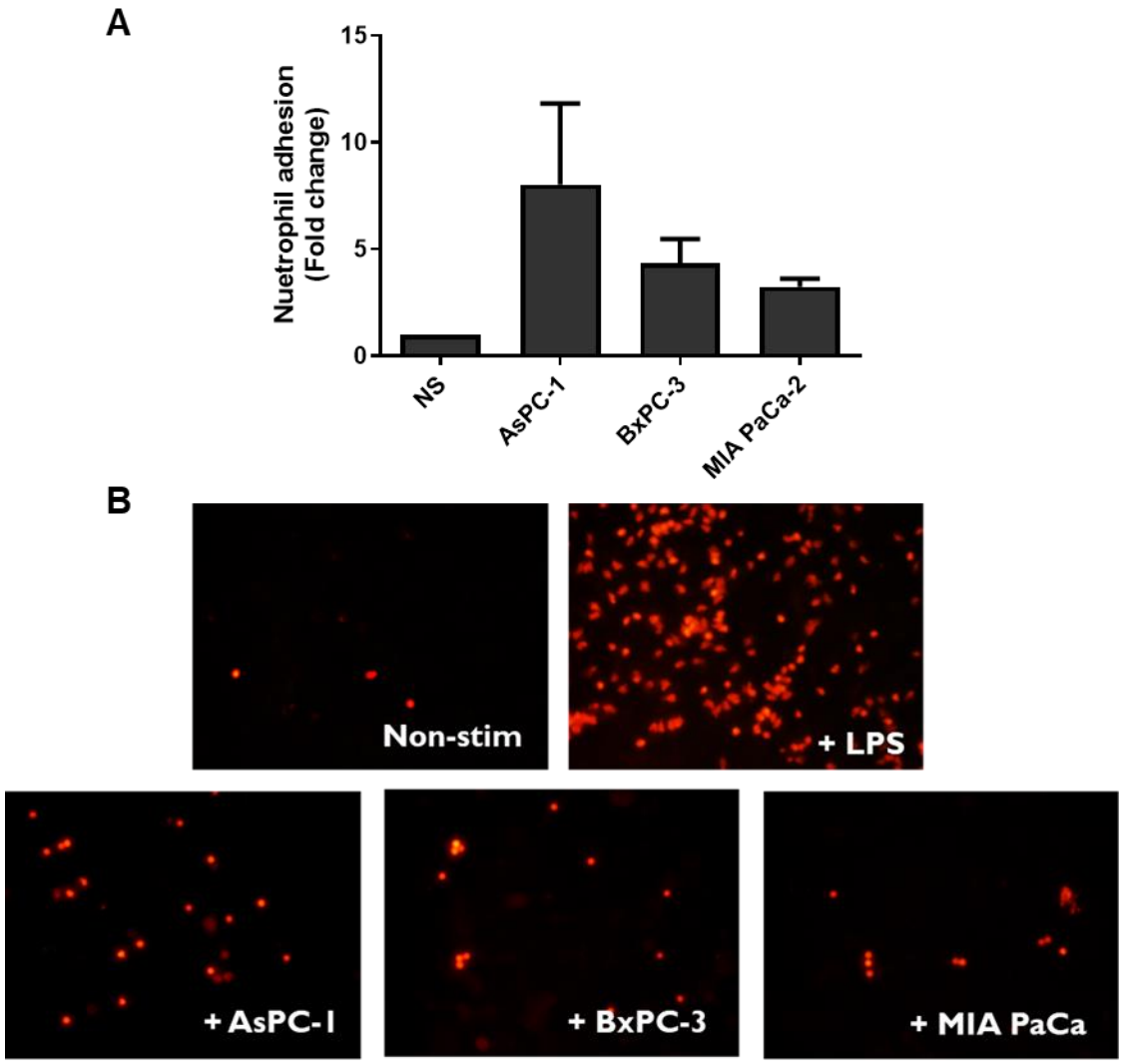

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Figure 5.3 Effect of PDEx on neutrophil adhesion to stimulated HMEC-1. HMEC-1 cells were grown to confluence in a 6-well plate and then treated with PDEx 10 µg/ml for 16 hours. After stimulation, cells were washed and incubated with CRMA-labelled neutrophils for 20 min at 37°C. After neutrophil binding, cells were thoroughly washed and imaged on an inverted microscope. A) Quantification of neutrophils bound to PDEx-stimulated HMEC-1. Data expressed as fold change in neutrophil adhesion relative to non-stimulated control. No significance between means as determined by one-way ANOVA with post-test Bonferroni's multiple comparison test. Data presented as mean ± SEM; n = 3. B) Representative images of neutrophil adhesion to PDEx-stimulated HMEC-1 from three independent experiments.

5.3.4 Effect of exosomes on activation of cell signalling pathways in HMEC-1 cells

Following on from the observation that ICAM-1 had a trend of upregulation in PDEx-stimulated HMEC-1, the signalling pathways involved were investigated by western blotting. The induction of ICAM-1 in endothelial cells involves the nuclear factor kappa B (NF-κB) transcription factor which is translocated from the cytoplasm to the nucleus upon activation by inflammatory stimuli [332]. Before nuclear translocation, NF-KB must be released from the inhibitory proteins IKBs, which bind to latent NF-KB in the cytoplasm. Upon stimulation, a series of signalling events are initiated which leads to the phosphorylation and degradation of IKB, subsequently releasing NF-KB in its active form [332]. In this study, cytoplasmic and nuclear extractions of PDEx-stimulated HMEC-1 cells were prepared to assess the levels of NF-KB p65 and IKBα, in the nucleus and cytoplasm respectively (Figure 5.4 & 5.5). The expression levels of NF-KB p65 and IKBα were measured relative to α-actinin or β-actin. Figure 5.4a shows similar expression levels of cytoplasmic IKBα in PDEx-stimulated compared to non-stimulated. On the other hand, there was significantly less cytoplasmic IKBα in 2.5 µg/ml LPS-stimulated HMEC-1 (0.32 ± 0.01-fold change; ** $P < 0.01$; n = 3) compared to non-stimulated. Figure 5.4b shows a representative blot of cytoplasmic IKBα from 3 independent experiments. In contrast, there was a trend increase in NFKB p65 observed in PDEx and LPS-stimulated HMEC-1 compared to non-stimulated (Fig 5.5a). There was a 2 to 4-fold increase in nuclear NFKB p65 in AsPC-1, BxPC-3 and MIA PaCa-2 exosome stimulated HMEC-1. LPS stimulation induced a 12-fold increase in nuclear NFKB in HMEC-1. A blot from two independent experiments is shown in Figure 5.5b.

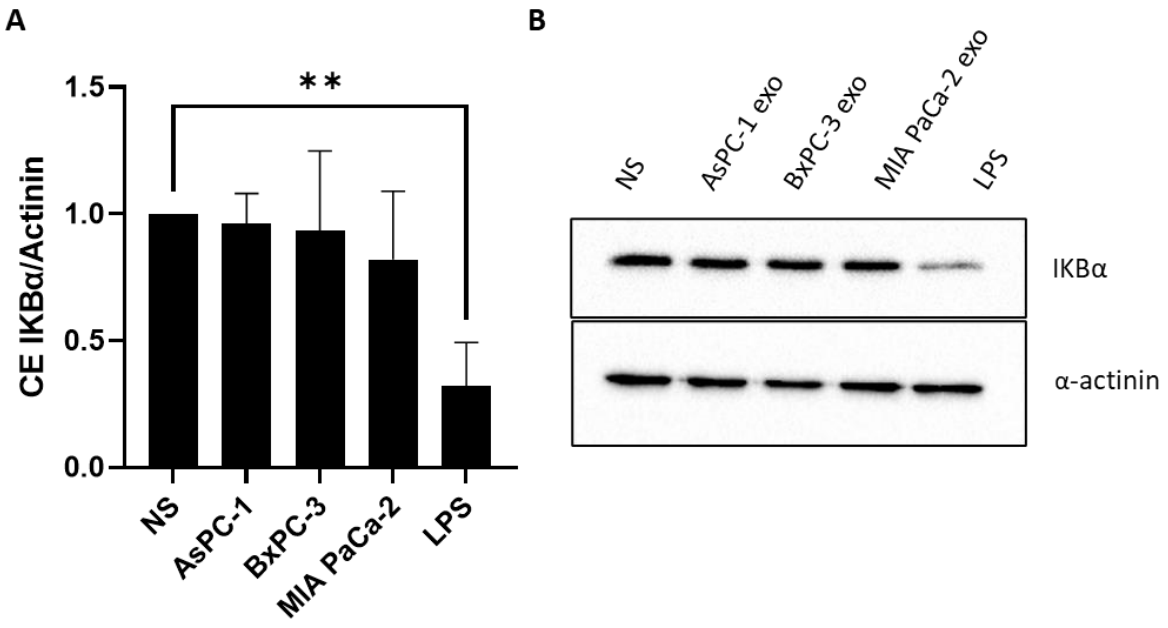

Figure 5. 4 Effect of PaCa-derived exosomes on cytoplasmic IKBα degradation in HMEC-1 cells. After treatment of cells with PDEx for 30 min, cytoplasmic extraction of HMEC-1 was prepared and western blotting performed to determine IKBα expression. Protein levels were quantified by densitometry. A) Quantification of cytoplasmic IKBα expression in HMEC-1. The expression levels of cytoplasmic IKBα were normalised and quantified to the loading control actinin. Quantification is expressed as fold change relative to non-stimulated. Minimal changes were observed in cytoplasmic IKBα levels in PDEx-stimulated, while 2.5 µg/ml LPS stimulation resulted in significantly less IKBα, indicative of degradation due to inflammatory activation of endothelial cells. CE = cytoplasmic extraction. NS = non-stimulated. Data expressed as mean ± S.D. Analysis was performed using one-way ANOVA with post-hoc multiple comparisons test; ** P < 0.01, *n = 3.* B) Representative blot of IKBα expression in cytoplasmic extractions of PDEx-stimulated HMEC-1 cells.

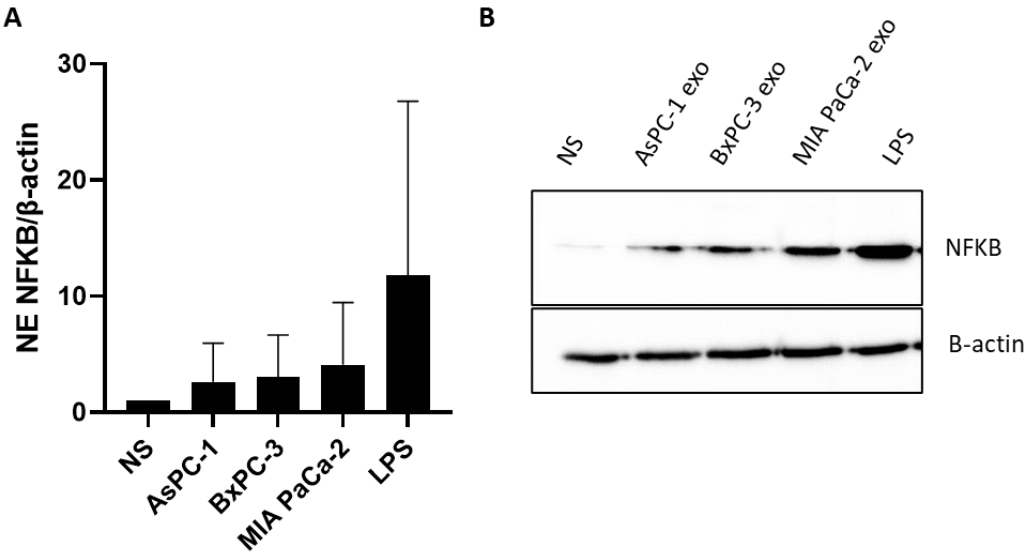

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Figure 5.5 Effect of PaCa-derived exosomes on NF-KB p65 nuclear translocation in HMEC-1 cells. After treatment of cells with PDEx for 30 min, nuclear extraction of HMEC-1 was prepared and western blotting was performed to determine NFKB p65 expression. Protein levels were quantified by densitometry. A) Nuclear extractions were used to assess levels of NFKB. The expression levels of NFKB were normalised and quantified to the loading control β-actin. Quantification is expressed as fold change relative to non-stimulated. NE = nuclear extraction. A trend increase in NF-KB translocation to the nucleus in HMEC-1 was observed with PDEx stimulation. Data expressed as mean ± S.D; n = 2. Non-significance between means was analysed using one-way ANOVA with post-hoc multiple comparisons test. B) One blot from two independent experiments of NFKB expression in nuclear extractions of PDEx-stimulated HMEC-1.

In addition to ICAM-1 induction signalling, western blotting was used to identify other signalling pathways known to be involved in endothelial cell activation. The signalling proteins assessed were p38 MAPK, Erk and Akt. The activation of p38 MAPK is involved in increased cellular adhesion molecules [333] and permeability [334, 335]. Activation of Akt and Erk pathways were also included to assess activation of TLR4, a known receptor for DAMPs [336, 337]. As mentioned previously in this thesis, DAMPs are of interest as they may potentially be mediators of PDEx-induced stimulation of activation responses in inflammatory cells. HMGB-1 has been shown to activate inflammation and activation in endothelial cells [338]. LPS was used as a positive control to assess TLR4 activation pathways.

Samples from cytoplasmic extractions of PDEx-stimulated HMEC-1 were used for analysis. Results show a slight upregulation in p-p38 in PDEx-stimulated HMEC-1 with a 2 to 3-fold increase compared to non-stimulated (n = 3; Figure 5.6a). LPS stimulation induced a 14-fold increase in p-p38 compared to non-stimulated. On the other hand, there was a trend decrease in p-Erk and p-Akt expression in PDEx-stimulated HMEC-1 (n = 2, Figure 5.6b & c). P-Akt expression was reduced by 0.3 to 0.5-fold in PDEx stimulated, while p-Erk expression was reduced by 0.5 to 0.6-fold compared to non-stimulated. LPS stimulation of HMEC-1 also resulted in a decrease in p-Akt (0.6-fold) and p-Erk (0.7-fold).

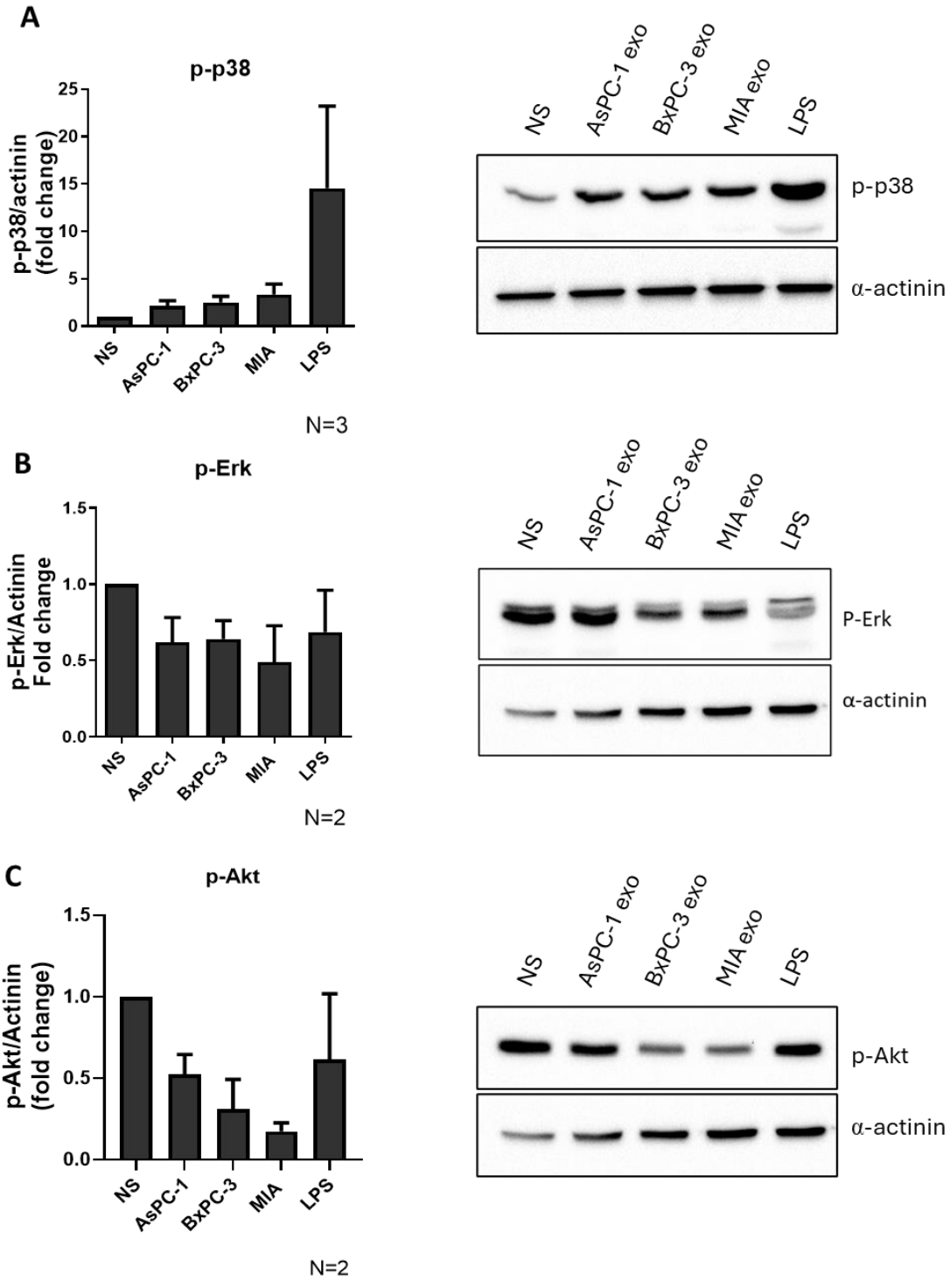

Figure 5.6 Effect of PDEx on signalling in HMEC-1 endothelial cells. Cells were stimulated with PDEx for 30 min and analysed for downstream signalling proteins A) p38 (n = 3) B) P-Akt (n = 2) and C) Erk (n = 2) involved in endothelial cell activation. Cytoplasmic extractions were prepared and used to perform western blots. Densitometry analysis was performed and quantification of protein expression is relative to non-stimulated (NS) HMEC-1. Protein expression was normalised to α-actinin. LPS served as positive control. Data expressed as mean ± SEM. Right pane: Representative blots of p-p38, p-Akt and p-Erk in PDEx-stimulated HMEC-1.

5.4 Discussion

This study investigated several activation responses in HMEC-1 cells when stimulated by PDEx including permeability, upregulation of ICAM-1, adhesion of neutrophils and intracellular signalling. Minimal effects on endothelial permeability and signalling were observed, however PDEx showed a great induction in ICAM-1 expression and increased neutrophil adhesion compared to non-stimulated HMEC-1.

The permeability assay used in this study did not seem to be an appropriate method to study endothelial activation in HMEC-1 in our hands. As evident with the positive control LPS, there were also no significant changes to permeability after stimulation (Figure 5.1). PMA on the other hand induced a large increase in HMEC-1 permeability. For this permeability assay to be successful, it is important for a uniform monolayer to be formed with resting cells which exhibit a cobblestone morphology. However, there were issues with the culture of HMEC-1 cells, where monolayers were not formed with the typical cobblestone morphology. Earlier passages of the cell lines were brought up, but the same issues prevailed. The lack of well-formed monolayers makes it difficult for this permeability assay as pre-existing disruptions to the barrier integrity indicate that the cells were already slightly activated. Thus, any changes induced by PDEx would be difficult to detect. This may also explain the great increase in permeability induced by PMA. PMA is a potent protein kinase C activator which results in increased endothelial permeability [339]. Slight openings of intercellular junctions from a poorly intact monolayer can make it easier for PMA to penetrate, thus exacerbating the activation and permeability response.

As the permeability assay did not seem to be ideal in detecting changes induced by PDEx, the expression of ICAM-1 was assessed. In contrast to the permeability results, PDEx induced a large upregulation of ICAM-1 in HMEC-1. Furthermore, this evidence was supported as shown by the increased neutrophil binding after treatment with PDEx, as ICAM-1 is known to mediate interactions with neutrophils via lymphocyte function-associated antigen 1 (LFA-1) [340]. This suggests that PDEx can increase upregulation of cellular adhesion molecules and facilitate interactions with other cells. In turn, the adhesion of neutrophils may result in further activation responses such as the release of NETs, which can further exacerbate endothelial activation and integrity. The release of NETs at the endothelial surface can also facilitate platelet adhesion and activation [209]. Thus, the upregulation of ICAM-1 in endothelial cells by PDEx can lead to a chain of other cellular activation predisposing to prothrombotic events. The effects on the upregulation of other cell adhesion molecules such as VCAM-1, and the following effects after neutrophils adhesion such as NET release will need further studies.

The trend upregulation of ICAM-1 expression observed by flow cytometry prompted investigation of the signalling mechanism of ICAM-1 induction. ICAM-1 induction in endothelial cells is dependent on the transcription factor NF-KB which is translocated to the nucleus upon activation with pro-inflammatory stimuli [341]. A trend showing increased NFKB p65 nuclear translocation was observed in PDEx stimulated, which was expected in the context of endothelial activation and upregulation of cell adhesion molecules. However with the low number of replicates (n = 2), it would be necessary for more experimental repeats before being able to conclude that PDEx results in an increase in NFKB p65 nuclear translocation. On the other hand, the levels of cytoplasmic IKBα in PDEx-stimulated were shown to exhibit similar levels compared to non-stimulated while LPS-stimulated showed a trend decrease. Decreased IKBα levels in the cytoplasm after pro-inflammatory, including LPS, stimulation is expected due to its activation leading to degradation [332]. With PDEx stimulation showing a trend increase in nuclear NFKB p65, it is expected for IKBα levels to be decreased as its degradation is responsible for the release of NFKB to the nucleus. Despite this, the comparable levels of IKBα in PDEx-stimulated and non-stimulated HMEC-1 may be due to a biphasic degradation of IKBα. Recent studies have shown a biphasic degradation IKBα where stimulation with TNF-α resulted in rapid degradation within minutes, its levels were restored in 1-2 hours, before another degradation phase at 6 hours [342, 343]. Thus, the similar levels of IKBα observed in PDEx stimulated compared to non-stimulated HMEC-1 may be due to a restoration of IKBα following an initial degradation after activation.

The signalling of activation markers p-p38, p-Akt and p-Erk were also investigated. Western blot shows a trend increase in p-p38 with treatment of PDEx. The activation of p38 has been shown to be involved in the upregulation of cell adhesion molecules [333], therefore the observed trend supports the upregulation of ICAM-1 by PDEx seen in this study. In addition, p38 also regulates endothelial cell permeability [334, 335, 344]. Thus, although the permeability imaging assay performed in this study was not able to show damaged endothelial barrier integrity even with LPS control, the western blot results can suggest that endothelial integrity is compromised as seen through the activation of p38.

