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Full Length Article

Breast cancer cell-induced platelet activation is compounded by tamoxifen and anastrozole *in vitro*

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ABSTRACT

Platelet-tumour cell interaction is implicated in the initiation of breast cancer-associated thrombosis, with hormone-therapy (Tamoxifen/Anastrozole), increasing this risk. However, recent *in vitro* research indicates that Tamoxifen inhibits platelet activation, while the effects of Anastrozole on platelet activation are not well characterised. This study investigated platelet activation caused by Tamoxifen or Anastrozole-treated breast cancer cells *in vitro*.

MCF7 and T47D cells were pre-treated with Tamoxifen or Anastrozole to mimic the effects of the drugs *in vivo*, and co-cultured with whole blood. Platelet activation was determined using flow cytometry. Platelet ($CD41a^+CD62P^+$) was determined using an interval gating strategy. Platelet morphology was visualised using scanning electron microscopy.

Our results support clinical findings, showing that hormone-therapy is associated with platelet activation. Tamoxifen-treated MCF7 cells increased P-selectin expression, with ultrastructural analysis showing fully spread platelets. Conversely, Tamoxifen-treated T47D cells decreased P-selectin expression with platelets showing signs of early aggregation. Anastrozole pre-treatment decreased P-selectin expression, with treated MCF7 cells inducing platelet membrane folds and lamellipodia extension, and treated T47D cells inducing platelet aggregation and fibrin network formation indicating hypercoagulation.

The findings support clinical studies. Hormone-therapy augments tumour cell-induced platelet activation, which may be linked to cell phenotype. This may have clinical implications for treatment strategies.

1. Introduction

Breast cancer is one of the most commonly diagnosed cancers in women worldwide, with an increasing incidence in third-world countries [1]. Increasing evidence points towards an association between tumour progression and platelet function [2,3]. Within the tumour microenvironment, leaky blood vessels allow for engagement between platelets and tumour cells, with platelets postulated to facilitate tumour cell intravasation [4]. In the circulatory system tumour cell-induced platelet aggregation confers protection against immunosurveillance [5,6], and high velocity shear forces [5]. Additionally, the release of platelet-stored growth factors, allows for extravasation and colonization of secondary sites [5]. Tumour cells facilitate this dynamic cross-talk with platelets by activating procoagulation mechanisms *via* the secretion of tumour-associated tissue factor, thrombin or adenosine diphosphate (ADP) [7,8]. Upon activation, platelets undergo rapid changes in morphology [9,10]. Early platelet activation includes retraction of granular contents into the centre of the platelet with the widening of the open canalicular system (OCS), forming pseudopodia extending into lamellipodia and filipodia [9,11]. This is followed by further extension and final release of granular contents from the OCS facilitating further adhesion and aggregation, prior to the final or spread phase [9,10]. α-Granular contents include membrane proteins (*e.g.* GPIb complex, GPIIb/IIIa, P-selectin), coagulation factors (*e.g.* Factors V, XI; fibrinogen, von Willebrand factor), growth factors and chemokines. These components are also stored within microparticles [12–14] and released during the activation process. These constituents mediate a range of functions [15,16] which ultimately facilitate tumour progression while contributing to hypercoagulation [13].

A hypercoagulable state is characteristic of cancer patients, shown

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by thrombocytosis, heightened expression of platelet activation markers and platelet-derived microparticles [6,17]. Breast cancer itself is a risk factor for cancer-associated thrombosis [18,19,20,21]. Treatment strategies for hormone-responsive breast cancer have improved mortality rates; however, they are associated with increased risk for thromboembolic events [19,20,22]. Tamoxifen, a selective oestrogen receptor modulator (SERM) and the predominant treatment prescribed for oestrogen-receptor positive breast tumours in pre- and post-menopausal women [22], increases survival and reduces the risk of recurrence of invasive and non-invasive breast cancer when used for a minimum of 5 years [23,24] but is associated with significant risk of thromboembolic complications [20,22,25–27]. Anastrozole, a third generation non-steroidal aromatase inhibitor (AI) that reduces oestradiol bioavailability is clinically more effective in reducing thrombotic complications in postmenopausal women [27]; however, risk remains elevated compared to that of tumour-free individuals, with combination AI and chemotherapy use heightening the risk of venous thromboembolism (VTE) [20].