For the other two signalling proteins assessed, p-Erk and p-Akt, both showed a trend decrease with PDEx stimulation. LPS stimulation also induced a trend decrease in p-Akt and p-Erk. There is a large variation in the response with LPS and more experiments would be needed to confirm the response as only two independent experiments were conducted. Stimulation of endothelial cells with LPS is expected to induce activation responses including phosphorylation of Akt and Erk. However, such response may vary depending on the concentration of LPS. A study has reported that endothelial barrier integrity is regulated by different concentrations of LPS through the PI3K/Akt signalling

pathway [345]. The study found that 1 μg/ml LPS resulted in increased phosphorylation of Akt in human pulmonary endothelial cells and was reduced with 10 μg/ml LPS, compared to non-stimulated. Thus, the reduced p-Akt observed in this study may have been due to the concentration of LPS (2.5 μg/ml) being over the threshold that results in an increase in Akt signalling pathway. Further experimental repeats would also need to be conducted to confirm the trend of reduced p-Akt and p-Erk seen in PDEx-stimulated HMEC-1. The PI3K/Akt signalling pathway is known for its roles in cell survival and anti-apoptotic effect [346]. A study has shown that the inhibition of Akt signalling in endothelial cells stimulated apoptosis [347]. Thus, PDEx may be stimulating endothelial cell apoptosis. Furthermore, Akt has also been shown to regulate other cellular functions. For example, tissue factor expression has been shown to be inhibited by the PI3K/Akt pathway [348, 349]. Interestingly VEGF was found to induce both AKT activation and tissue factor expression, and PI3K/Akt inhibition resulted in increased VEGF-induced tissue factor expression [348]. As VEGF can be derived from tumours, the PI3K/Akt pathway and its inhibition may be involved in the regulation of tissue factor expression on endothelial cells in cancer.

Future studies

As mentioned earlier in the discussion, there were some difficulties with the culture of the HMEC-1 where it appeared to be slightly activated and did not exhibit the typical cobblestone morphology of a well-formed monolayer. In future studies, it would be beneficial to use primary endothelial cells such as human umbilical vein endothelial cells (HUVECs). Unlike HMEC-1, HUVEC cells are not immortalised, thus they allow for closer representation of *in vivo* situations. While there were several observed trends in this study, more studies would be needed to confirm and gain statistical significance.

Another area of interest which was not able to be investigated in this study due to time constraints, is the effect of PDEx on the conversion of endothelial cells to a prothrombotic phenotype. Several pro-coagulant factors can be upregulated in response to inflammatory stimulation, as described earlier. These include examining the effect on tissue factor and thrombomodulin expression, and vWF release. The natural anti-coagulant protein, thrombomodulin, is involved in antithrombotic mechanism through its effect on thrombin inhibition and its downregulation is often observed in activated endothelial cells inducing a procoagulant phenotype [350]. Tissue factor being the primary initiator of the coagulation cascade, it would be interesting to detect tissue factor induction with PDEx, along with the roles of the PI3K/Akt pathway in the regulation of tissue factor expression which has been suggested in previous studies. Finally, vWF release from WPBs exocytosis is increased after activation of endothelial cells, and its interactions with platelets has been shown to mediate thrombotic events [351].

Chapter 6

The effect of anti-platelet treatment on tumour infiltration and vasculature in pancreatic cancer

Publication:

This chapter of the thesis includes a small part of work that has been previously published (described in the introduction), as well as additional unpublished data which was done by this author. The published paper is attached in the Appendix at the end of the thesis.

1. Elaskalani O, Domenichini A, **Abdol Razak NB**, E Dye D, Falasca M, Metharom P. *Antiplatelet Drug Ticagrelor Enhances Chemotherapeutic Efficacy by Targeting the Novel P2Y12-AKT Pathway in Pancreatic Cancer Cells.* Cancers (Basel). 2020 Jan 20;12(1):250. doi: 10.3390/cancers12010250.

6.1 Introduction

The work presented in this thesis thus far has investigated the effects of pancreatic cancer-derived exosomes on cells known to have roles in coagulation and thrombosis, one of which was platelets. The activation of platelets in cancer is well-documented, and it is also known to mediate interactions with leukocytes and endothelial cells [352]. As briefly discussed in an earlier chapter of this thesis, tumour cell-induced platelet aggregation has been described in many cancers and a number of mechanisms have been described, such as the expression of tissue factor, podoplanin or secretion of ADP by tumour cells. The activation of platelets can induce the expression CD154 which can interact with CD40 on endothelial cells leading to various inflammatory responses, such as upregulation of adhesion molecules, ICAM1 and VCAM1, and secretion of chemokines [353, 354]. All together, these promote the recruitment of leukocytes [355]. Furthermore, activated platelets also release soluble CD154 which can interact with endothelial cells, and induce the expression of E-selectin and P-selectin [352]. The interaction between platelets, leukocytes and the endothelium has been reported to be essential in the propagation of venous thrombosis. In a mouse model of DVT, the recruitment of leukocytes was initiated by a proinflammatory endothelium, a vital process in the development of venous thrombosis which was supported by activated platelets [155]. Thus, collectively the co-operation of leukocytes, endothelial cells and platelets plays an essential role in thrombosis and this interaction is likely to be more prominent in cancer due to cancer-induced platelet activation. **Given the well-known activation of platelets in cancer, this study aimed to investigate the effect of platelet inhibition with a clinically available anti-platelet drug, ticagrelor, and examine its effect on tumour vasculature and macrophage infiltration in a xenograft model of pancreatic cancer.** The results will give insight into the potential benefits of targeting a key player of thrombosis in cancer. This study was done in a mouse model of pancreatic cancer, which is relevant for the study of cancer-associated thrombosis, due to the hypercoagulability and increased incidence of VTE in pancreatic cancer.

The work presented in this chapter is derived from a larger animal study which has been published and was primarily led by co-investigator (and former PhD student), Omar Elaskalani. In the study led by Omar, one of the objectives was to investigate the effect of ticagrelor on tumour outcome in mice with pancreatic cancer. Tumour outcome was assessed by tumour weight and volume. The treatment of ticagrelor led to a trend of reduced volume of tumours, compared to without treatment, while there were minimal changes to tumour weight (Publication Figure 6a). In addition, the effect of antiplatelets in immunocompetent mice was also examined. For this, a syngeneic mouse model was also established where mouse MT4-2C cells in Matrigel were injected into the right flank of C57BL6 mice. The treatment of ticagrelor led to a greater reduction of tumour volume

in the syngeneic mouse model (Publication Figure 7), indicating that a fully developed immune system did not impact the effect on tumour growth by ticagrelor.

Another parameter that was assessed in the study by Omar Elaskalani is the haematological parameters. The treatment of ticagrelor did not have a significant impact on the red blood cell or platelet count (Figure 6.0). All other haematological parameters, namely white blood cells, granulocytes, monocytes and lymphocytes were significantly reduced. However, it should be noted that gemcitabine, which is the standard of care for pancreatic cancer, was also included as treatment in Omar's study. Compared to gemcitabine treatment, there were no significant differences, rather a slight increase in all the blood counts of ticagrelor-treated mice. While chemotherapeutic drugs such as gemcitabine and antiplatelet agents are known to be associated with lower platelet and white blood cell count [356, 357], these results suggest that ticagrelor has a slightly less adverse and suppressive effect on immune cell numbers than gemcitabine.

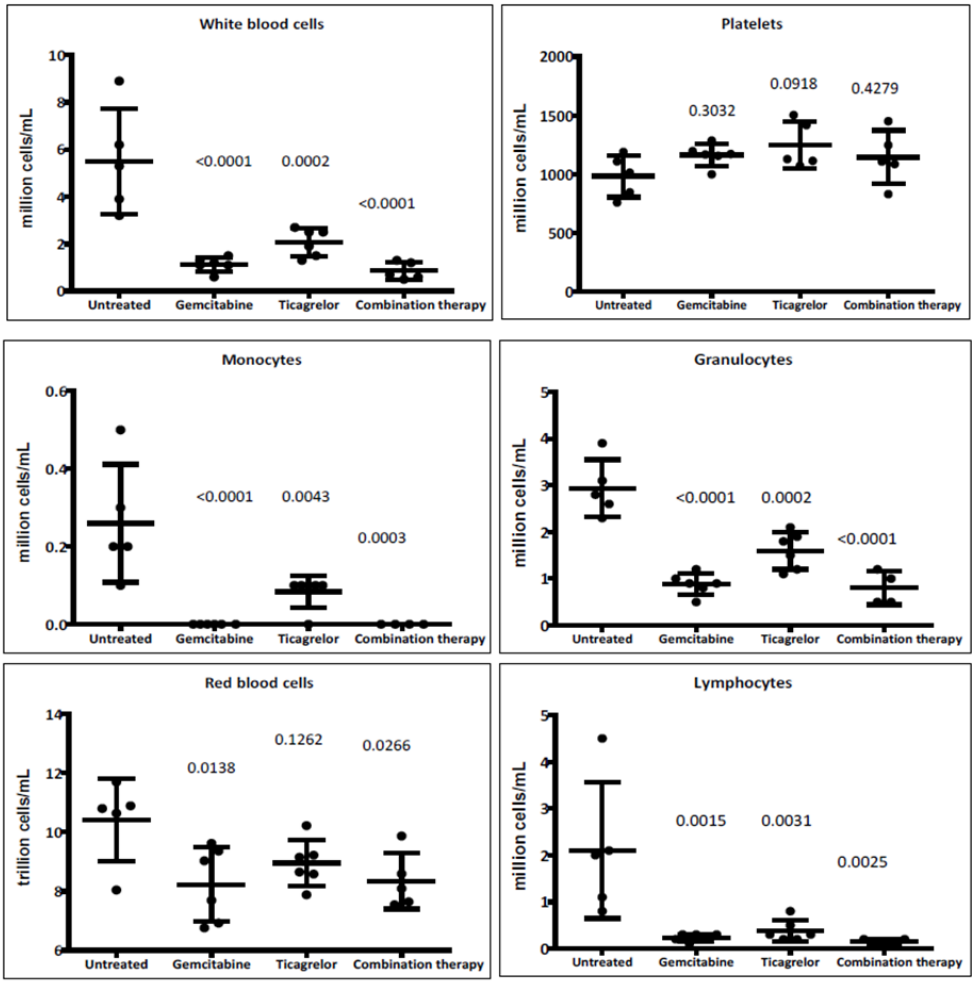

Figure 6.0 *(See figure legend on the next page)*

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Figure 6.0. Haematological parameters of mice bearing BxPC-3 tumours with different treatment groups. Untreated refers to mice receiving no drug treatment. Combination therapy refers to a combination of gemcitabine and ticagrelor treatment. The number of white blood cells, platelets, monocytes, granulocytes, red blood cells and lymphocytes were counted using a haematology analyser BC-VET2800. Significance was determined using ordinary one-way ANOVA with post-hoc Sidak's Multiple comparison test, *n = 8* for untreated, *n = 8* for gemcitabine, *n = 7* for ticagrelor and *n = 7* for combination. P value above each treatment represents the significance of the difference between the treatment and untreated group. This figure was reproduced with permission from Omar Elaskalani's thesis (submitted in 2019).

The author contributed to Omar's study through assistance with animal work and conducting a TGFβ-1 ELISA on the mouse plasma. These results, along with the immunofluorescence analysis of the tumours, will be presented and discussed in this chapter. **In addition to the impact on tumour outcome described in Omar's study, this study aims to give further insight into the inhibition of platelets in cancer by assessing the effects on plasma TGFβ-1 levels, macrophage infiltration and angiogenesis.**

6.2 Materials and methods

The work presented in this chapter has come from a larger study that was primarily conducted by Omar Elaskalani. Omar established a xenograft mouse model of pancreatic cancer with subcutaneous injection of pancreatic cancer BxPC-3 cells. Female NOD-SCID mice were injected with 2.5 x 10^6 in the right flank. When tumours were palpable, they were divided into treatment groups including untreated and ticagrelor-treated (50 mg/kg). At the end of the mice study, blood and tumour samples were collected. A TGF-β1 ELISA using the mice plasma, and immunofluorescence of tumour sections were conducted by this author for this study.

TGF-β1 ELISA

Mice blood was collected from the vena cava of mice under anaesthetics. The blood was anticoagulated with 5 mM ethylenediaminetetraacetic acid and centrifuged at 15 min for 15,000 x *g* without brake to obtain plasma. Samples were stored at -80°C until ready for analysis. An ELISA was performed using a Mouse TGF-β1 ELISA Kit (Biosensis BEK-2095-1P). Samples were subjected to acid activation in accordance with the manufacturer's instructions. A total of four mice plasma samples from each treatment group were included in the assay. The concentrations of TGF-β1 were calculated from the standard curve generated at 450nm using a plate reader.

Immunofluorescence of tumour sections

Tumours were obtained and embedded in optimal cutting temperature compound (Tissue-Tek) and stored at -80°C until cutting. Frozen tumours were cut 10 µm thick and fixed with ice-cold acetone for 10 min at 4°C. Sections were blocked in blocking solution (10% fetal bovine serum and 1% bovine serum albumin in PBS) and then stained overnight at 4°C with the following primary antibodies: Alexa Fluor 594-conjugated anti-CD31 (diluted 1:50; Biolegend) to visualise blood vessels, and Alexa Fluor-647 anti-F4/80 (diluted 1:100; Biolegend) to visualise macrophage infiltration. Samples were washed three times for 5 minute each, in PBS then mounted using ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen). Images were captured using a Nikon A1+ point scanning confocal microscope at 20X magnification. A total of 5 tumours (one tumour from each mouse) per treatment group were sectioned and stained for analysis. For the presentation in figures, images were edited in PowerPoint. The same colour contrast and saturation were applied to the same group of images to promote better visualisation of CD31 and nuclei staining.

6.3 Results

6.3.1 Anti-platelet ticagrelor led to decreased TGF-β1 levels

The anti-platelet drug, ticagrelor, is widely used in patients with coronary artery disease to reduce the risk of blood clots. In this study, mice bearing BxPC-3 tumours were treated with vehicle or ticagrelor (50 mg/kg). We were interested in determining the effect of ticagrelor treatment on the plasma levels of transforming growth factor beta 1 (TGF-β1), a cytokine known to facilitate cancer metastasis [358], of which platelets are the main source in the circulation. TGF-β1 levels in plasma were quantified using an ELISA. The treatment of mice bearing BxPC-3 tumour with ticagrelor (50 mg/kg) led to significantly lower plasma levels of TGF-β1 (1954 ± 39 pg/ml; *** $P < 0.001$, $n = 4$) compared to control mice without treatment (2092 ± 213 pg/ml, $n = 4$) (Figure 6.1). The plasma levels of TGF-β1 were also examined in NOD/SCID mice without tumours. Results show that the presence of a tumour led to a significant increase in TGF-β1 levels (2092 ± 213 pg/ml; *** $P < 0.001$, $n = 4$) compared to mice without tumour (764 ± 108 pg/ml, $n = 4$). Furthermore, not only did ticagrelor treatment significantly reduce TGF-β1 levels in mice with tumours, but it was reduced to levels that were seen in mice with no tumours. This indicates that ticagrelor has a potent effect on TGF-β1 levels in tumour-bearing mice, as it was able to be brought down to levels comparable to that seen in mice with no tumours.

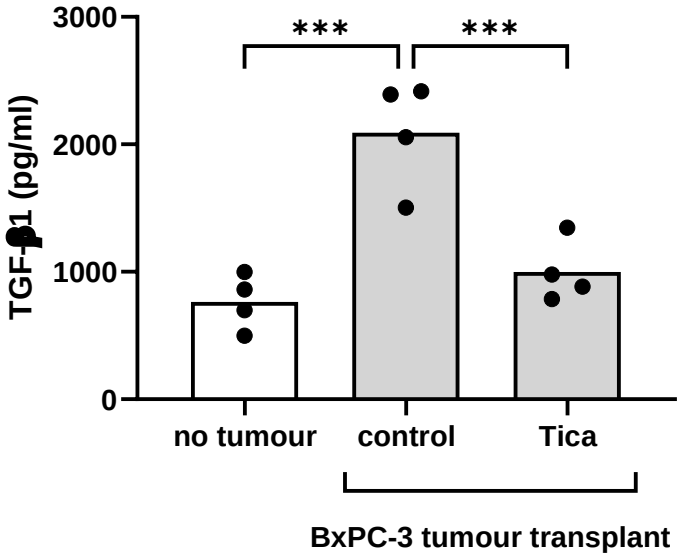

Figure 6.1. Quantification of TGF-β1 in mice with BxPC-3 tumours treated with ticagrelor, and in aged-matched NOD/SCID mice without tumours. Ticagrelor resulted in a significant decrease in TGF-β1 compared to control mice without treatment. Each dot represents one mouse. Data analysis was performed using one-way ANOVA with post-hoc Bonferroni's multiple comparison test; *** $P < 0.001$, $n = 4$.

6.3.2 The effect of anti-platelet on tumour infiltration and vasculature

To assess the impact of platelet inhibition on angiogenesis, tumour vasculature was assessed by endothelial cell staining. The impact on tumour infiltration of macrophages was also assessed to determine any association with tumour growth.

6.3.2.i Effect of anti-platelet on macrophage infiltration

As briefly mentioned in an earlier chapter (Chapter 3), macrophages have the ability to demonstrate plasticity in cancer. This plasticity results in M1 anti-tumour and M2 pro-tumour macrophages. Macrophages are the most abundant immune cell infiltrate in cancer [359]. Tumour infiltration of macrophages was assessed by immunofluorescence staining of tumour sections with Alexa Fluor-647 anti-mouse F4/80 antibody. Immunofluorescence revealed that macrophages were present in large numbers in the BxPC-3 xenograft tumour. The number of macrophage infiltrate, assessed by surface area quantification, showed no significant changes with ticagrelor treatment (% surface area F4/80+: control 21 ± 1.7% vs ticagrelor 16 ± 2.4%; ns, $n = 5$) (Figure 6.2).

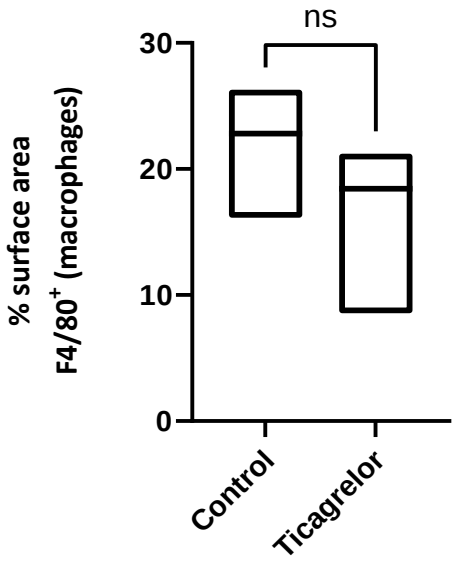

Figure 6.2. Surface area quantification of F4/80-positive staining in BxPC-3 xenograft tumour sections. Tumour sections were stained with anti-F4/80 antibody. Immunofluorescence images were taken from randomly selected fields and the F4/80-positive macrophages were quantified using Image J. A total of 5 tumours (one tumour per mouse) from each treatment group were included in the analysis and quantification. Control refers to tumours from mice without ticagrelor treatment. Significance between means was determined using an unpaired t-test. There was no significant difference between the control and ticagrelor treatment (n = 5).

6.3.2.ii Effect of anti-platelet on tumour vasculature

Tumour vasculature was assessed by immunofluorescence staining for endothelial marker CD31 using Alexa Fluor 594-conjugated anti-CD31 antibody. Quantification of the vessel density by CD31-positive surface area in tumour sections revealed that the vasculature in BxPC-3 xenograft tumour was significantly reduced in mice with ticagrelor treatment (% surface area CD31+: control 4.8 ± 0.5% vs ticagrelor 2.7 ± 0.3%; * $P < 0.05$, n = 5) (Figure 6.3). A closer look at the vasculature structure in the untreated control group show blood vessels that are characteristic of tumour vasculature (Figure 6.4). These include a chaotic, and disorganised structure with a lack of conventional hierarchy of blood vessels that is found in normal tissue [360]. Blood vessels also have inconsistent diameter along the same vessel, and uneven shape (Figure 6.4a). Continuous and long segments of blood vessels were visible, suggesting a more developed vasculature (Figure 6.4a,b and d). However, short segments were also visualised, as well as punctate vessels which may be the cross-section or lumen of a blood vessel (Figure 6.4c,d). In addition, endothelial sprouts which can be identified by finer structures that are sprouting off larger vessels (Figure 6.4b, arrow), was also observed.

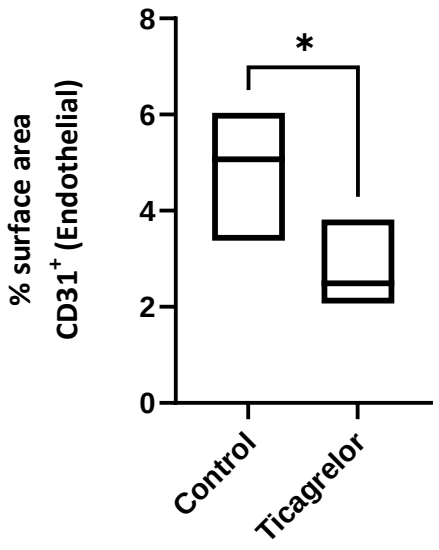

Figure 6.3. Quantification of CD31-positive endothelial cells in BxPC-3 xenograft tumours. Immunofluorescence images of tumour sections were obtained from both control and ticagrelor treated group. Immunofluorescence images were taken from randomly selected fields and the surface area of CD31-positive endothelial cells was quantified using Image J. A total of 5 tumours (one tumour per mice) from each treatment group were included in the analysis. Control refers to tumours from mice without ticagrelor treatment. Significance between means was determined using unpaired t-test; * $P < 0.05$ ($n = 5$).

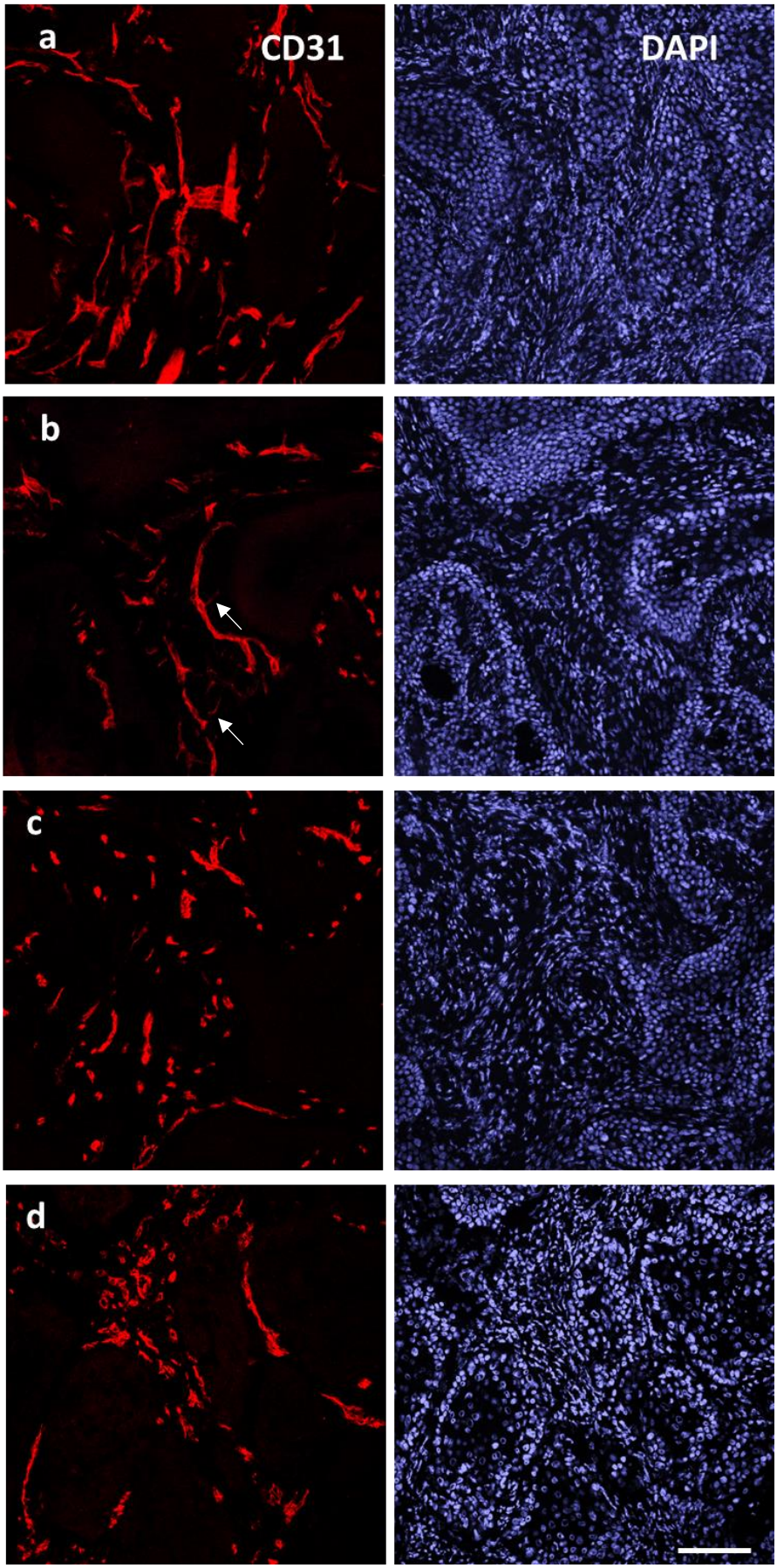

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Figure 6.4. Immunofluorescence microscopic images of tumour vasculature in BxPC-3 tumours from untreated xenograft models. Blood vessels were visualised by immunofluorescence staining with endothelial cell marker CD31. Tumour sections were imaged under 20x magnification. Images were taken from a total of 5 tumours (one tumour from 5 different mice) from each treatment group. Representative images are shown. Scale bar = 100 µM.

In tumours from ticagrelor-treated mice, the presence of continuous vessels was lacking (Figure 6.5). Many of the blood vessels imaged appeared discontinuous with short segments or as punctate vessels. The diameter of the blood vessels also appeared thinner compared to those in the control group. However, it should be noted that the appearance of blood vessels in histological sections is greatly influenced by the thickness of the section, which in this case was 10 µm. Thinner sections tend to show shorter segments, while thicker sections (40-60 µm) can allow better visualisation of the vascular network [361]. Despite this, comparison of the control and ticagrelor-treated group showed key differences with less dense and short, punctate vessels with thinner diameters in tumours from the ticagrelor-treated group.

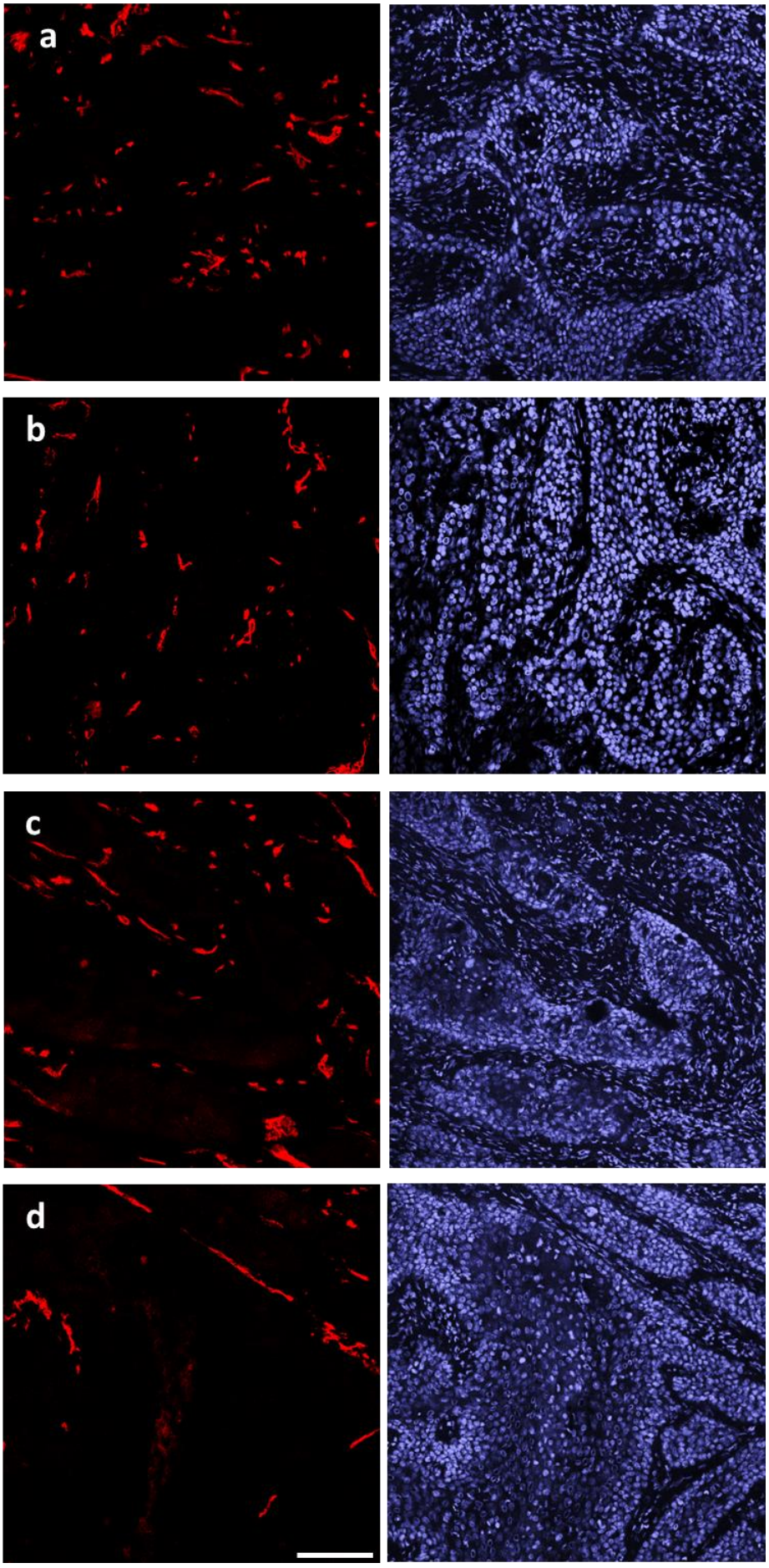

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Figure 6.5. Immunofluorescence microscopic images of tumour vasculature in BxPC-3 tumours from ticagrelor-treated xenograft models. Blood vessels were visualised by immunofluorescence staining with endothelial cell marker CD31 (left panel) and nuclei was stained with DAPI (right panel) Tumour sections were imaged under 20x magnification. Images from five tumours (one tumour per mouse) were taken. Representative images are shown. Scale bar = 100 µM.

6.3.3 The effect of ticagrelor treatment on intratumoural spaces

During the collection of tumour tissue samples from mice, it was observed that the tumours from the control group were soft and larger in comparison to the tumours from ticagrelor treated. In contrast, tumours of ticagrelor-treated mice were more solid and smaller in size. Furthermore, in immunofluorescence imaging of the tumours, the differences in nuclei staining were also strikingly different (Figure 6.6). The nuclei staining of control tumour sections appeared to be less dense with the presence of many holes or intratumoural spaces. Tumours from ticagrelor-treated on the other hand, showed more dense staining of nuclei and little to no intratumoural spaces were observed. To obtain quantitative analysis, the intratumoural spaces were counted. Any intratumoural spaces observed were counted from 9-10 sections per mouse. The total number of spaces were divided by the number of sections for each mouse. A total of 5 mice were included in the analysis for both control and ticagrelor-treated groups. The intratumoural space count was less in ticagrelor-treated group (1.04 ± 0.2) compared to control (2.50 ± 1.4) (Figure 6.7, n = 5)

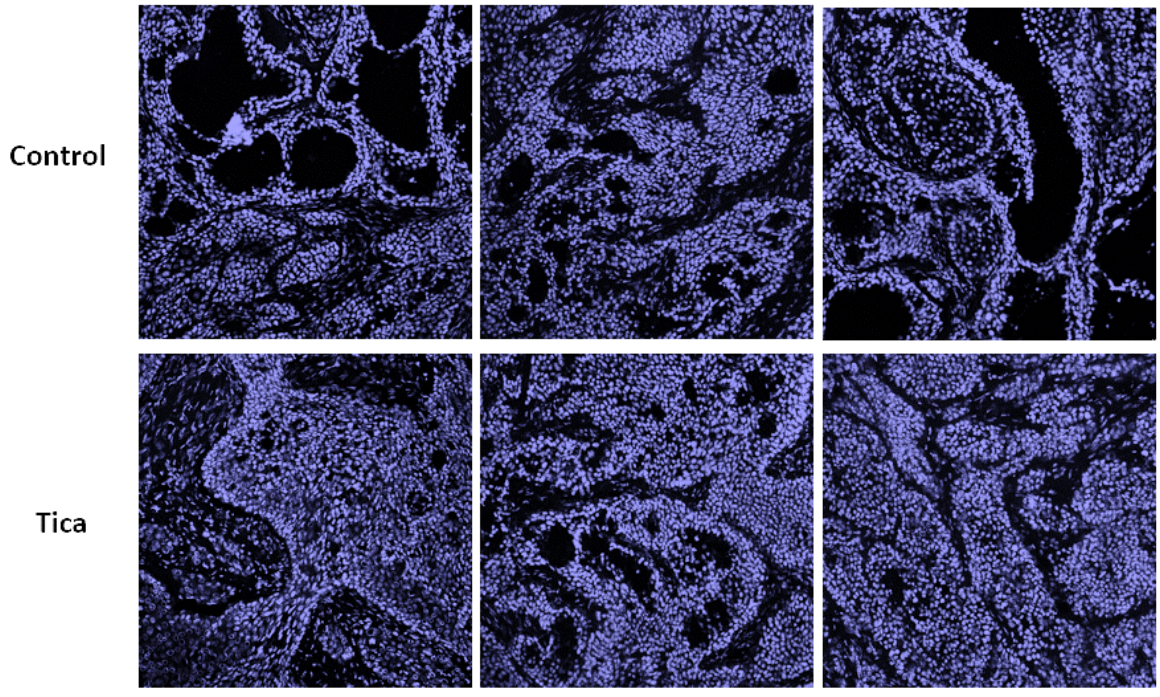

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Figure 6.6 Representative images of nuclei staining in control and ticagrelor treated group. Tumour sections were stained with DAPI and imaged on Nikon confocal microscope. Evidence of large intratumoural spaces with control staining, which are not as evident in ticagrelor treated. Tica = ticagrelor. A total of 5 tumours (one tumour from five mice) from each treatment group were included in the analysis.

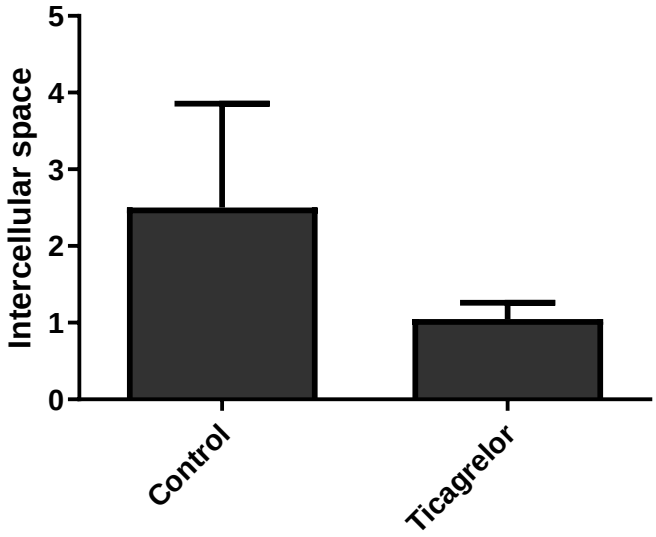

Figure 6.7 Quantification of the intratumoural spaces observed in BxPC-3 xenograft tumours. Intratumoural spaces were counted in tumour sections from mice in untreated and ticagrelor-treated groups. The average count from 9 to 10 tumour sections was calculated for each mouse. A total of 5 mice from each treatment group were included in the analysis. Data expressed as mean ± SEM. Significance between means was determined using unpaired t-test. There was no significant difference between control and ticagrelor treated groups (n = 5).

6.4 Discussion

This study explored the effect of an anti-platelet, ticagrelor, on plasma TGF-β1 levels, macrophage infiltration and tumour vasculature in a xenograft mouse model of pancreatic cancer. The findings are summarised as follows: Ticagrelor treatment in mice resulted in significantly lower TGF-β1 levels in mice compared to the control group, and a trend of reduced macrophage infiltration was observed. Key differences were observed in the tumour vasculature, with structures in ticagrelor-treated exhibiting short and punctate vessels. Finally, there was a trend of increased intratumoural spaces observed in the control group. Furthermore, the data from the published study relevant to

this study, showed that all haematological parameters except for platelets and red blood cells were significantly reduced, and a trend reduction in tumour volume.

Ticagrelor is widely used as an anti-platelet in cardiovascular disease patients to reduce the risk of thrombosis. The drug acts on the P2Y12 receptor, which is activated by adenosine diphosphate (ADP) [362]. ADP is a strong platelet agonist and has been implicated in the activation of platelets in cancer. Tumour cells expressing ADP, as well as the release of ADP from dense granules of activated platelets, constitute a positive activation loop and activation of nearby platelets. Thus, the use of ticagrelor leads to a profound effect on platelet activation.

Platelets are the main source of TGF-β1 in the circulation. Our results show that ticagrelor treatment in mice showed a stark decrease in TGF-β1 levels in plasma. This effect may be largely due to the inhibition of ADP activating P2Y12 receptors on platelets. The activation of P2Y12 receptor on platelets by ADP leads to potentiation of degranulation, including alpha granules, which are a major source of TGF-β1. Apart from the inhibition of direct platelet activation, tumour cell-induced platelet activation may also be restricted. Pancreatic cancer cells also express P2Y12, and its growth *in vitro* was suppressed by ticagrelor (Publication Supplementary Fig. S3). Thus, the inhibition of P2Y12 in tumour-bearing mice may have also led to reduced tumour cell-induced platelet activation, further contributing to the reduced secretion of TGF-β1 from activated platelets. It is interesting however, that platelet count was not impacted by ticagrelor treatment, yet still resulted in a significant reduction in TGF-β1. This may suggest a lower than normal activation of platelets in ticagrelor-treated, resulting in a reduced release of TGF-β1 and detection in plasma.

The effect of ticagrelor in significantly reducing white blood cell, but not platelet count is intriguing. Macrophages and neutrophils are known to play roles in facilitating cancer progression [363, 364]. Patients with advanced cancer often present with high levels of neutrophilia [183]. A reduction in their numbers with ticagrelor treatment may reflect an anti-tumour inflammation response, which may also be contributing to the observed reduced tumour growth.

In the immunofluorescence analysis, there were no significant differences in the quantification of macrophage infiltration to tumours in both groups. Macrophages were chosen to be analysed in these tumours instead of neutrophils, as neutrophils are known to be early responders and are not expected to be present in the late stages of cancer. The haematological analysis of the mouse however shows that there was a significant reduction in monocytes, with almost zero levels in the blood of ticagrelor treated. This suggests that tumour macrophage infiltration to tumours was maintained with ticagrelor treated, and likely all the macrophages within the blood had infiltrated the tumours. Macrophages have been shown to express P2Y12, and *in vitro* studies show inhibition

with ticagrelor led to enhanced tumour cell phagocytosis and induced an anti-tumour phenotype [365]. Thus, the maintained infiltration of macrophages observed in ticagrelor-treated may be inducing an anti-tumoural effect and contributing to the decreased tumour volume that was observed.

One key difference that was observed is the changes to the characteristics and density of the tumour vasculature with ticagrelor treatment. There was a significant reduction in the density of tumour vasculature, as well as distinctly different visual characteristics to the vasculature where the vessels were thin, short and punctate. In addition, there was no evidence of endothelial sprouting as was observed in the control group. These results suggest that the inhibition of platelets resulted in a disruption to the tumour vasculature. The formation of blood vessels in tumours, known as angiogenesis, is important for tumour growth, and this process can be facilitated by growth and angiogenic factors released from platelets [366]. Among the angiogenic factors in platelets is TGFβ-1, which was shown to be reduced in ticagrelor-treated mice in this study. Together these results suggest that the disruption to tumour vasculature observed in ticagrelor-treated may be due to the inhibition of platelet activation and its release of angiogenic factors such as TGFβ-1.

Another distinct observation in this study was the presence of intratumoural spaces in the control group. This would explain the soft and spongey texture of the control tumours, compared to the solid and dense tumours in ticagrelor-treated tumours which was observed during the time of tissue sample collection. There was a large variation seen with the control group, however this may be due to different sections of the tumour, where some sections completely lack the presence of intratumoural spaces. An explanation for these spaces may be areas for blood containing nutrients for the tumour's growth. This would explain the slight increased tumour size of the control group. A supporting observation in this study is the formation of better vasculature in control compared to ticagrelor-treated. In addition, the endothelial cells of tumour vasculature are known to be defective, with openings that promote leakiness, resulting in the accumulation of blood lakes within the tumour [316]. In contrast, the lack of vasculature and vessels observed in ticagrelor-treated would mean less blood supply to the tumour and could explain the lack of blood lakes and decreased tumour growth, as observed in this study.

The TICONC (Ticagrelor-Oncology) study by Wright and colleagues [367], was the first study to investigate the effects of ticagrelor treatment on platelet activation within a population of patients with metastatic breast and colorectal cancer. The study found that colorectal cancer patients receiving ticagrelor had significantly reduced levels of spontaneous platelet aggregation, compared to baseline. In breast cancer patients, ticagrelor significantly reduced platelet integrin activation

assessed by fibrinogen binding. These results suggested that ticagrelor treatment in cancer patients could lead to a more rested state in platelets. This may also explain the reduced levels of TGF-β1 seen in ticagrelor-treated in this study, as despite platelet counts being similar to the control group, there was less plasma TGF-β1, possibly from less secretion of TGF-β1 as platelets are more rested.

In future studies, to help further understand the role of platelets in promoting thrombosis in cancer, the impact of ticagrelor treatment on NET generation will be investigated. Studies have shown that the activation of platelets are important for the generation of NETs, and these interactions facilitate thrombus formation. Due to insufficient plasma samples (after conducting TGF-β1 ELISA) we were unable to conduct analysis for NET markers. Several NET markers can be assessed in the plasma such as cell-free DNA, myeloperoxidase, neutrophil elastase and citrullinate histone H3 [368]. In addition, the same markers can be used in immunofluorescence to assess for the presence of intratumoural NETs. These studies may highlight the importance of platelet activation (via ADP-induced activation) in NET generation, and whether ticagrelor treatment can reduce the presence of NETs and NET markers, which serve as a platform for thrombotic events.

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Chapter 7

Discussion, limitations and future directions

As a general conclusion, this thesis aimed to investigate the effects of pancreatic cancer-derived exosomes (PDEx) on the activation of cells that are known to play roles in thrombosis, namely neutrophils, platelets, and endothelial cells. While other studies have looked at the impact of cancer-derived exosomes or EVs on these cells individually *in vitro*, this thesis allowed a broader insight on the ability of PDEx to promote thrombosis through the assessment of the direct activation of all three cells. Furthermore, the effect on the inhibition of one of the main players of thrombosis (also known to play roles in cancer progression), platelets, in a pancreatic cancer xenograft model was assessed to investigate its effect on tumour outcome, as well as tumour vasculature and macrophage infiltration.

Summary of findings

Overall, our studies found that PDEx were capable of inducing direct stimulation of neutrophils to release neutrophil extracellular traps (NETs). This NET release mechanism was found to be rapid, occurring within 30 minutes. In the platelet system, the novel phenomenon of platelet priming by PDEx was observed. This is a unique finding, which to the author's knowledge, has not been reported before. These PDEx-primed platelets were able to stimulate NET release, in the same manner which was observed by PDEx-induced NETs. That is a rapid mechanism with strong NET-inducing capacity. Furthermore, it was observed that PDEx did not induce direct activation of platelets. However, another functional mechanism of PDEx was suggested, the expression of ectonucleotidase CD73 on PDEx, which may possibly be dampening platelet activity through ATP cleavage. In the endothelial cell system, results suggest that PDEx can stimulate the expression of ICAM-1. Accordingly, the stimulation of endothelial cells by PDEx resulted in increased neutrophil adhesion. Finally, in the *in vivo* study, platelet inhibition by ticagrelor, in a BxPC-3 xenograft model resulted in reduced tumour growth and the disruption of tumour vasculature.

Limitations

The isolation of cancer-derived exosomes by differential ultracentrifugation used in this thesis has some limitations. While its greatest advantage is the capacity to isolate exosomes from large sample volumes, the disadvantages are low yield and low purity [173]. The low yield is particularly an issue, as often just enough exosomes were isolated from a single isolation preparation based on the micrograms of exosomal protein needed for the experimental assay. This also meant that a dose-response effect was not able to be examined, nor was it possible to prepare large amount of

exosomes (50 μg/ml) to examine the effect of a high concentration of PDEx. It would be desirable to see that the effect of PDEx increases with concentration used, and if there is a threshold for activation in other cells.

A great limitation of this thesis was the difficulty in working with primary neutrophils due to their sensitive nature. Neutrophils are known to be notoriously hard to isolate from blood and study in a pristine inactivated state due to their highly sensitive nature [369]. This was also evident in our hands in several instances, for example the variation in NET release with different blood collection methods (Figure 3.4), and the strong protease activity upon lysis resulting in sample degradation after storage. In addition, several attempts for phospho-tyrosine blots showed evidence of tyrosine phosphorylation occurring in non-stimulated neutrophils (data not shown) which indicated the presence of pre-activated neutrophils. In such cases, it would be difficult to detect an activation response upon stimulation. This together with the highly potent protease activity observed in isolated neutrophils were the greatest challenges, which significantly impacted the progress of the research in delineating the signalling pathways that are activated by PDEx through western blotting. Despite the many attempts and efforts made to optimise conditions for western blots, it was found to be a major limitation which led to a change in direction of the research.