Recent studies highlight that platelets may play a greater part in venous thrombosis than previously described [28,29]. Clinically, elevated platelet count, platelet hyperaggregability, and heightened levels of $CD62P^+$ (P-selectin) microparticles are suggested as risk factors for VTE in cancer patients [17,28]. *In vitro* studies have confirmed the ability of mammary carcinoma cell lines to induce platelet activation demonstrated by P-selectin expression (exposure) [27], ultrastructural alterations [30], and aggregation *via* GPIb-IX and GPIIb/IIIa activation [31,32]; and platelet-dependent induction of metastasis [2,27]. Tamoxifen has been extensively investigated; however, contradictory laboratory results have been produced, some of which do not reflect the prothrombotic clinical situation [20,22,25–27]. Tamoxifen-induced platelet activation is linked to its ability to facilitate Ca^{2+} entry [33–35]. Nevertheless, recent studies show that despite this Tamoxifen inhibits washed platelet aggregation, particularly under collagen stimulation [26]. At high concentrations Tamoxifen pre-treatment of platelets decreases MCF7 cell metastatic potential and inhibits induced platelet activation as identified by P-selectin expression (exposure) [27]. These discordant results may reflect differences in hormone-therapy dosage and experimental method in platelet isolation, with the loss of plasma in the preparation of washed platelets linked to studies refuting Tamoxifen-induced platelet activation and aggregation [13].

To clarify contradictory results regarding Tamoxifen-induced platelet activation; and determine the effect of Anastrozole-induced platelet activation, we present the effects of physiological doses of Anastrozole and Tamoxifen on luminal phenotype MCF7- and T47D-induction of platelet activation. Pre-treatment of breast cancer cells allows for mimicking the cumulative effect of the drugs, as would occur *in vivo*, followed by exposure to whole blood. While aggregometry remains the gold standard for investigating platelet aggregation in disease states, we describe an early stage in the coagulation process that may impact tumour processes, by assessing platelet activation. Briefly, as previously described by our laboratory, we use an index that describes P-selectin expression in relation to the number of platelets expressing the marker [10,36]. However, as noted previously, the lack of P-selectin expression could erroneously be interpreted as inactivation, whereas it could reflect progression to late stage activation [10]. Thus, we couple our analysis with ultrastructural assessment of platelet morphology to better understand the phenomenon of platelet activation in light of hormone-therapy.

2. Methods

2.1. Cell culture

MCF7 and T47D breast cancer cells, obtained from ATCC (Virginia, USA) were cultured in 75 cm^2 Nunc culture flasks to passage numbers of 36 and 29 respectively. MCF7 cells were propagated in DMEM (Dulbecco's Modified Eagles Medium, Lonza, Walkersville, MD, USA), with 10% FBS (Foetal Bovine Serum, Gibco, Life Technologies, CA, USA), 0.1% P/S (Penicillin/Streptomycin, Sigma-Aldrich, St. Louis, MO, USA). T47D cells were propagated in RPMI (Roswell Park Memorial Institute medium) with 0.2 units/ml bovine insulin, 10% FBS and 0.1% P/S. Cells were cryopreserved in 10% DMSO (Dimethyl sulfoxide, Saarchem, Johannesburg, South Africa), with 60% FBS and 30% DMEM at −80 °C for subsequent experimentation.