In the endothelial cell studies (Chapter 5), it would have been preferred to also include primary endothelial cells as they would have a better representation of the responses observed in *vivo* conditions. Particularly for the permeability assay, HUVECs may have been able to show a better activation response, as this was not seen with HMEC-1 even with LPS as the positive control. In addition, it has been reported that HMEC-1 cells are less responsive to stimulus, whereby 20 times more of the TNF-α concentration was required to induce maximum response observed in HUVEC [370]. It was also suggested that the use of more than one type of endothelial cell type in studies is recommended, as HMEC-1 and HUVEC were shown to respond differently to exogenous growth factors [371].

In the *in vivo* study described in Chapter 6, a xenograft model of pancreatic cancer was used. With an interest in cancer-induced NET as a mechanism for thrombosis in cancer, this model would be less preferred to study the effect on NET generation as it used immunodeficient mice. Given the important roles of both neutrophils and platelets in cancer-associated thrombosis, it would be superior to use immunocompetent mice.

Significant findings

The first significant finding in the thesis was that human neutrophils can be directly activated by PDEx to release NET. This contrasts with the first report of breast cancer 4T1-induced NET release from mice neutrophils with G-CSF treatment [145]. This study did not assess NET release from mice neutrophils without G-CSF treatment, to determine if G-CSF was important in facilitating NET release. Since the result of PDEx-induced NET was found in this thesis, one other study in 2023 has shown that A549 lung cancer cell-derived EVs could induce NET release from human neutrophils, both from healthy controls as well as patients with gastroesophageal adenocarcinoma [259]. Interestingly, NET formation was significantly greater in cancer patients than in healthy controls. Furthermore, *in vivo* studies also confirmed NET formation was stimulated by tumour-derived exosomes as the pre-injection of exosomes before tumour cell inoculation led to increased presence of NETs and metastasis [259].

In chapter 4, it was observed that platelets were not activated by direct stimulation with PDEx, this is in contrast to the study by Gomes et al. who were the first to report a tissue factor-independent activation of platelets by breast cancer-derived exosomes [280]. Since then, no other studies have reported a TF-independent pathway activation of platelets by cancer-derived exosomes or EVs. This may be a phenomenon specific to breast cancer cells, or may be due to inadequate washing of the platelets in the study by Gomes et al. Their platelet isolation method describes only one wash of the platelets, in contrast to three washes which was performed in our laboratory for this study. In addition, only the TF-positive MDA-MB-231, but not MCF-7, induced platelet aggregation in the absence of plasma [280]. Hence there may have been contamination of minute traces of thrombin from plasma, which would be sufficient to induce platelet aggregation.

Despite no evidence of direct platelet activation by PDEx, it led to the discovery of functional CD73 on PDEx, which may explain the slight dampening effect that was observed in platelet activation in Chapter 4. More experiments are needed to confirm these observations. If confirmed, it would be a new mechanism to be investigated concerning exosome-derived CD73 and its role in adenosine generation and immunosuppression in the tumour microenvironment. In recent years, studies have provided evidence for tumour-derived exosomes carrying CD73 with different functions such as their ability to suppress T cells [286, 372], mediate immunosuppression by macrophages [373], or polarisation of macrophages to promote angiogenesis [374]. Studies of the role of exosome-derived CD73 are emerging and represent a relatively novel mechanism by which tumour-derived exosomes can promote immunosuppression. In the case observed in our studies, CD73-generated adenosine may be inhibiting normal activation of platelets, and may be a regulating mechanism in cancer

where exosome-primed platelets can travel through the circulation in an inactivated state, before reaching the distant site of metastasis and thrombosis, then exerting their effects through activation of other cells.

The third and most novel finding is that PDEx treatment of platelets led to its priming, which resulted in significant NET release. To the author's knowledge, this has not been reported elsewhere. Earlier studies have found that platelets can take up and deliver angiogenic factors and tumour-derived cytokines [375, 376]. In 2015, Best et al. first described tumour-educated platelets which showed the potential to use platelets as biomarkers since they contain an altered RNA profile [291]. It is likely that tumour-derived exosomes contribute to these effects through the transfer of their contents. Since then, other studies have also described the use of tumour-educated platelets as markers in other cancers [377, 378]. However, no studies have shown the impact of educating or priming platelets by tumour-derived exosomes, on mediating interactions with and influencing activation of other cells.

Future directions

A key question that remains unanswered is the mediating factor through which PDEx are inducing the activation of neutrophils and endothelial cells. Despite the many years since the discovery of NETs and their implications in cancer [145], it is surprising that the specific mediating factor and signalling pathways of NET in cancer have not been delineated. Understanding these would be pivotal to uncovering specific targets to inhibit NET formation in cancer which may have benefits to both thrombosis and metastasis. A potential candidate are the DAMP molecules, known to activate a wide range of immune and inflammatory responses. In the context of inflammation, DAMPs are most abundantly stored in platelets and can mediate the recruitment and activation of neutrophils. Thus, identifying DAMPs as the mediating factor of PDEx-induced NET could allow for the inhibition of cancer-associated thrombosis and metastasis.

Experimental work from an earlier chapter (Chapter 4) suggested that pancreatic cancer-derived exosomes may carry ectonucleotidase CD73 which could be responsible for the diminished ATP release from activated platelets. While exosome-activated platelets did not result in increased release of ATP, exosome-derived CD73 may be responsible for the generation of adenosine which would have consequences on the tumour microenvironment through an immunosuppressive effect. It would be interesting to further investigate the effects that exosome-derived CD73 may contribute to immunosuppression and cancer progression.

The complex interplay between PDEx, neutrophils, platelets and endothelial cells

The results from each chapter of this thesis have demonstrated that the effects of PDEx on different cells are vast, which consequently can translate to complex interactions *in vivo*. There is likely a role for every cell involved in thrombosis and inflammation, to be influenced by cancer-derived exosomes. In effect, the mediated changes induced by PDEx on cells can in turn affect adhesion and activation of other cells, promoting a vicious cycle between immune and vascular cells in cancer, thus exacerbating prothrombotic interactions and facilitating thrombosis. Examples can be drawn from each chapter of this thesis and are summarised in Figure 7.1. We have also previously shown that NETs can serve as a platform for the direct activation of platelet aggregation [41]. In addition to the interactions depicted in Figure 7.1, other interactions which have been reported can further contribute to and exacerbate events predisposing to thrombosis. For example, in an *in vivo* model of thrombosis, neutrophil adhesion to endothelial cells led to NET formation [155]. Furthermore, NET release has also been shown to activate endothelial cells by upregulation of adhesion molecules and expression of tissue factor expression [379]. Thus, it appears to be an incredibly complex interplay of various cells which can either be activated or regulated by the pancreatic cancer-derived exosomes. These activation mechanisms combined from different cell types are involved in an activation loop, ultimately leading to the propagation of cancer-associated thrombosis.

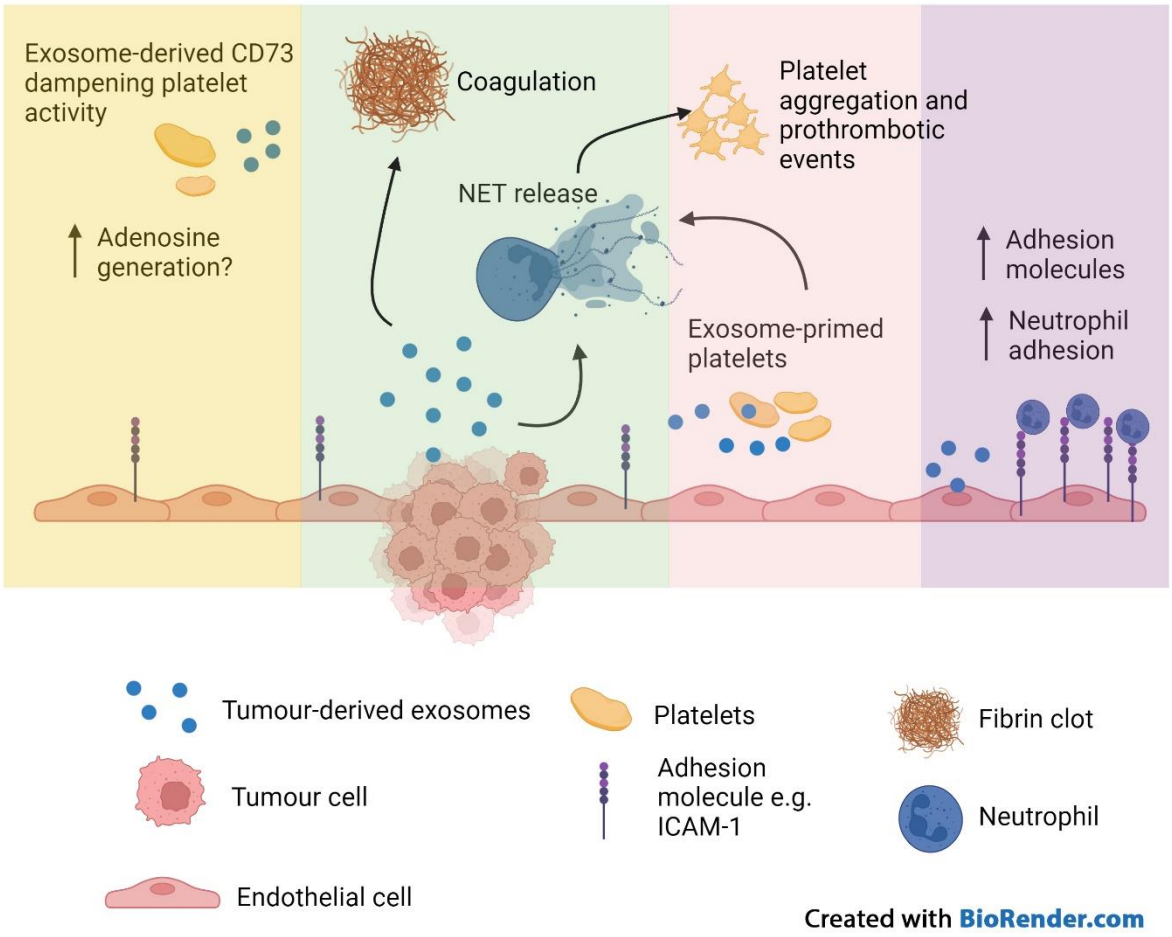

Figure 7.1 Schematics of the complex interactions between platelets, neutrophil and endothelial cells under the effect of PDEx. Tumour cells secrete exosomes which directly stimulate NETs and coagulation (Panel 2). Exosomes do not directly induce activation of platelets but priming of platelets with exosomes led to NET formation (Panel 3). The release of NET can cause platelet adhesion and aggregation (Panel 3). In endothelial cells, exosomes can promote adhesion of neutrophils by upregulation of adhesion molecules such as ICAM-1 (Panel 4). CD73 on cancer-derived exosomes may dampen platelet activity through ATP cleavage which may result in increased adenosine generation.

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Appendix

cancers

MDPI

Review

Cancer-Associated Thrombosis: An Overview of Mechanisms, Risk Factors, and Treatment

Norbaini Binti Abdol Razak [1,2], Gabrielle Jones [1], Mayank Bhandari [3], Michael C. Berndt [4] and Pat Metharom [1,2,*]

1 School of Pharmacy and Biomedical Sciences, Curtin University, Perth 6100, Australia; n.abdolrazak@postgrad.curtin.edu.au (N.B.A.R.); gabrielle.jones@postgrad.curtin.edu.au (G.J.)
2 Curtin Health Innovation Research Institute, Curtin University, Perth 6100, Australia
3 Fiona Stanley Hospital, Perth 6150, Australia; Mayank.Bhandari@health.wa.gov.au
4 School of Medicine, Curtin University, Perth 6100, Australia; m.berndt@curtin.edu.au
* Correspondence: pat.metharom@curtin.edu.au; Tel.: +61-08-9266-9271

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Abstract: Cancer-associated thrombosis is a major cause of mortality in cancer patients, the most common type being venous thromboembolism (VTE). Several risk factors for developing VTE also coexist with cancer patients, such as chemotherapy and immobilisation, contributing to the increased risk cancer patients have of developing VTE compared with non-cancer patients. Cancer cells are capable of activating the coagulation cascade and other prothrombotic properties of host cells, and many anticancer treatments themselves are being described as additional mechanisms for promoting VTE. This review will give an overview of the main thrombotic complications in cancer patients and outline the risk factors for cancer patients developing cancer-associated thrombosis, focusing on VTE as it is the most common complication observed in cancer patients. The multiple mechanisms involved in cancer-associated thrombosis, including the role of anticancer drugs, and a brief outline of the current treatment for cancer-associated thrombosis will also be discussed.

Keywords: venous thromboembolism; thrombosis; cancer

1. Introduction

Armand Trousseau first reported on the relationship between thrombosis and cancer in 1865. Since then, numerous studies have established that thrombosis is a common complication for cancer patients, contributing to the second-leading cause of mortality in cancer patients [1,2]. Thrombotic complications in cancer can vary from arterial or venous thromboembolism to disseminated intravascular coagulation [3,4]. Despite the well-known association between cancer and thromboembolic disease, the mechanisms that promote thromboembolic events in cancer patients are not clear and appear to be multifaceted [5]. Cancer patients are generally in a hypercoagulable or prothrombotic state, as they usually present with abnormalities in each component of Virchow's triad, thus contributing to thrombosis. The three components are a stasis of blood flow, endothelial injury, and hypercoagulability, the latter including abnormalities in the coagulation and fibrinolytic pathway and platelet activation. The specific mechanisms leading to abnormalities in Virchow's triad in cancer patients, particularly the effect on the host haemostatic system to promote the prothrombotic state, are not well understood and may be tumour specific as different cancer types have varying risk rates for cancer-associated thrombosis. This review will give an overview of the main thrombotic and bleeding disorders in cancer (arterial and venous thrombosis and chronic disseminated intravascular coagulation), the risk factors for developing cancer-associated thrombosis, and the

multiple mechanisms (direct and indirect) thought to promote cancer-associated thrombosis. A brief outline of the current treatment of cancer-associated thrombosis will also be discussed.

2. Types of Cancer-Associated Thrombosis

2.1. Venous Thromboembolism

Venous thromboembolism (VTE) comprises deep vein thrombosis (DVT) and pulmonary embolism (PE). The development of VTE is often initiated in the valve sinus where a number of features surrounding these valves make the site prone to thrombosis. These include abnormal and reduced blood flow, reduced shear stress, and hypoxia leading to an intact but dysfunctional endothelium [6]. In addition, platelets and leukocytes tend to become trapped in valve pockets [7]. In cancer patients, tumours can compress veins, resulting in venous stasis, thus encouraging thrombosis. VTE contributes significantly to morbidity and mortality of cancer patients, with a fatal PE being 3 times more common in cancer patients compared to non-cancer patients [8,9]. Cancer patients have a 5- to 7-fold increased risk of developing VTE [10,11] and those who develop VTE at diagnosis of cancer or within the year tend to have a significantly worse prognosis compared with cancer patients without VTE [12]. A diagnosis of VTE is a serious complication of cancer that adversely affects a patient's quality of life and reduces overall survival rates [13,14]. It is estimated that approximately 4–20% of cancer patients will experience VTE at some stage, the rate being the highest in the initial period following diagnosis. Annually, 0.5% of cancer patients will experience thrombosis compared with a 0.1% incidence rate in the general population [15].

2.2. Arterial Thrombosis

Although there are fewer data available on arterial thrombosis in cancer compared with on VTE, it is nonetheless observed in cancer. There have been multiple case reports suggesting acute arterial thrombosis in the setting of a new malignancy [16]. Navi et al. recently investigated the association between cancer patients and risk of arterial thrombosis in a large retrospective matched-cohort study. The incidence rate of arterial thrombosis at 6 months was 4.7% in cancer patients compared with 2.2% in the matched controls [17].

The pathogenesis of arterial thrombosis differs substantially from venous thrombosis as it typically occurs with endothelial damage. An atherosclerotic plaque is prone to thrombosis when it presents as a lipid-rich core with a thin fibrous cap. A thrombus can form over a ruptured plaque or an intact plaque that has superficial endothelial erosion [18]. Platelet activation that is persistent at the site of rupture promotes thrombosis by the exposure of procoagulant molecules within the plaque core. In contrast to the low venous shear rates in VTE development, the high shear rates in stenosed arteries contribute to a thrombus that is predominantly composed of platelets, as they are the only blood component capable of adhering at high shear. The high shear rates can also activate platelets, thus further promoting thrombosis [19]. The resulting intraluminal thrombosis often manifests as myocardial infarction or stroke. Furthermore, tissue factor found in atherosclerotic plaques appears to play an important role in the initial development of thrombosis following plaque rupture [18]. It should be noted, however, that arterial thrombosis in cancer can occur in the absence of an atherosclerotic plaque such as that observed in cardiovascular patients, where systemic hypercoagulation is induced by several secreted factors from cancer cells, such as thrombin and vascular endothelial growth factor (VEGF), thereby promoting platelet activation and coagulation [20].

Many chemotherapeutic agents are known to be prothrombotic and there are multiple case reports documenting an association between chemotherapy and arterial thrombosis. Platinum-based agents (cisplatin), vascular endothelial growth factor (VEGF) inhibitors (bevacizumab), and VEGF tyrosine kinase receptor inhibitors (sorafenib/sunitinib/pazopanib) have been associated with increased rates of arterial thrombosis [20]. Other major risk factors for arterial thrombosis include vessel damage which results from hypertension, atherosclerosis, or vascular anomalies. These factors contribute

to arterial thrombosis by inducing turbulence and altering blood flow, hence allowing for platelet adhesion which plays an important role in pathogenesis [18]. Furthermore, a recent meta-analysis has shown that the major risk factors for arterial thrombosis are significantly associated with VTE [21], suggesting that the two thrombotic disorders are simultaneously activated by biological stimuli.

2.3. Chronic Disseminated Intravascular Coagulation

Thrombotic complications observed in cancer are not limited to VTE or arterial thrombosis, with other more severe manifestations of the procoagulant state such as disseminated intravascular coagulation (DIC) and thrombotic microangiopathy (TMA) [12,22,23]. DIC is a severe yet rare complication of cancer that manifests itself as a consumptive coagulopathy resulting in microvascular thrombosis with tendency for severe bleeding, thrombocytopaenia, and organ failure [24,25]. Bleeding is thought to be due to hyperfibrinolysis that dominates microvascular thrombosis [26]. The incidence of DIC in solid tumours reported in a clinical study was 7% [27], with other reports indicating high incidence of up to 85% in acute promyelocytic leukaemia [28] Treating cancer patients with acute DIC is often very difficult with the majority of patients dying within 1–4 weeks [24]. DIC and thrombogenesis in cancer patients tends to involve defects in the three constituents of normal host defense against thrombosis: (1) blood flow leading to stasis, (2) balance of the procoagulant and anticoagulant proteins within the blood, resulting in the activation of circulating procoagulant proteins, and (3) vessel wall activation, resulting in an increase in its procoagulant contribution [12]. The clinical presentation of DIC in cancer patients is often less severe and has a more delayed onset, but following the clinical presentation, DIC progresses in a gradual yet chronic manner whereby systemic activation of coagulation occurs [22]. Eventually, this process can result in the exhaustion of coagulation factors and platelets, and bleeding may occur as the first clinical symptom to indicate DIC [22].

DIC is often difficult to distinguish from thrombotic microangiopathy (TMA), which manifests as thrombotic purpura and haemolytic uremic syndrome [23]. TMA shares the same clinical consequences as DIC and, thus, also leads to microvascular thrombosis with increased tendency for bleeding and organ failure. However, although DIC and TMA are similar and associated with each other, it is important that they be differentially diagnosed as treatments for TMA and DIC are different [25]. DIC is brought about by the marked activation and consumption of the coagulation system (triggered by substances like tissue factor, inflammatory cytokines, and activation leukocytes) which subsequently activates secondary fibrinolysis [25]. However, TMA onset is initiated by the marked activated and consumption of platelets in response to numerous factors which in turn lead to activation and subsequent injury to vascular endothelial cells [25].

3. Risk Factors for Cancer-Associated Thrombosis

Cancer is a well-known and established risk factor for thromboembolic events; however, there are several other risk factors known to increase a cancer patient's risk of developing thrombosis. In cancer patients, arterial and venous thrombosis are considered clinical manifestations of a multifactorial systemic disease and result from a number of risk factors which can be divided into the individual patient and cancer-associated risks [29]. Such risk factors include age, ethnicity, immobility, cancer type, and chemotherapy, will be discussed briefly below. Due to VTE being much more frequently clinically observed in cancer patients relative to arterial thrombosis, this section will concentrate on risk factors for VTE.

3.1. Individual Patient Risk Factors

3.1.1. Age

In the general population, incidence rates for VTE increase exponentially with age [30,31]. A large prospective study found that individuals aged 85 years and older have an almost 10-fold higher incidence rate (6.96 per 1000 person-years) compared with those aged 45 to 54 years of age

(0.72 per 1000 person-years) [31]. Likewise, increasing age is a risk factor for VTE in the cancer population. In retrospective cohort studies, cancer patients aged ≥65 years old have a greater likelihood of developing VTE compared with younger patients [32,33]. Similarly, in patients undergoing cancer surgery, the risk of VTE increased with age, compared with those aged less than 60 years (OR = 2.6, 95% CI: 1.2–5.7). In a retrospective cohort study undertaken by Vergati and colleagues that included cancer patients that were undergoing chemotherapy, the elderly patients (>70 years) had an almost 2-fold increased risk of developing VTE compared with the young to middle-aged patients (≤70 years), with incidence rates of 11% and 6%, respectively [34]. Increasing age, irrespective of cancer, is accompanied by factors that increase one's risk of thrombosis, including decreased exercise, increased immobility, and systemic activation of coagulation (reviewed in [35]).

3.1.2. Sex

Few studies have looked at the overall effect of sex in a cancer cohort and the risk of developing VTE. Retrospective studies show that females are at greater risk for VTE [32], while male patients are more likely to develop arterial thromboembolism [33]. In contrast, Chew et al. [36] found that sex did not predict thromboembolism in any of the cancers included in their study.

3.1.3. Race

In a large retrospective study by Khorana et al. [32], the rates of VTE occurrence in cancer patients of different ethnicities were examined. A significant association with VTE was found in black patients, who had the highest rate of VTE (5.1%), followed by whites and Hispanic patients (4.0%). The lowest rates were observed in Asian/Pacific Islander patients (3.3%), consistent with Chew et al. [36]. Moreover, the rate of VTE in black patients increased at a greater rate (36.7%) than in other ethnicities (26.8%). In contrast, a large retrospective study found no significant differences in the incidence rates of PE and DVT between blacks and whites [37].

3.1.4. Comorbidities

Multiple studies have identified an association between medical comorbidities and an increased risk of cancer-associated thrombosis. Comorbid conditions such as renal failure, respiratory disease, heart disease, obesity, and acute infection have been found to be associated with an increased risk of developing VTE in cancer patients, with infection identified as one of the most strongly associated risk factors for VTE [9,32].

3.1.5. Immobility

Immobility plays a role in predisposing cancer patients to VTE. Mobility in cancer patients is clinically assessed by performance status, and higher rates of VTE were observed in cancer patients with poor performance status [38,39]. In another study, bed rest of greater than 3 days was associated with significantly higher rates of VTE [40]. It is believed that immobility increases the chance of VTE through stasis of the venous blood flow [41].

3.1.6. Previous History of VTE

A previous episode of VTE is a major risk factor for developing VTE. Cancer patients with a history of VTE have a 6- to 7-fold increased risk of VTE recurrence when compared with cancer patients with no history of VTE [40,42].

3.2. Cancer-Associated Risk Factors

The rates of cancer-associated thrombosis (CAT) can be impacted by a number of cancer-related risk factors including cancer site, stage, histological subtype, and time since diagnosis. It is important to note that comparison of VTE rates amongst different patients in the literature is often difficult due

to varying differences in study design, patient populations, methods for data collection, and follow-up periods [42].

3.2.1. Site of Cancer

The primary site of the cancer is frequently identified as a risk factor for VTE, with cancers of the pancreas, uterus, lung, stomach, and kidney, and primary brain tumours associated with an increased risk of VTE [43]. A recent meta-analysis found that pancreatic cancer displayed the highest rate of VTE [44], while other studies suggest that the highest incidence rates occur in mucin-producing adenocarcinomas of the pancreas, lung, and gastrointestinal tract [45]. In contrast, the rates of increased VTE in cancer patients may reflect the frequency of cancer within the population as a whole, with a higher incidence of CAT observed in women with ovarian, breast, and lung cancer and higher incidence observed in men with prostate, colorectal, and lung cancer [45]. Irrespective of these differences, it is universally agreed upon that the incidence of VTE is not equivalent in different cancer types, which may suggest cancer-specific mechanisms playing a role in cancer-associated thrombosis.

3.2.2. Stage of Cancer

Patients with advanced-stage cancer appear to be at a greater risk of developing VTE [9]. In a Danish population-based cohort study, the risk of cancer patients developing VTE increased with cancer stage, and the calculated adjusted relative risks for stage I, II, III, and IV cancer were 2.9, 2.9, 7.5, and 17.1, respectively [46]. Similarly, a study observing oncologic patients who underwent surgery reported that patients with advanced-stage cancer had a higher risk of developing VTE [40]. A population-based study also found that patients with distant metastases are at a greater risk of VTE compared with patients without distant metastases. This study reported an initial 4-fold increased risk in cancer patients without metastases compared with non-cancer patients, which increased to 58-fold in patients who had solid tumours with distant metastases [11]. More recently, data from the Vienna Cancer and Thrombosis Study comparing the effect of local, regional, and distant cancer stages on development of VTE found a significantly higher risk of VTE in patients with regional and distant disease, when compared with patients with local disease [47].

3.2.3. Histology of Cancer

Histological subtypes of some types of cancer have been linked to an increased risk of VTE. For example, histological subtypes of lung and ovarian cancer show varying degrees of increased risk for VTE, while other histopathological subtypes of colon and breast cancer are nonpredictive for cancer-associated VTE incidence [14]. Studies have shown an increased risk of adenocarcinoma compared with squamous cell carcinoma in patients with non-small cell lung cancer [36,48]. Some studies have reported that the mucin-producing adenocarcinomas, e.g., pancreas, lung, and gastrointestinal tract, have the highest incidence of cancer-associated VTE [45]. On the other hand, there was no significant difference in the incidence of VTE in different histological subtypes of breast and colon cancer [49,50].

Ahlbrecht et al. published results from the Vienna Cancer and Thrombosis study and reported that tumour grade was associated with an increased risk of VTE in a cohort of patients' variety of solid tumours [51]. Patients with high-grade tumours (G3 and 4) had 2-fold increased risk of developing VTE compared with those with low-grade tumours (G1 and 2). Thus, tumour grading may be another valuable histopathologic parameter to stratify patients into VTE risk groups.

3.2.4. Time after Diagnosis

The immediate period following a cancer diagnosis is when the risk of developing VTE is highest [43]. This could be explained by the fact that many therapeutic interventions occurring during this period of time, such as chemotherapy, carry their own increased risk [43]. Numerous studies have demonstrated that the initial period following a cancer diagnosis is linked to a higher risk of VTE with

the greatest risk during the first three to six months following diagnosis, while other research suggests that the risk of VTE is greatest within the first year following diagnosis [9,14,52].

3.3. Cancer-Treatment-Associated Risk Factors

3.3.1. Surgery and Hospitalisation

The prothrombotic state of malignancy is often provoked by cancer therapies and treatments, including surgery. Historically, the observed incidence of VTE has been greatest in cancer patients admitted to the hospital, either for surgery or acute medical illness [53]. VTE is a common complication for those patients who undergo surgery, with cancer surgery increasing the risk of postoperative DVT 2-fold and that of fatal PE greater than 3-fold when compared with similar procedures carried out on non-cancer patients [40]. Over the years, the risk for thrombotic complications as a result of cancer surgery has been reduced due to improved surgical techniques, increased mobility following surgery, improvement of prophylaxis, and improvements in perioperative care [40]. Cancer-related surgery of the pelvis and abdomen is associated with a higher risk for VTE development in patients, while orthopaedic surgery of the lower limbs is also considered a high-risk surgery, particularly in Western populations [54,55].

3.3.2. Chemotherapy

Chemotherapy is an important risk factor for VTE amongst cancer patients and may explain its increasing incidence over the past few decades [53]. Cancer patients have a 6- to 7-fold increased risk of developing chemotherapy-associated thrombosis [53]. A predictive model for chemotherapy-associated VTE was developed and ranks patients with stomach and pancreatic cancers as very high risk compared with other cancer types. These patients are assigned 2 points, and a score greater than 2 classifies a patient into the high-risk VTE group [56]. A study undertaken by Khorana et al. determined that VTE rates were significantly higher in cancer patients over the 12 months following the initiation of chemotherapy compared with in non-cancer patients [53]. In cohort studies, patients who were receiving or were recently exposed to chemotherapy were found to be at increased risk of developing thrombosis [46,57]. Many types of chemotherapy drugs used to treat cancer have been associated with an increased risk of thrombotic events. Cisplatin is a platinum-based chemotherapeutic drug widely used in several malignancies and is usually used in combination with other drugs. The first evidence of cisplatin inducing increased arterial vascular toxicity and thrombotic potential was in 1986 [58], not long after its FDA approval in 1976. Substantial risk of both venous and arterial thromboembolic events has been reported in patients after treatment with cisplatin-based chemotherapy [59,60]. Combination chemotherapy with cisplatin leads to an approximately 2-fold increase in thrombotic complications in gastroesophageal cancer patients compared with patients receiving combination therapy with another platinum-based drug, oxaliplatin [61]. Other immunosuppressive or cytotoxic chemotherapies, such as L-asparganase, thalidomide, lenalidomide, and tamoxifen, have also been reported to increase the risk of VTE [62].

3.3.3. Angiogenesis Inhibitors

The importance of angiogenesis in tumour growth, invasion, and metastasis has led to the use of inhibitors that target blood vessel formation. Bevacizumab is a monoclonal antibody targeting circulating vascular endothelial growth factor (VEGF), which can be released from cancer cells and activates the endothelium. The effect of bevacizumab appears to have a greater impact on increasing the risk of arterial rather than venous thromboembolic events. While it is unclear if this drug increases the risk of VTE [63], several meta-analyses and large clinical trials have found bevacizumab to increase the risk of arterial thromboembolic events when compared with chemotherapy alone [64,65].

3.3.4. Central Venous Catheters

Central venous catheters (CVC) are vital for a number of aspects of cancer therapy, including delivery of intravenous drugs and collecting blood samples [14,66]. Their use, however, can result in the formation of catheter-related thrombosis, which is a serious complication that can interrupt the infusion of chemotherapy treatment, blood products, or intravenous medications, as well as cause serious morbidity including PE and postphlebitic syndrome [66,67]. It is estimated that the incidence of catheter thrombosis is between 5 and 30%, but it is thought to be underestimated because clinical signs of catheter thrombosis appear to be vague and nonspecific [66]. Indwelling CVC have been linked to an increased risk for developing VTE, and it is estimated that the rate of symptomatic catheter-related DVT is between 0.3 and 28%. However, this number dramatically increases to approximately 27 to 66% using venography [14].

4. Mechanisms of Cancer-Associated Thrombosis

The molecular mechanisms leading to the predisposition of cancer patients to thromboembolic events are poorly understood. However, several mechanisms that can promote a hypercoagulable state are seen in cancer patients, thereby increasing the risk of thromboembolic events. This section will focus on both direct (Figure 1) and indirect (Figure 2) mechanisms where a number of studies which the readers are referred to are pancreatic or stomach cancer studies.

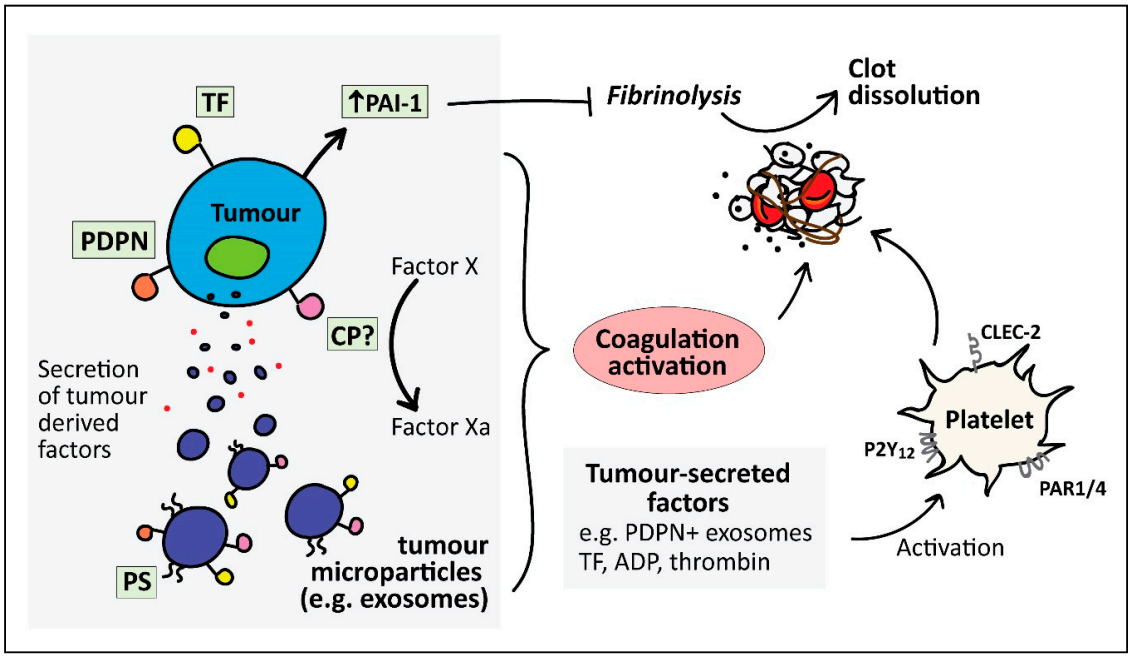

Figure 1. Direct mechanisms involved in cancer-associated thrombosis. Direct activation of coagulation and platelets can occur through several factors expressed on or released from cancer cells. These include the expression of tissue factor (TF), the key initiator of the coagulation cascade, which can also be released by TF-positive microparticles. Podoplanin (PDPN) expression can directly cause platelet activation and aggregation via the C-type lectin-like receptor 2 (CLEC-2) receptor on platelets. Plasminogen activation inhibitor-1 (PAI-1), a key inhibitor of fibrinolysis, is highly expressed in cancer cells. Cancer cells also secrete platelet agonists such as ADP and thrombin, thus further promoting platelet activation through P2Y12 and protease-activated receptors 1 and 4 (PAR1/4), respectively. Phosphatidyl serine (PS) expressed on tumour microparticles may also promote coagulation as PS serves as a surface for formation of coagulation complexes. Cancer procoagulant (CP) has been shown to directly activate coagulation by activating Factor X.

4.1. Direct Mechanisms for Cancer-Associated Thrombosis

4.1.1. Tissue Factor

Tissue factor (TF) is the best characterised tumour-derived procoagulant protein. It is a 47 kDa transmembrane protein that primarily initiates the extrinsic pathway of the coagulation cascade, resulting in thrombin generation which activates platelets and initiates blood clotting [68]. TF is abundantly expressed on subendothelial cells such as fibroblasts, pericytes, and vascular smooth muscle cells, and triggers haemostasis upon vascular injury. However, TF is not expressed on normal quiescent endothelium. In contrast, malignant tissue involving endothelial and tumour cells constitutively expresses TF. Despite many observations of TF expression in several types of cancers, the association of tumour TF expression with risk of VTE has only been observed in pancreatic and ovarian cancer [69,70]. Furthermore, TF expression has been shown to increase with advanced stages of pancreatic cancer and poorer prognosis [71,72]. Apart from TF expression in pancreatic tumours, TF is also present on the surface of microvesicles that are released by pancreatic tumours, which was also found to be associated with increased VTE in pancreatic cancer patients [73].

4.1.2. Microparticles (MP)

MP released from cancer cells can serve as direct and indirect contributors to the prothrombotic mechanism in cancer. MP are small (0.1–1 μm diameter) membrane vesicles that are released from apoptotic or activated normal cells, or resting malignant cells. Early studies showed that breast and hepatocarcinoma cell lines showed procoagulant activity both in vivo and in vitro, which was associated with tumour-shed vesicles [74]. More recently, circulating MP in several cancer types have been shown to accelerate thrombus formation in vivo [75]. The procoagulant activity of MP has been attributed to the surface expression of active TF [75–77], as well as the presence of phosphatidylserine, which provides a negatively charged surface that supports the assembly of coagulation complexes [78]. Recently, Stark et al. reported phosphatidylethanolamine externalisation from pancreatic cancer MP as an important player in cancer-associated DVT [79]. In addition, Geddings and colleagues showed that TF-positive MP enhanced platelet activation and increased thrombosis in mice [76]. The association of TF-positive MP and VTE incidence has only been observed in pancreatic cancer patients [80].

4.1.3. Podoplanin

Cancer-associated fibroblasts express podoplanin [81,82], a protein known to cause activation and aggregation of platelets, referred to as tumour-cell-induced platelet activation, through the C-type lectin receptor 2 (CLEC-2) [83]. Podoplanin expression has been reported in several pancreatic cancer cell lines, HPAF-II, HPAC and PL45 [84], and BxPC-3 (unpublished laboratory data). In an inferior vena cava (IVC) stenosis model of DVT, CLEC-2 depletion in platelets leads to reduced venous thrombosis, which was restored after transfusion of wild-type platelets. Increased podoplanin levels in the IVC wall after stenosis correlated with the degree of thrombosis [85]. However, the association of tumour-associated podoplanin and VTE in cancer patients has not been established apart from in patients with brain cancer [86]. It has been suggested that cancer cells expel podoplanin into the bloodstream in order to have an effect on thrombosis at distant sites [87]. Supporting this, tumour-derived MPs that bear podoplanin have been detected in the blood of patients with pancreatic and colorectal cancer [88].

4.1.4. Plasminogen Activator Inhibitor-1 (PAI-1)

PAI-1 is a key inhibitor of fibrinolysis which has been shown to be highly expressed in pancreatic cancer cells [89]. Increased PAI-1 in plasma results in reduced fibrinolytic activity, thus increasing the risk of thrombosis [90]. A study conducted in 1992 found excess PAI-1 in pancreatic cancer patients which correlated with thromboembolic developments [91]. However, the role of the fibrinolytic system and PAI-1 in cancer-associated thrombosis has not been well studied. A study

investigating the role of PAI-1 in a murine xenograft A549 cell tumour model found significantly increased thrombi and shortened occlusion times when treated with an anti-VEGF drug, bevacizumab, which also increased PAI-1 levels in the tumour and plasma [92]. Interestingly, the increased thrombotic effect of bevacizumab was significantly reduced by a PAI-1 inhibitor, suggesting a role of PAI-1 in cancer-associated thrombosis. However, further studies are required to elucidate its role and mechanism.

4.1.5. Cancer Procoagulant (CP)

CP is a cysteine protease that was firstly isolated from rabbit malignant tissue and reported to induce direct coagulation activation by directly activating factor X, without a requirement for coagulation factor VII [93]. It was later isolated from human carcinomas carrying procoagulant activity [94]; however, a study in breast cancer patients found no association of CP with procoagulant markers [95]. The purity of the CP preparations used in earlier studies that established CP as a procoagulant protein was later questioned as potential contamination of TF/factor VIIa complex [96]. Hence, Raasi et al. conducted a study to examine the presence of TF associated with CP by sequencing proteins that reacted with anti-TF monoclonal antibodies. Although there were cross-reactive proteins, none resembled the molecular weight or sequence of TF [97]. The role of CP as a procoagulant in cancer has gone largely unstudied and more studies would be required to confirm the role of CP as a protein that activates coagulation and its association with cancer-associated thrombosis.

4.1.6. Tumour-Derived Platelet Agonists

Adenosine diphosphate (ADP) and thrombin are well-known platelet aggregation agonists. Cancer cells are known to secrete ADP which causes platelet activation and aggregation via the P2Y1 and P2Y12 receptors [98]. Thrombin is also generated by pancreatic tumours [99] and has been found to be increased in plasma of patients with pancreatic cancer [100], thus implicating a role of these tumour-derived products in platelet activation and coagulation in pancreatic cancer.

4.2. Indirect Mechanisms of Cancer-Associated Thrombosis

Both cancer-derived factors and associated mechanisms that can activate or facilitate interactions with host cells can subsequently promote thromboembolic events. The host cells that have main roles in promoting thrombosis in cancer include platelets, leukocytes, and endothelial cells. A summary of indirect mechanisms that can promote thrombosis in cancer is depicted in Figure 2.

4.2.1. Microparticles

As described above, TF-positive MP observed in cancer patients are derived from cancer cells. In addition, they can also be released from activated endothelial cells and monocytes in response to cancer. Inflammatory cytokine release from cancer cells can cause activation of endothelial cells and monocytes and stimulate the release of TF-positive MP; however, the relative contribution of tumour cells and host cells to the total amount of TF-positive MP observed in cancer patients is unknown.

4.2.2. Inflammatory Cytokines

Tumour cells synthesise and secrete various inflammatory cytokines which are generally thrombogenic, capable of promoting a procoagulant phenotype in host endothelial cells [101]. In addition, the tumour presence causes a reactive response in host inflammatory tissues leading to excess cytokine release. The most well-defined proinflammatory cytokines that have been shown to exert prothrombotic effects are tumour necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β). TNF-α and IL-1β can induce the expression of TF and von Willebrand factor on vascular endothelial cells [102]. In a mouse tumour model, TNF-α induced TF expression on endothelial cells. TNF-α and IL-1β have also been shown to downregulate and attenuate antithrombotic regulators such as

thrombomodulin [103,104], a receptor on endothelial cells that binds with thrombin and activates protein C, a potent anticoagulant protein. The release of nitric oxide and prostacyclin—inhibitors of platelet adhesion and activation—from endothelial cells has also been shown to be suppressed after exposure to TNF-α and IL-1β [105,106]. Furthermore, TNF-α and IL-1β strongly impair the antithrombotic response of endothelial cells by stimulating the production of the fibrinolysis inhibitor, PAI-1.

Proangiogenic and growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and granulocyte colony stimulating factor (G-CSF) also play a role in regulating the procoagulant phenotype of host cells. VEGF is secreted by various tumour cells and can induce TF expression on macrophages [107]. G-CSF has shown to lead to an increase in endothelial activation markers (thrombomodulin and von Willebrand factor) and coagulation markers, suggesting an increase in haemostatic activation [108]. Basic fibroblast growth factor has been shown to increase TF expression on endothelial cells [109].

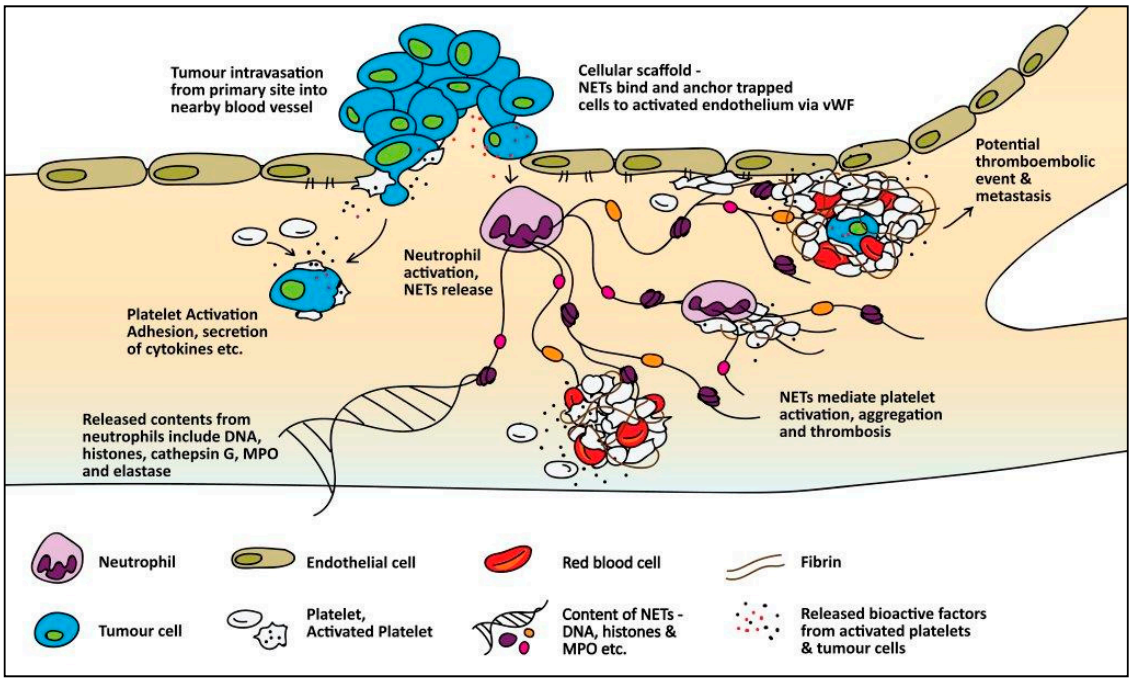

Figure 2. Indirect mechanisms promoting thrombosis in cancer. Tumours can be highly metastatic, resulting in cancer cell dissemination and intravasation into nearby blood vessels. Inflammatory cytokine secretion from tumour cells can cause activation of platelets and promote a procoagulant phenotype in endothelial cells. Cancer-derived factors also stimulate neutrophils to release neutrophil extracellular traps (NETs). NETs serve as a scaffold that can physically entrap platelets, or activate platelets through NET-associated histones, ultimately leading to profound platelet activation, fibrin deposition, and entrapment of red blood cells, exacerbating clot formation.

4.2.3. Adhesion Molecules

Cancer cells have the capacity to express specific adhesion molecules allowing for attachment to blood vessel walls and interactions with blood cells, as well as the activation of the procoagulant properties of host cells, primarily the endothelial cells, leukocytes, and platelets. The attachment of tumour cells to endothelial cells is significant in localising the initiation of clotting near the blood vessel wall and the subsequent formation of a thrombus. Several adhesion molecules have been described for the adhesion of different types of tumour cells to endothelial cells. For example, under flow conditions, HT-29M colon carcinoma cells used E-selectin to roll and adhere on activated endothelial cells. Vascular cell adhesion molecule-1 was required for the adhesion of A375M cells 29M [110]. These specific cancer and endothelial cell interactions can enhance aggregate formation which can promote clotting through

perturbation of blood flow. Furthermore, endothelial cells and activated platelets express P-selectin to which cancer cells can bind; however, the ligand for P-selectin on cancer cells is unclear. In addition, cancer cells may facilitate interaction with platelets via three mechanisms [101]. These are through the integrin αV/β, with the platelet integrin αIIb/β3, or P-selectin on platelets binding to either glycoprotein s-Le(x) on mucin-producing carcinoma, or sulphatides, which are expressed on some cancer cells. The enhanced interactions between cancer cells and platelets and endothelial cells can promote cell–cell aggregate formation, leading to perturbation of blood flow, therefore promoting blood clotting and vessel occlusion.

4.2.4. Neutrophil Extracellular Traps

The release of neutrophil extracellular traps (NETs) in response to pancreatic-cancer-derived factors has recently been reported in vitro [111]. NETs are a DNA-associated mesh of histones and neutrophil-derived proteases, which were first identified for their antimicrobial functions [112]. However, NETs have recently attracted interest for their ability to promote venous and arterial thrombosis in mice [113–115]. A significant reduction in venous thrombi was observed when NETs were targeted with DNAse I and neutrophil elastase inhibitor [113,114]. Cancer-associated NETs may further facilitate interactions with, or activation of, host cells to promote thromboembolic events. For example, NET-associated histones can activate endothelial cells and subsequently increase von Willebrand factor release (a glycoprotein important for platelet adhesion and aggregation in thrombosis) [114,116]. NETs can also serve as a platform for direct platelet adhesion and aggregation [6,117], which is vital for thrombus formation. A recent study by Mauracher et al. found that increased citrullinated histone H3 (a biomarker for NET formation) was associated with an increased incidence of VTE in patients with cancer, while other NET biomarkers (cell-free DNA and nucleosomes) were associated with high risk of VTE during the first 3–6 months [118]. These data suggest the significance of NETs in the pathogenesis of cancer-associated thrombosis.

4.2.5. Mucins

Several types of cancers are a mucin-producing carcinoma with aberrant expression and altered glycosylation of several mucins [119,120]. The heavily O-linked glycosylation sites on mucins serve as ligands for selectins [121]. Mucins have been shown to interact with blood cells via selectins, resulting in the formation of microthrombi. Mucins alone, purified from xenografted tumours, were not capable of directly activating platelets in vitro; however, mucins incubated with whole blood caused platelet activation, an effect that was found to be mediated by leukocyte L-selectin [122]. In addition, purified mucins when injected intravenously into mice resulted in widespread platelet-rich intravascular microthrombi [122]. Shao and colleagues provided further mechanistic insight on mucin-induced microthrombi [123]. The reciprocal interaction between platelets and neutrophils, with L-selectin and P-selectin glycoprotein ligand-1 on neutrophils and P-selectin on platelets, was essential for the release of cathepsin G from neutrophils [123], a known agonist of platelet aggregation [124].

4.2.6. Hypoxia

Tumours provide a highly hypoxic microenvironment [125,126], a condition that promotes endothelial dysfunction. In response to hypoxia, endothelial cells produce elevated levels of phospholipase A2, leading to the excessive production of prostaglandins and synthesis of platelet-activating factor (PAF) [127]. PAF is not only a potent platelet agonist but also activates neutrophils, promoting their adhesion to the endothelium under hypoxic conditions [128,129]. Indeed, blocking of PAF resulted in decreased neutrophil adhesion to hypoxic endothelial cells [129]. Moreover, hypoxia causes Weibel–Palade bodies present within endothelial cells to exocytose, resulting in the release of von Willebrand factor and overexpression of P-selectin, thereby increasing the indirect procoagulant response through endothelial cells. In addition, hypoxia can also increase ADP generated by tumours [130], further exacerbating platelet activation.

4.2.7. Damage-Associated Molecular Patterns (DAMPs)

DAMPs are released by dying tumour cells or through cell stress pathways that do not necessarily lead to cell death. Their role has just started to become understood in cancer [131]. They represent a broad array of molecules such as histones, High Mobility Group Box 1 (HMGB1), S100 proteins, and heat shock proteins, which are localised within the cell and only released upon cell death or during cell stress. The release of DAMPs initiates a host response via innate immune pattern recognition receptors to coordinate protective responses. However, DAMPs may also have detrimental effects for the host, triggering chronic inflammation and immune cell activation [132], which may ultimately have consequences for thrombosis in addition to benefiting tumour growth and survival. Indeed, increased circulating nucleosomes are observed in plasma of cancer patients compared with in healthy controls. The DAMPs which have so far been recognised to have potent procoagulant activity are histones and HMGB1. The implications of circulating histone and HMGB1 in the blood circulation of cancer patients are increased platelet activation and aggregation [133,134] and activation of neutrophils, particularly the initiation of the release of NETs [135], which have been shown to exert major consequences for thrombosis as described above. Furthermore, higher extracellular DNA levels were observed in patients with PE which was specific for PE amongst other potential diagnoses [136], establishing a link between extracellular DNA and thromboembolic complications. However, these studies have not been conducted comparing patients with and without cancer.

4.2.8. Cancer-Associated Chemotherapy

Despite a large body of evidence suggesting that cisplatin-based chemotherapy predisposes patients to thromboembolic events, the mechanism of cisplatin-associated thrombosis is not fully understood. Lechner et al. reported that cisplatin treatment of two human endothelial cell lines resulted in endothelial cell apoptosis, with the progressive release of procoagulant endothelial microparticles that generated thrombin independently of TF [137]. Chemotherapy is thought to increase the risk of VTE through direct drug-induced damage to the endothelium as well as by increasing the expression of TF procoagulant activity of monocytes and macrophages, which induces a procoagulant response from the host cells [35,138]. Another prothrombotic mechanism involving chemotherapy is its direct hepatotoxicity, which consequently leads to a decline in the plasma levels of natural anticoagulant proteins, including protein S, protein C, and antithrombin [35,139]. Chemotherapy is also responsible for inducing apoptosis of both tumour and host endothelial cells, causing cytokine release, which can increase both the expression and activity of TF (reviewed by Haddad and Greeno [45]).

4.2.9. Coagulation Gene Defects

In a study observing thrombophilic genes in gastrointestinal cancers, Pihusch and colleagues found an increased presence of the factor V Leiden and prothrombin gene G20210A mutation in those who subsequently developed thromboembolic disease [140].

4.2.10. Decreased Coagulation Inhibitors

An early study investigating the haemostatic balance in pancreatic cancer found that the coagulation inhibitors antithrombin III, heparin cofactor II, protein C, free protein S, and thrombomodulin were significantly decreased during the progression of pancreatic cancer after diagnosis [141].

5. Patient Management

Cancer patients have a significantly higher risk of developing VTE as compared to non-cancer patients. This is due to a combination of cancer-related, treatment-related, and patient-related factors. The pathophysiology of cancer-associated thrombosis is multifactorial and poorly understood. Patients with cancer-associated thrombosis have significantly shorter overall survival than cancer

patients without thrombosis. All cancer patients should be periodically assessed for VTE risk. The risks of cancer-associated thrombosis increase with hospitalisation, infection, chemotherapy, blood transfusions, erythropoiesis-stimulating agents, the presence of medical comorbidities, and the presence of central venous catheters. Cancer patients undergoing major abdominal or pelvic surgery should be offered postoperative VTE prophylaxis for up to 4 weeks. Healthcare professionals should educate patients about the signs and symptoms of VTE. A high index of suspicion is required for the early diagnosis of cancer-associated thrombosis, and early initiation of treatment can improve survival.

6. Cancer-Associated Thrombosis Therapy

A detailed discussion concerning therapeutic management of cancer-associated thrombosis is beyond the scope of this review. The reader is referred to several exhaustive reviews on this topic, which cover the use and appropriateness of thromboprophylaxis in cancer patients undergoing surgery and in the ambulatory patient setting, as well as clinical strategies for acute and extended treatment (>6 months) and treatment issues within special populations (patients with recurrent VTE while on anticoagulation, patients with thrombocytopenia, patients with brain tumours, catheter-related thrombosis, and incidental cancer-related thrombosis) [142,143]. Low-molecular-weight heparin has been shown to be superior to warfarin in reducing the risk of current VTE in patients with cancer-associated thrombosis and is the recommended current standard thromboprophylactic treatment [142–144] and first-line therapy for acute cancer-associated thrombosis in several existing major clinical guidelines [145,146]. For VTE and cancer, the CHEST guideline and expert panel report [146] suggests usage of low-molecular-weight heparin over vitamin K antagonists (moderate-quality evidence, grade 2B), and low-molecular-weight heparin over direct-acting oral anticoagulants, dabigatran (Grade 2C), rivaroxaban (Grade 2C), apixaban (Grade 2C), or edoxaban (Grade 2C). Until recently there has been a paucity of evidence with respect to the use of direct-acting oral anticoagulants for treating cancer-associated thrombosis and whilst their use relative to low-molecular-weight heparin would have practical advantages, concerns remain around the lack of reversal agents to rapidly allow haemostasis and the lack of widely available assays to monitor their anticoagulant activity [142]. Recently, the first large randomised prospective clinical trial, the Hokusai VTE Cancer study, was published comparing the safety and efficacy of dalteparin (low-molecular-weight heparin) with edoxaban (a direct-acting Factor Xa inhibitor) [147]. Oral edoxaban was non-inferior to subcutaneous dalteparin with respect to a composite outcome of recurrent VTE or major bleeding. The rate of recurrent VTE was lower with edoxaban but with a higher rate of major bleeding. In a smaller clinical trial with a similar patient profile to Hokusai VTE Cancer study, Young et al. published the results of SELECT-D trial in which they compared oral Factor Xa inhibitor (rivaroxaban) with low-molecular-weight heparin in cancer patients with VTE. They concluded that rivaroxaban was associated with low VTE recurrence but higher clinically relevant nonmajor bleeding [131].

7. Conclusions

In recent years there have been significant advances in our understanding of the molecular mechanisms associated with the increased risk of VTE in cancer, although there remain significant gaps in our knowledge of the causes of and best approaches for thromboprophylaxis in cancer-associated thrombosis. More research in this field should lead to a better understanding of the pathophysiology and optimal therapeutic approaches for the prevention of cancer-related thrombosis.

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Antiplatelet Drug Ticagrelor Enhances Chemotherapeutic Efficacy by Targeting the Novel P2Y12-AKT Pathway in Pancreatic Cancer Cells

Omar Elaskalani [1,2], Alice Domenchini [3], Norbaini Binti Abdol Razak [1], Danielle E. Dye [1], Marco Falasca [3] and Pat Metharom [1,2,4,*]

1 Platelet Research Laboratory, School of Pharmacy and Biomedical Sciences, Curtin Health and Innovation Research Institute, Faculty of Health Sciences, Curtin University, Bentley Campus, Kent Street, Bentley, Building 305, Perth, WA 6102, Australia; omar.elaskalani@postgrad.curtin.edu.au (O.E.); n.abdolrazak@postgrad.curtin.edu.au (N.B.A.R.); Danielle.dye@curtin.edu.au (D.E.D.)
2 Platelet Research Group, Perth Blood Institute, West Perth, WA 6005, Australia
3 Metabolic Signalling Group, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, WA 6102, Australia; alice.domenchini@curtin.edu.au (A.D.); marco.falasca@curtin.edu.au (M.F.)
4 Western Australian Centre for Thrombosis and Haemostasis, Health Futures Institute, Murdoch University, Perth, WA 6150, Australia
* Correspondence: pat.metharom@curtin.edu.au; Tel.: +61-(08)-9266-9271

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Abstract: *Background:* Extensive research has reported that extracellular ADP in the tumour microenvironment can stimulate platelets through interaction with the platelet receptor P2Y12. In turn, activated platelets release biological factors supporting cancer progression. Experimental data suggest that the tumour microenvironment components, of which platelets are integral, can promote chemotherapy resistance in pancreatic ductal adenocarcinoma (PDAC). Thus, overcoming chemoresistance 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 axis and a well-known antiplatelet drug, could be a therapeutic option for PDAC. *Methods:* We investigated a functional P2Y12 receptor and its downstream signalling in a panel of PDAC cell lines and non-cancer pancreatic cells termed hTERT-HPNE. We tested the synergistic effect of ticagrelor, a P2Y12 inhibitor, in combination with chemotherapeutic drugs (gemcitabine, paclitaxel and cisplatin), in vitro and in vivo. *Results:* Knockdown studies revealed that P2Y12 contributed to epidermal growth factor receptor (EGFR) activation and the expression of SLUG and ZEB1, which are transcriptional factors implicated in metastasis and chemoresistance. Studies using genetic and pharmacological inhibitors showed that the P2Y12–EGFR crosstalk enhanced cancer cell proliferation. 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 chemodrugs. In vivo, a combination of ticagrelor with gemcitabine significantly reduced tumour growth, whereas gemcitabine or ticagrelor alone had a minimal effect. *Conclusions:* 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.

Keywords: antiplatelet drug; chemoresistance; P2Y12; pancreatic cancer; ADP

1. Background

Pancreatic cancer has the lowest survival rates, where approximately 9% of patients survive five years after diagnosis [1]. Pancreatic ductal adenocarcinoma (PDAC) is the most common malignancy of the pancreas, accounting for >90% of pancreatic cancer cases [2]. Late diagnosis, the prevalence of metastasis and chemotherapy resistance account for the poor survival rates. Pancreatic cancer is also associated with thrombotic complications [3]. The elevated risk is attributed to the high metastatic rate and the ability of pancreatic cancer cells to activate platelets and the coagulation cascade [4]. 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 its profound adverse effects [5]. Anticoagulants have been examined as an adjuvant in combination with chemotherapy in PDAC, and despite a significant reduction in symptomatic venous thrombosis, there was no major increase in overall survival [6–8]. Recently, a clinical trial has been initiated to examine clopidogrel (antiplatelet, P2Y12 inhibitor) in combination with gemcitabine in PDAC patients (NCT02404363).

Several pathways in cancer cells can cause chemoresistance, including increased drug efflux, reduced drug cellular uptake, accelerated drug deactivation and activation of alternative oncogenic signalling pathways. Moreover, activation of the epithelial–mesenchymal transition (EMT), a cell developmental programme, in cancer cells further promotes chemotherapy resistance in PDAC [5]. We recently showed that platelet-derived factors, predominantly ADP/ATP, were responsible for an increased level of SLUG, an EMT transcriptional factor, which regulated the expression of cytidine deaminase (CDD) and equiliberative nucleoside transporter 1 (ENT1), and contributed to gemcitabine resistance [9].

The Gi-coupled P2Y12 receptor plays a crucial role in platelet function. P2Y12 downstream signalling in platelets is mediated through PI3-kinases, AKT, extracellular signal regulated kinases (ERK), Src kinases, small G protein Rap1 and G protein-gated inwardly rectifying potassium channels (GIRK) [10,11]. ADP is an important platelet agonist. It activates platelets through Gq-coupled P2Y1 and Gi-coupled P2Y12 receptors [12]. Solid tumours secrete more ADP (and ATP) compared to normal tissues, especially under hypoxia, which often occurs in solid tumours as they outgrow their blood supply [13]. ADP released by tumours activates platelets P2Y12 and P2Y1, which in turn releases growth factors to support tumour growth and metastasis [14]. P2Y12 is a target for several clinically available antithrombotic drugs [15]. Inhibition of platelet P2Y12 has been shown to reduce cancer growth and metastasis in ovarian, melanoma and lung cancer mice models [14,16]. However, the expression and signalling of P2Y12 in cancer cells are poorly investigated. The receptor is primarily expressed in platelets and brain tissue, with some reports showing P2Y12 in glioma, astrocytoma and breast cancer cell lines [17]. Here, we validated the expression of a functional P2Y12 in a panel of PDAC cells. Since several P2Y receptors have been shown to activate oncogenic EGFR signalling [18–20], we hypothesised that inhibition of P2Y12 may reduce epidermal growth factor receptor (EGFR) signalling and cancer growth. Our results show that ticagrelor, a clinically available P2Y12 inhibitor, exerted an anticancer effect and synergised with chemotherapeutic agents in vitro and in vivo.

2. Methods and Materials

2.1. Cell Lines

AsPC-1, BxPC-3, MiaPaCa-2, CFPAC-1, PANC1 and hTERT-HPNE cell lines were from ATCC® (Manassas, VA, USA). The murine Kras-driven pancreatic cancer cell line MT4-2D was kindly provided by Professor David. Tuveson (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). All cells tested negative for *Mycoplasma*. AsPC-1 and BxPC-3 cells were maintained in RPMI-1640 medium. MiaPaCa-2, PANC-1 and MT4-2D were maintained in DMEM medium. CFPAC-1 cells were maintained in IMDM medium. All culture media were supplemented with 10% fetal bovine serum

(FBS, from Bovogen Biologicals, Melbourne, 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, Waltham, MA, USA), puromycin (750 ng/mL, ThermoFischer, Waltham, MA, USA) and 5 mM D-glucose. Unless specified, all reagents were obtained from Gibco® Life Technologies (Melbourne, Australia).

2.2. Reagents

Paclitaxel, ADP, Apyrase, Tween-80 and polyethylene glycol-300 (PEG-300) were purchased from Sigma-Aldrich, (St. Louis, MO, USA). Cisplatin and erlotinib were obtained from Selleckchem (Pittsburgh, PA, USA). Gemcitabine was obtained from Eli Lilly (Indianapolis, IN, USA). Ticagrelor was obtained from Sigma-Aldrich, Selleckchem and Pure Chemistry Scientific Inc. (Burlington, MA, USA). PSB-0739 and MRS 2179 were from Tocris Bioscience (Bristol, UK). Cultrex basement membrane Type-3 was from Trevigen, Inc. (Minneapolis, MN, USA). Matrigel was obtained from Corning Life Sciences (Corning, NY, USA)

2.3. 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 for 72 h. In an experiment 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 the tetrazolium dye MTT as previously described [21]. The half-maximal inhibitory concentration (IC_{50}, μM) of ticagrelor was calculated using GraphPad Prism 8.0 Software (San Diego, CA, USA). 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 [22].

2.4. 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 antibodies were as follows; EPR18611 (Abcam, Cambridge, UK), 4H5L19 (ThermoFischer), and NBP2-61749 (Novus Biologicals, Littleton, CO, USA). Rabbit anti-CDD was obtained from Santa Cruz Biotechnology (Dallas, TX, 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 using centrifugation. Twenty micrograms of protein from cell lysates, and ten micrograms from the platelet lysate were loaded per lane. Proteins were analysed using immunoblotting after an SDS-PAGE. It is important to mention that P2Y12 in the positive control (i.e., platelets) and test cell lines were subjected to the same experimental conditions to prepare lysates. P2Y12 can form homo, oligomers and heterodimers, and each form can be glycosylated and thus may appear at different molecular weights in immunoblots based on experimental conditions, cell types and detecting antibodies [23–27].

To investigate the 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 a sample loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 6.8) supplemented with protease/phosphatase Inhibitor Cocktail (Cell Signaling Technology®, Danvers, MA, USA). Lysates

were then analysed using SDS–PAGE and immunoblotted for the relevant protein. Densitometry readings maybe found in supplemental data (Figure S6).

2.5. 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 (Hilden, Germany). Silencer® negative control (sequence: proprietary, catalogue #AM4635) was obtained from Ambion® (ThermoFisher). P2Y12 and Silencer siRNA (25 nM) were prepared using a DharmaFECT1 transfection agent (1 µL per well) (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 a DharmaFECT1 transfection agent (1 µL per well) in serum/antibiotic-free media. The transfection mixture was then added and the plate was incubated. After 24 h, the media were then replaced with complete media and incubated for 48 h. Cells were washed in PBS and then lysed with radioimmunoprecipitation assay buffer (RIPA) containing protease inhibitors).

2.6. 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 the 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 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 an ADP assay kit (Abcam) 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).

2.7. 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 an Amplite fluorometric Caspase-3/7 Assay Kit (AAT Bioquest, Sunnyvale, CA, USA) according to the manufacturer's instructions. The increase of Ex/Em = 350/450 nm was measured using the EnSpire Multimode plate reader. A NucView® 488 Caspase-3 Assay Kit (Biotium, Fremont, CA, USA) was used to detect caspase-3 activity within live cells.

2.8. In Vivo Tumour Growth

Female NOD-SCID and C57BL6 mice aged 5–6 weeks were from the Animal Resources Centre (Murdoch, WA, Australia) and housed in specific pathogen-free conditions at the Life Science Research Facility, Curtin University. All experiments were performed according to the Australian Code of Practice as per the University Animal Ethics Committee (approval number ARE2018-34). A BxPC-3 xenograft model was established via the subcutaneous injection of 2.5×10^6 cells in 100 µL RPMI/Cultrex basement membrane Type-3 (1:1) in the right flank of NOD-SCID mice. When tumours became palpable (50–100 mm^3), the animals were randomly divided into four groups (vehicle control, ticagrelor, gemcitabine, ticagrelor plus gemcitabine). For the syngeneic model, MT4-2D cells (2.5×10^5) in 100 µL RPMI/Matrigel (1:1) were injected in the right flank of female C57BL6 mice as previously described [28]. After two days, the mice were randomly divided into four groups (vehicle control, ticagrelor, gemcitabine, ticagrelor plus gemcitabine). Ticagrelor (50 mg/kg) was prepared in 4% DMSO, 30% PEG + 5% Tween 80 + ddH2O.

Gemcitabine (25 mg/kg) was prepared in 0.9% NaCl. Mice were given either ticagrelor or a vehicle via oral gavage (200 µL) twice a day every 12 h, five days a week (Monday–Friday) in addition to either gemcitabine or 0.9% NaCl via intraperitoneal injection (IP, 150 µL) once a week. The tumour diameters

were monitored with a surgical calliper every three days. Tumour volumes were calculated using the formula = (width^2 × length)/2.

2.9. Transforming Growth Factor Beta 1 (TGF-β1) ELISA

Mouse blood was collected via vena cava (under anesthetics) into ethylenediaminetetraacetic acid (EDTA, 5 mM final concentration). Plasma was prepared by centrifuging the sample for 15 min at 1500× *g* at 4 °C without brake, aliquoted and stored at −80 °C until further analysis. The levels of TGF-β1 were measured using a Mouse TGF-β1 ELISA Kit (Biosensis BEK-2095-1P). Samples were subjected to acid activation according to the manufacturer's instructions. TGF-β1 concentrations in the samples were calculated from a standard curve generated at 450 nm using a plate reader.

2.10. Statistical Analysis

Data were analysed using GraphPad PRISM 8.0 software (GraphPad Software, San Diego, CA, USA). Results are expressed as the mean ± standard error (SEM). Student's *t*-test, one-way ANOVA or two-way ANOVA were used to examine the significance of the mean as appropriate and as indicated in the figure legend. The *p*-values and statistical significance are reported as per the style of New England Journal of Medicine: <0.033 (*), <0.002 (**) and <0.001 (***).

2.11. Ethics Approval

The studies involving the use of animals were approved by the Curtin University Ethics committee (approval number ARE2018-34).

3. Results

3.1. P2Y12 Expression in PDAC Cells

P2Y12 expression in cancer and normal pancreatic ductal epithelial cells was detected at the same molecular weight as P2Y12 in platelets with an anti-P2Y12 monoclonal EPR18611 (Figure 1A). The results were confirmed using two additional different anti-P2Y12 antibodies (4H5L19 and NBP2-61749, Figure S1A). P2Y12 protein expression level was measured relative to α-actinin (Figure 1B). P2Y12 was overexpressed (>5-fold difference) in the PDAC cell lines AsPC-1, BxPC-3, MiaPaCa-2 and PANC-1 when compared to normal cells, hTERT-HPNE. The P2Y12 expression was the lowest in the well-differentiated cell line CFPAC-1 compared to those that were poorly differentiated (AsPC-1, MiaPaCa-2, PANC1) or the moderate-to-poorly differentiated BxPC-3 cell line. Similarly, EGFR was highly expressed in all PDAC cell lines compared to hTERT-HPNE (Figure 1C). A positive correlation between P2Y12 and EGFR expression in PDAC (R = 0.49, *p*-value = 2×10^{-12}) was also demonstrated by the gene expression profiling interactive analysis (GEPIA) (Figure S4A) [29].

Since AKT is phosphorylated downstream of P2Y12 activation in platelets and glioma C6 cells [30,31], we investigated ADP-induced AKT signalling in PDAC cells. Our results showed that P2Y12 inhibitors, ticagrelor (Figure 1D) and PSB-0739 (Figure S1B), but not a P2Y1 antagonist (MRS 2179, Figure S1C) markedly reduced ADP-induced phosphorylated-AKT. Additionally, we measured the level of extracellular ADP secreted by PDAC cells and normal pancreatic cells, hTERT-HPNE; however, both tumour and non-tumour pancreatic cells produced comparable ADP secretion (Figure 1E). Since ADP, ATP and adenosine are known to induce transactivation of the mitogenic receptor EGFR [13], we hypothesised that ADP may induce prosurvival signals in tumour cell lines, but not in hTERT-HPNE, which expresses a relatively low level of P2Y12 and EGFR. To test this hypothesis, AsPC-1, BxPC-3 and hTERT-HPNE were treated with ADP. As a result, the activation of EGFR, AKT and ERK was significantly enhanced in PDAC cells, but not in hTERT-HPNE (Figure 1F).

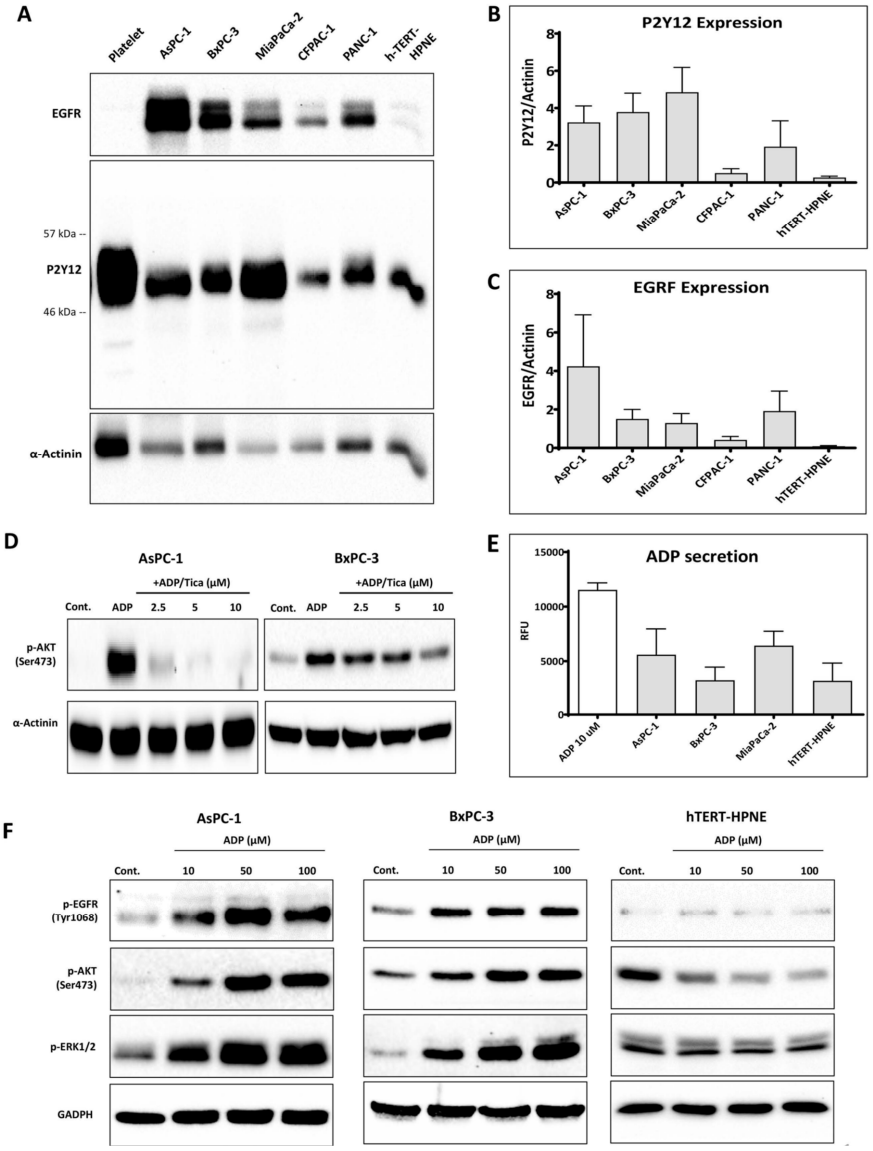


Figure 1. The P2Y12 receptor, activated by ADP, triggers AKT activation in PDAC cells. (**A**) Immunoblots show the expression of P2Y12 and EGFR in PDAC cells (AsPC-1, BxPC-3, MiaPaCa-2, CFPAC-1 and PANC-1) and the normal pancreatic duct cells h-TERT-HPNE. Platelets were used as a positive control for 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 five 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, Hercules CA, USA) and represented as columns using GraphPad Prism 8 (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 and 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 section. The columns represent the mean of relative fluorescence units (RFU) from three independent experiments. (**F**) Western blot analysis of phospho-EGFR Y1068 (p-EGFR Y1068), phospho-AKT S473 (p-AKT S473) and phospho-ERK 1/2 (p-ERK1/2) 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.

3.2. P2Y12 Regulated Phospho-EGFR, PDAC Cell Proliferation and EMT Markers SLUG and ZEB1

Since we observed that ADP induced EGFR activation in PDAC cells, we wanted to assess whether P2Y12 is required for EGFR phosphorylation. We first investigated four different siRNA sequences (Figure S2A,B) and selected the best-performing sequence (Hs_P2RY12_4 FlexiTube) for subsequent studies. Immunoblot results showed that P2Y12-siRNA significantly reduced the level of p-EGFR in AsPC-1 and BxPC-3 cells grown in complete media (Figure 2A,B), and attenuated the proliferation of AsPC-1 and BxPC-3, compared to control siRNA transfected cells (Figure 2C). Additionally, P2Y12 knockdown significantly reduced the expression of EMT transcription regulators SLUG and ZEB1 in AsPC-1, and the SLUG level in BxPC-3 (Figure 2A,B).

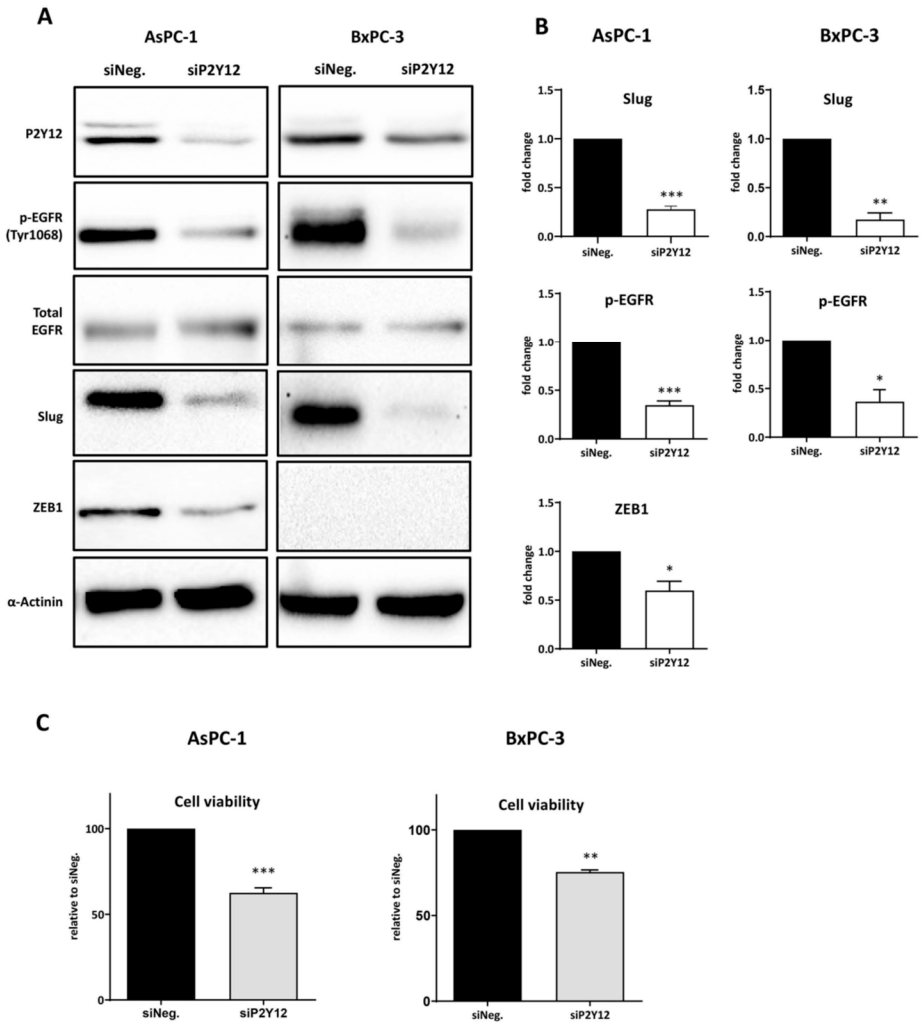


Figure 2. P2Y12 regulates EGFR activation, SLUG and ZEB1 expression and enhances PDAC cells viability. (**A**) Immunoblots showing the expression of P2Y12, p-EGFR Y1068, EGFR, SLUG and ZEB1 in AsPC-1 and BxPC-3 after P2Y12-specific siRNA treatment. Cancer cells were seeded and treated with P2Y12 siRNA or a negative control siRNA, as described in the Methods and Materials section. (**B**) The columns represent the fold change of protein levels (p-EGFR Y1068, SLUG and ZEB1) relative to the negative control siRNA-treated cells, normalised to 1 ($n \geq 3$). Statistical analysis was performed using one sample *t*-test (GraphPad Prism 8) comparing the mean of siP2Y12 treatment with the normalised value 1. (**C**) Cell viability of AsPC-1 and BxPC-3 cells following knockdown of P2Y12 compared with the negative control siRNA-treated cells ($n \geq 3$), calculated using one sample *t*-test (Graphpad Prism 8) and the normalised control group mean of 100%. Data are presented as mean ± SEM. *** $p < 0.001$, ** $p < 0.002$, * $p < 0.033$. siNeg: siRNA negative control.

3.3. Ticagrelor Reduced EGF-Induced AKT Activation in PDAC Cells

P2Y12 is known to signal through AKT in platelets. As a result, P2Y12 inhibition reduces AKT activation in response to a variety of platelet agonists [30]. Therefore, we hypothesised that in PDAC cells, the inhibition of P2Y12 may reduce AKT activation in response to EGF. As shown in Figure 3A and Figure S1D, P2Y12 inhibitors ticagrelor (5 μM) and PSB-0739 (20 μM) reduced AKT and ERK activation in response to EGF (10 ng/mL, 30 min), while apyrase (5 U/mL) failed to show a significant effect. Ticagrelor and apyrase did not show a consistent inhibition of EGF-mediated EGFR phosphorylation. To further investigate P2Y12-EGFR crosstalk, we examined whether inhibition of EGFR may reduce ADP-mediated AKT activation. Erlotinib, an EGFR inhibitor, at 5 μM markedly reduced ADP-induced EGFR and AKT phosphorylation in AsPC-1 and BxPC-3 (Figure 3B). Since AKT is downstream of both EGFR and P2Y12, we tested whether ticagrelor can potentiate the anticancer activity of erlotinib. Figure 3C shows that the addition of ticagrelor increased the inhibitory effect of erlotinib. The synergism was evaluated using the CI method [22] (Table 1A,B). The CI values are classified 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 [32]. At erlotinib concentrations of 0.001–1 μM, there was synergism, with CI values of less than 0.7 in AsPC-1 cells. In BxPC-3 cells, the CI values showed a slight synergism to nearly additive (CI 0.8–1.1) at 0.001–10 μM of erlotinib (Figure 3C). Immunoblot results showed that ticagrelor potentiated erlotinib-mediated AKT inhibition in AsPC-1 and BxPC-3 cells grown in 1% FBS (Figure 3D).

Table 1. Combination index (CI): synergism (CI < 1), additive effect (CI = 1) and antagonism (CI > 1).

A. AsPC-1		**B. BxPC-3**	
Erlotinib (μM)	**CI**	**Erlotinib (μM)**	**CI**
0.001	0.43	0.001	0.88
0.01	0.42	0.01	0.84
0.1	0.5	0.1	0.91
1	0.62	1	0.8
10	1.4	10	1.1

3.4. Ticagrelor Suppresses PDAC Cell Growth In Vitro

We next investigated the effect of ticagrelor on the proliferation of PDAC and normal pancreatic cells (Figure 4A, Table 2). Across PDAC cell lines, the IC_{50}'s were less than 10 μM, while ticagrelor up to 20 μM did not show any cytotoxicity effect on the normal cells, hTERT-HPNE. To further validate the cancer 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 (Figure S2C). This was a promising result as previous studies have established that ticagrelor plasma concentrations up to 10 μM are clinically tolerated [33]. PSB-0739, another P2Y12 inhibitor, also showed growth inhibitory effects (Figure S2D). As AKT is downstream of P2Y12 and is a known inhibitor of apoptosis [34], we investigated the effect of ticagrelor on apoptosis and AKT activity. Ticagrelor was found to induce a dose-dependent increase of caspase 3/7 (Figure 4B,C). Additionally, in non-serum-starved PDAC cells, ticagrelor caused a dose-dependent reduction in the expression level of phosphorylated AKT (Figure 4D,E).

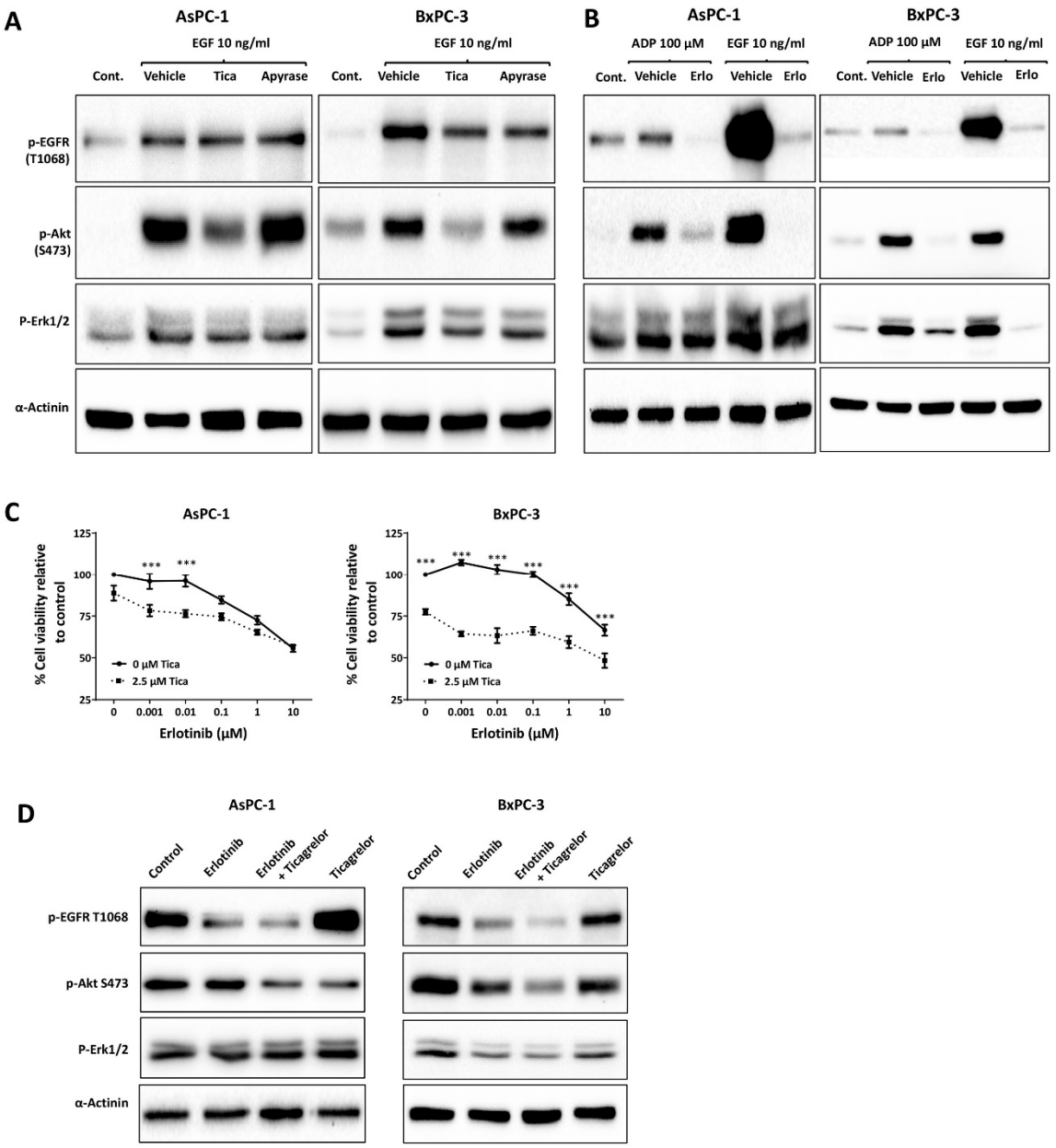


Figure 3. Ticagrelor attenuated EGF-stimulated AKT activation and potentiated the anticancer activity of erlotinib. (**A**) Immunoblots showing the expression of p-EGFR Y1068, p-AKT S473 and p-ERK1/2 in serum-starved AsPC-1 and BxPC-3 cells treated with EGF (10 ng/mL) combined with ticagrelor (5 μM) or apyrase (5 U/mL) for 1 h. (**B**) Immunoblots showing the expression of p-EGFR Y1068, p-AKT S473 and p-ERK1/2 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 (GraphPad PRISM 8.0) was used to examine the significance of the mean; $n = 5$, *** $p < 0.001$. Table 1A,B show the combination index (CI) values calculated using Chou–Talalay's method and can be interpreted as follow: CI < 0.9, synergism, CI > 1.1, antagonism, CI = 0.9–1.1, additive. (**D**) Immunoblots show the expression of p-EGFR Y1068, p-AKT S473 and p-ERK1/2 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 three independent experiments.

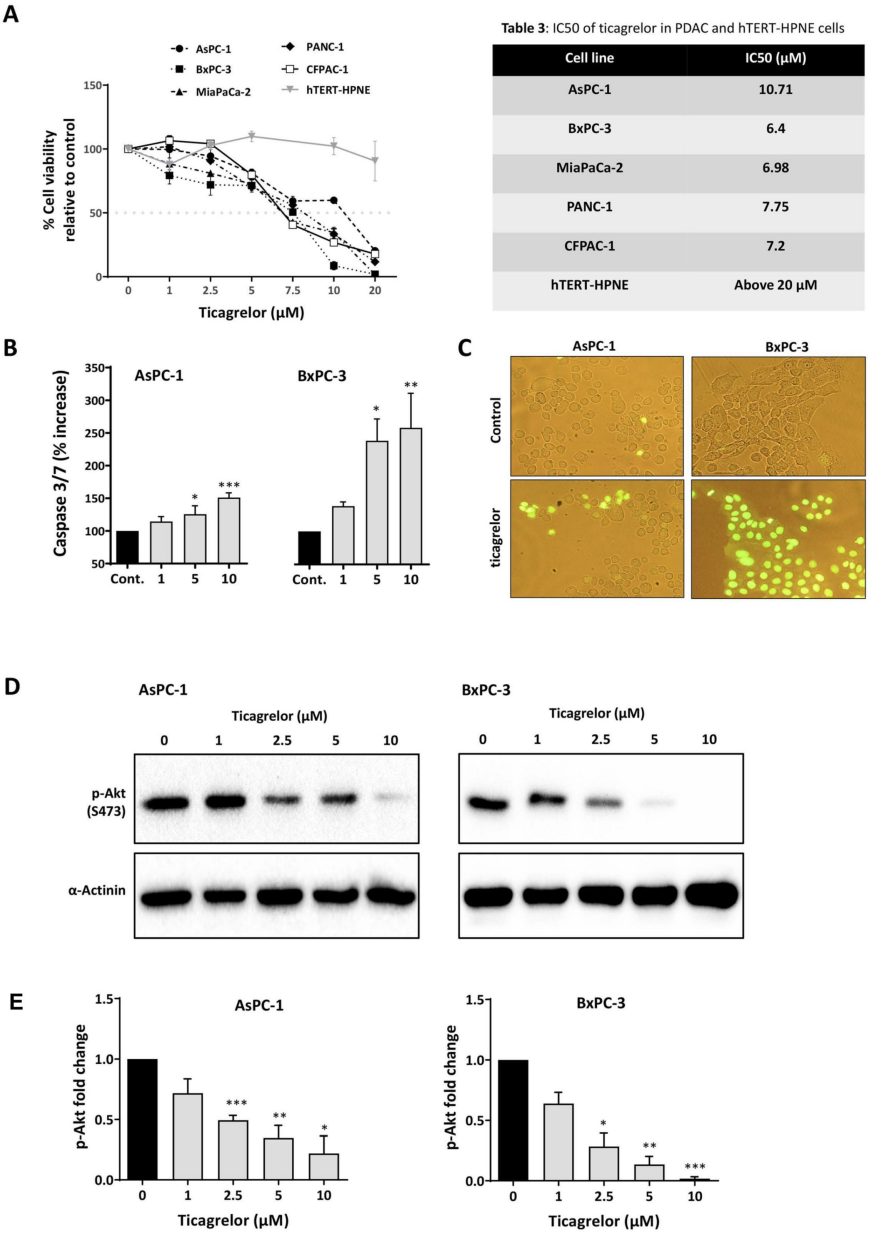


Table 3: IC50 of ticagrelor in PDAC and hTERT-HPNE cells

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

Figure 4. Ticagrelor treatment reduced PDAC cell viability and enhanced 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 ($n \geq 3$). Table 2 shows the IC_{50} (μM) of ticagrelor calculated using GraphPad PRISM 8 and log (inhibitor concentration) versus the normalised response (variable slope). (**B**) AsPC-1 and BxPC-3 cells were treated with ticagrelor (0–10 μM) for 12 h and analysed for apoptosis (caspase 3/7 activation). The columns represent the fold change in the level of activated caspase 3/7 relative to the control vehicle-treated cells, measured as described in the Methods and Materials section. Data are presented as mean ± SEM. One-way ANOVA with post-hoc Dunnett's multiple comparison test was used to examine the significance of the mean; $n \geq 4$, *** $p < 0.001$, ** $p < 0.002$, * $p < 0.033$. (**C**) Detection of caspase 3 activity in live cells treated with vehicle or ticagrelor 10 μM for 12 h. The caspase 3 substrate, once cleaved by caspase 3, formed a DNA dye, which stained the nucleus bright green. (**D**) Immunoblots showing 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 the control vehicle-treated cells ($n \geq 3$). Data are presented as mean ± SEM One-way ANOVA with post-hoc Dunnett's multiple comparison test was used to examine the significance of the mean. *** $p < 0.001$, ** $p < 0.002$, * $p < 0.033$.

Table 2. IC_{50} of ticagrelor in PDAC and hTERT-HPNE cells.

Cell Line	IC_{50} (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

3.5. Ticagrelor Synergised with Chemotherapy in PDAC Cells In Vitro

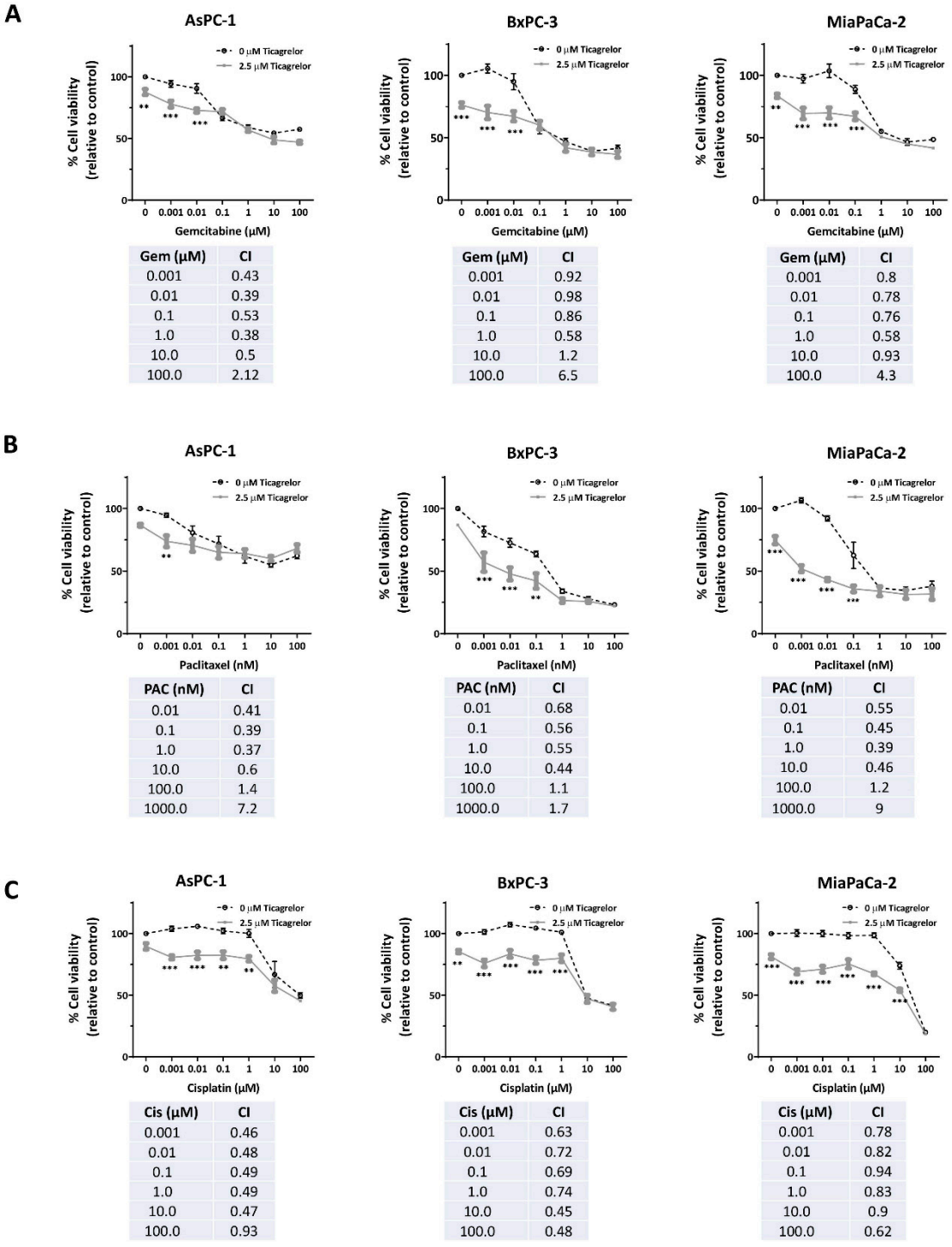
We next investigated, in vitro, the antitumour potential of ticagrelor in combination with PDAC chemotherapeutic drugs. Ticagrelor at a clinically relevant concentration (2.5 μM) [35,36] was combined with different concentrations of gemcitabine, paclitaxel and cisplatin. Dose-dependent growth inhibition was observed in AsPC-1, BxPC-3 and MiaPaCa-2 after 72 h of exposure to the chemotherapeutic agents (Figure 5A–C). AsPC-1 cells displayed the least sensitivity to the combined treatment (Figure 5A–C). The addition of ticagrelor improved the efficacies of the drugs in all tested PDAC cells. The combination therapies were largely synergistic, especially at low chemodrug concentrations (Figure 5A–C).

3.6. 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 [5]. Therefore, we tested the effect of gemcitabine in combination with ticagrelor on tumour growth, where in the xenograft model, BxPC-3 were transplanted into immune-deficient mice (NOD/SCID). After the tumour became palpable (3 weeks), mice were randomly distributed into four groups (vehicle control, gemcitabine, ticagrelor, gemcitabine plus 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 [37]. Therefore, ticagrelor was administered twice a day every 12 h. As shown in Figure 6A and Figure S3A, only the combination therapy consistently and significantly reduced tumour growth. The mean tumour volume from the group was also significantly reduced compared to the gemcitabine treatment alone. Ticagrelor and gemcitabine as single agents had minimal effects on tumour growth.

Platelets are the main source of transforming growth factor-beta 1 (TGF-β1) in the circulation, which plays a crucial role in cancer metastasis [38,39]. Therefore, we examined the level of TGF-β1 in mice with no tumours and in mice with BxPC-3 xenografts treated with a vehicle, gemcitabine, ticagrelor or in combination. As shown in Figure 6B, ticagrelor alone or in combination with gemcitabine significantly reduced the plasma level of TGF-β1.

We previously showed that platelets promote CDD expression in pancreatic cancer cells [9]. CDD is known to deactivate gemcitabine and increase drug resistance. In treating pancreatic cancer patients, gemcitabine is given together with nab-paclitaxel as the latter has been shown to reduce CDD expression in tumour tissues [40,41]. In our study, as shown in Figure 6C, we found the addition of ticagrelor noticeably decreased the expression level of CDD in the tumour tissue from mice treated with ticagrelor or ticagrelor in combination with gemcitabine.



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

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

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

Figure 5. Ticagrelor synergised 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; $n \geq 3$, *** $p < 0.0001$, ** $p < 0.001$. The tables below the graphs show the CI values calculated using Chou–Talalay's method and can be interpreted as follow: CI < 0.9, synergism, CI > 1.1, antagonism, CI = 0.9–1.1, additive.

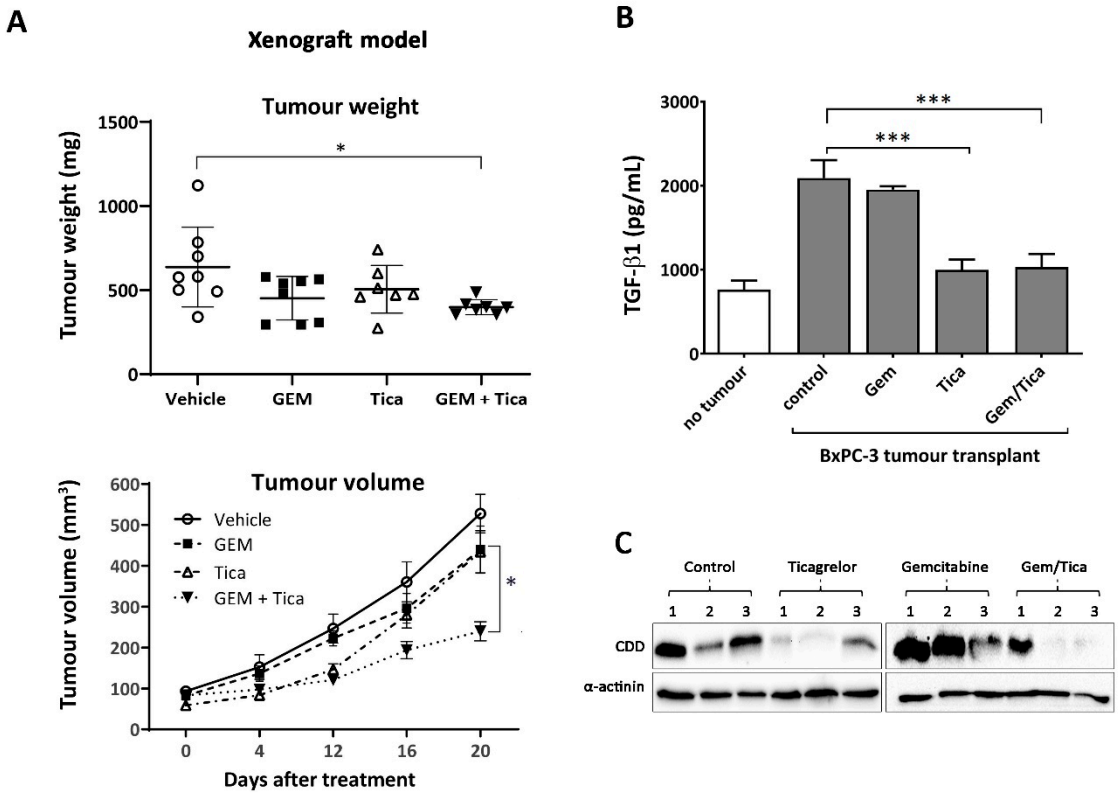


Figure 6. Combined treatment of ticagrelor (Tica) and gemcitabine (GEM) reduced tumour growth in vivo. (**A**) Effect of different treatments on tumour weight and volume in each NOD/SCID mouse bearing BxPC-3 tumours. The significance of the difference between tumour weight and volume in mice bearing BxPC-3 tumours treated with a vehicle control or gemcitabine, ticagrelor or gemcitabine plus ticagrelor was tested using ordinary one-way ANOVA (weight) or two-way ANOVA (volume) with post-hoc Dunnett's multiple comparison test; $n = 8$ for vehicle, $n = 8$ for gemcitabine, $n = 7$ for ticagrelor, $n = 7$ for gemcitabine plus ticagrelor. The significance of the difference between the tumour volumes was calculated using the two-way ANOVA Dunnett's multiple comparison test to examine the significance of the mean. *** $p < 0.001$, * $p < 0.033$. (**B**) The level of plasma TGF-β1 in NOD/SCID mice without tumours, or age-matched NOD/SCID mice with BxPC-3 tumours treated with vehicle, gemcitabine, ticagrelor or ticagrelor plus gemcitabine ($n = 4$). The significance of the difference between the TGF-β1 level was calculated using the two-way ANOVA Dunnett's multiple comparison test to examine the significance of the mean. *** $p < 0.001$, * $p < 0.033$. (**C**) CDD expression in BxPC-3 tumour tissues extracted from mice treated with a vehicle control, ticagrelor, gemcitabine or ticagrelor plus gemcitabine ($n = 3$).

Additionally, we examined the effect of the combined therapy on the growth of pancreatic cancer cells in immunocompetent mice. As shown in Figure 7 and Figure S3B, this treatment combination significantly reduced tumour growth, indicating that ticagrelor's effect on tumour growth was not affected by a fully developed immune system. Importantly, the addition of ticagrelor to gemcitabine did not produce any noted adverse effects as the final weights and the haematological parameters were similar between the gemcitabine and combined therapy groups [42].

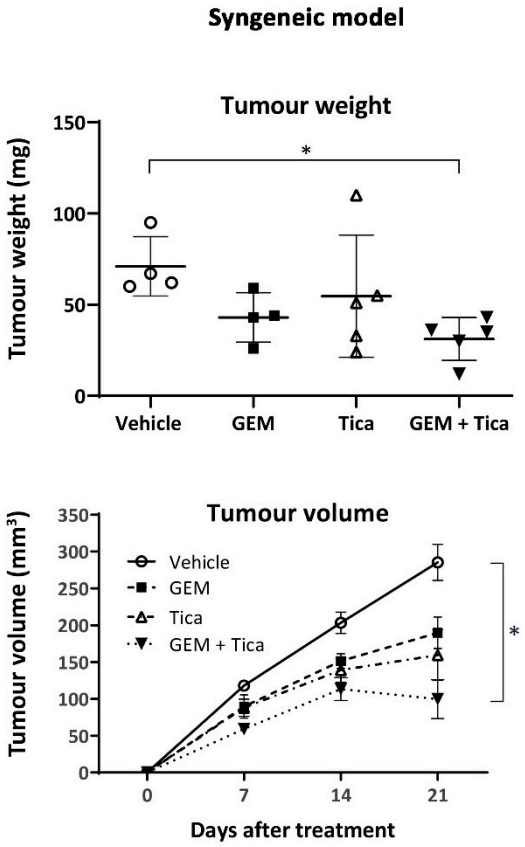


Figure 7. Ticagrelor potentiated gemcitabine activity in the syngeneic tumour mouse model. Effect of different treatments on tumour weight and volume in a syngeneic pancreatic cancer mouse model (C57BL6/J mice transplanted with MT4-2D). The significance of the difference between the tumour weight and volume in a syngeneic pancreatic cancer mouse model treated with vehicle control or gemcitabine, ticagrelor or gemcitabine plus ticagrelor was tested using ordinary one-way ANOVA (weight) or two-way ANOVA (volume) with post-hoc Dunnett's multiple comparison test; $n = 4$ for vehicle, $n = 4$ for gemcitabine, $n = 5$ for ticagrelor, $n = 5$ for gemcitabine plus ticagrelor. The significance of the difference between the tumour volumes was calculated using two-way ANOVA Dunnett's multiple comparison test to examine the significance of the mean. * $p < 0.033$.

4. Discussion

Targeting ATP receptors in cancer cells or the ADP receptor P2Y12 in platelets have attracted overwhelming interest from cancer researchers recently [13,16]. However, the role of ADP and P2Y12 in cancer cells remains poorly investigated. Through functional and molecular studies, we demonstrated that the P2Y12 receptor was expressed in PDAC cells and was required for cancer cell proliferation. Targeting the P2Y12 receptor with ticagrelor repressed cancer cell growth and its presence synergised with several chemotherapeutic agents in vitro. The combination of ticagrelor and gemcitabine significantly reduced tumour growth in both the xenograft and syngeneic tumour mouse models tested.

P2Y12 belongs to a family of purinergic (P2) G-protein coupled receptors GPCRs [30]. We confirmed the expression of the P2Y12 protein in a group of pancreatic cancer cell lines. Using the anti P2Y12 monoclonal antibody EPR18611 (Abcam) against a specific peptide sequence of P2Y12 that does not share significant homology with P2Y1 or P2Y13, we were able to detect P2Y12 in cancer cells at the same molecular weight as in platelets (Figure 1A). The antibody performance was validated using a knockdown strategy where four different sequences of siRNA were used to suppress the expression of P2Y12 compared to siNeg-treated cancer cells (Figure S2A). The expression of P2Y12 was also confirmed using two different anti-P2Y12 antibodies (Figure S1). The gene expression was also validated using qPCR (Figure S5).

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 through EGFR transactivation [19,20]. Here, our data clearly showed that extracellular ADP induced EGFR activation in PDAC cells. P2Y12 contributed to EGFR activation, potentially via several mechanisms. First, P2Y12 may induce EGFR transactivation through Src and matrix metalloproteases (MMP) axis as demonstrated with P2Y1 and P2Y2 [18,20]. Second, P2Y12 could regulate EGFR activation by promoting EGFR association with another member of the EGFR family, the human epidermal growth factor receptor 3 (HER3). Thus, inhibition of the purinergic receptor could reduce HER3 activation, and subsequently EGFR activation [19,43]. Third, P2Y12 could induce EGFR activation via Src, then the transactivated EGFR forms a multireceptor complex with P2Y12, leading to an increase in the downstream oncogenic signaling [44]. 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 essential in platelet activation [30]. Our results demonstrated a similarity between P2Y12 signalling in platelets and PDAC cells since only the inhibition of P2Y12, but not P2Y1, reduced ADP-mediated AKT phosphorylation. Previous studies have indicated that P2 signalling promotes cancer invasion and chemotherapy resistance by supporting EMT in cancer cells [45]. Signalling through ATP, for example, can promote the upregulation of the mesenchymal transcriptional factor SNAIL1 in prostate cancer cells [46], whereas we previously demonstrated that ADP signalling promotes SLUG upregulation in PDAC cells [9]. SNAIL1 and SLUG (SNAIL2) belong to a family of mesenchymal transcriptional factors that 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) [5]. Here, our results showed for the first time that P2Y12 mediated the expression of the EMT-related factors SLUG and ZEB1. Interestingly, analysis of RNA sequencing expression data from tumours and normal samples from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) projects using the web-based tool GEPIA revealed a positive correlation between the expression of P2Y12 and ZEB1 (Pearson correlation coefficient = 0.76, $p < 0.05$) in PDAC patients (Figure S4B,C). As recent data indicate that ZEB1 is a critical player in pancreatic cancer metastasis [47], substantially more investigations are needed to elaborate on the role of the P2Y12–ZEB1 axis in cancer invasion and metastasis.

Ticagrelor can attenuate AKT activation in platelets in response to a variety of platelet stimulants [48]. Here, our data suggested a novel effect for ticagrelor in PDAC cells through the reduction of the activity of EGF downstream effectors AKT and ERK. Inhibition of the P2Y1 also reduced EGF-induced AKT and ERK activation (Figure S1E), indicating that both P2Y1 and P2Y12 contributed 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 have been due to the off-target effects of ticagrelor on ENT1. Ticagrelor is known to increase extracellular adenosine through the inhibition of ENT1-mediated adenosine cellular uptake [49]. Extracellular adenosine can also induce EGFR activation [50], thus the effect of ticagrelor on EGFR activation may be disrupted by the presence of adenosine.

The effects of ticagrelor on PDAC cell proliferation and apoptosis observed here are in accordance with a recent study where it was shown that P2Y12 protected platelets from apoptosis via the AKT-dependent inactivation of apoptosis regulators Bak and Bax [34]. Furthermore, our results showing minimal impact of ticagrelor on normal pancreatic duct cells were comparable to the previous Food and Drug Administration (FDA) data indicating ticagrelor up to 20 μM had a negligible toxicity on hepatocytes in vitro [33]. Ticagrelor has been reported to have several off-target effects that may be related to tumour growth [33,49,51,52]. Therefore, it is possible that the anticancer activity of ticagrelor is the result of a multitarget effect. Importantly, our results in the mouse models suggest that ticagrelor plus gemcitabine exerted a significant antitumour effect. As antitumour immune responses

can be restrained by tumour-educated platelets [53], the tumour-suppressive impact of ticagrelor in the syngeneic model may be partly ascribed to its inhibitory effect on platelet function.

Ticagrelor use is associated with an increased bleeding risk. However, its short-term activity and recent FDA approval of a reversal agent may make it a more favourable choice than other antiplatelet agents in the context of cancer-associated thrombosis [5]. It is important to mention that several clinical trials failed to show a survival benefit of using anticoagulants for the primary or secondary prevention of venous thrombosis in pancreatic cancer, despite a reduction in the incidence of thrombotic events at the expense of the bleeding risk [6–9]. Therefore, antiplatelet medication, such as ticagrelor, may provide an alternative strategy to mitigate cancer-associated thrombosis, as well as conferring a direct anti-tumour activity, as highlighted by our study.

5. Conclusions

The tumour micro-environment, of which platelets are integral, is involved in cancer metastasis and chemotherapy resistance. Extensive research has reported that extracellular ADP in the tumour micro-environment can stimulate platelets through interaction with the platelet receptor P2Y12. In turn, activated platelets release biological factors supporting cancer progression. Our study revealed that pancreatic cancer cells also expressed a functional P2Y12, which is required for cell proliferation by promoting EGFR-dependent and independent AKT-mediated survival signalling. Subsequently, we demonstrated that blocking P2Y12 with the clinically available antiplatelet drug, ticagrelor, reduced cancer cell proliferation, activated apoptosis, and synergised with several chemotherapeutic agents in vitro. The addition of ticagrelor to gemcitabine significantly reduced tumour growth in both wildtype and immune-compromised xenograft mouse models, indicating that ticagrelor may be a promising option in PDAC therapy, and potentially for other cancers with similar pathologies and underlying mechanisms.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/1/250/s1, Figure S1: Verification of P2Y12 expression using two different anti-P2Y12 antibodies, Figure S2: Verification of P2Y12 knockdown and its effect on the downstream target SLUG using four different siP2Y12 sequences, Figure S3: Image of all extracted tumour masses from mice, Figure S4: Correlation of P2Y12 expression with EGFR, ZEB1 and SLUG, Figure S5: P2Y12 gene expression in pancreatic cancer cell lines, Figure S6: Western blot files with densitometry data.

Author Contributions: O.E. and P.M. designed the research and analysed data. O.E. performed the majority of the experiments. A.D., N.B.A.R., D.E.D. and P.M. performed experiments. M.F. provided vital reagents and a critical review. O.E. drafted and N.B.A.R., A.D., D.E.D. and M.F. contributed to parts of the manuscript. O.E. and P.M. finalised the article. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no potential conflicts of interest

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CDD	Cytidine deaminase
CI	Combination index
Cis	Cisplatin

EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ENT1	Equiliberative nucleoside transporter 1
Gem	Gemcitabine
GEPIA	Gene expression profiling interactive analysis
GPCRs	G-protein coupled receptors
MMP	Matrix metalloproteases
Pac	Paclitaxel
PDAC	Pancreatic ductal adenocarcinoma
Tica	Ticagrelor

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