2.2. Cell seeding

Cryopreserved MCF7 and T47D cells were thawed, washed once in 1 ml Phosphate Buffered Saline (PBS) and resuspended in 1 ml respective media. The trypan blue viability assay coupled with a TC20 Automated Cell Counter (Bio-Rad, Hercules, CA USA) was used to determine cell viability. Cells were seeded in duplicate at a concentration of 1×10^5 cells/well into 24-well plates (Nunc, Roskilde, Denmark) and incubated in 200 µl respective media for 24 h at 37 °C and 5% CO_2 for cell adherence and formation of a monolayer.

2.3. Hormone-therapy treatment

The maximum effective concentrations of Anastrozole (Sigma Aldrich, Saint Louis, USA; A273) and Tamoxifen (Sigma Aldrich; T5648), were determined using the LDH cytotoxicity assay (Thermofisher Scientific, Waltham, USA) and Neutral Red cell viability assay [37]. Tamoxifen and Anastrozole were tested at ranges from 0.5 to 2.5 µM and 1–10 µM, respectively. This correlates with other cellular studies conducted in which concentrations mimicked that of treatment regimens given to women with breast cancer [38–40]. Based on ensuing results and corresponding with other studies, the maximum effective concentration of Tamoxifen was determined to be 2 µM and Anastrozole, 1 µM [38–40].

Cells were thus rinsed with PBS and incubated in 200 µl 1 µM Anastrozole or 200 µl 2 µM Tamoxifen in respective media for 24 h at 37 °C. Controls included normal media and the diluent (0.1% DMSO).

2.4. Blood preparation and co-culture

Healthy female volunteers between 19 and 30 years old, and between days 1–10 of their menstrual cycle (low levels of circulating oestrogen and progesterone, lessening hormonal effects on breast cancer cells) participated in this study. Exclusion criteria included: pregnancy; contraceptive use; autoimmune diseases or immunodeficiency; history of cancer; cancer; smoking; and consumption of anti-platelet and anti-coagulation medication (*e.g.* aspirin and warfarin) in the previous 72 h. Ethics was approved by University of the Witwatersrand (#M160826).

Peripheral whole blood ($n = 5$) was collected in two 3.2% sodium citrate vacuette coagulation tubes. The first 2 ml of blood drawn was discarded to exclude mechanically activated platelets. Following drug treatment, breast cancer cells were rinsed twice with PBS and incubated with 200 µl whole blood (WB) for 2.5 min.

WB samples underwent erythrocyte lysis with 3 ml ammonium chloride (NH_4Cl) buffer for 10 min at room temperature for subsequent analysis [10]. For the positive control WB was incubated with 0.1 U/ml human thrombin-α (SANBS, South Africa), for 5 min to induce platelet activation; the negative control included WB not exposed to breast cancer cells.

2.5. Scanning electron microscopy (SEM)

Twenty microliters of sample was placed on glass coverslips (10 mm round, Lasec, Johannesburg, South Africa) in 24-well plates and was incubated at 37 °C, 5% CO_2 for 5 min to facilitate adhesion to the coverslip [10]. Samples were washed in 0.1 M PBS on a microplate

shaker for 20 min, fixed in 2.5% formaldehyde/glutaraldehyde for 15 min, rinsed thrice with 0.1 M PBS, secondarily fixed in 1% osmium tetroxide and rinsed in PBS. Cells were dehydrated through a series of ethanol (30%, 50%, 70%, 90%, and three times absolute ethanol), and dried using hexamethyldisilazane. Samples were mounted onto aluminium stubs, coated by carbon evaporation and examined using a FEI Nova 600 Scanning Electron Microscope (acceleration voltage 30 kV) at the Microscopy and Microanalysis Unit (University of the Witwatersrand) for qualitative assessment.

2.6. Flow cytometry

Samples were centrifuged at 200 ×*g* for 5 min and the resulting pellet resuspended in 150 µl Tyrode's buffer for double-labelling with APC-conjugated mouse anti-human CD41a (BD Pharmingen, 559777) and FITC-conjugated mouse anti-human CD62P (BD Pharmingen, 555523) at 1:20, for each antibody, as determined *via* titration. Samples were fixed in 1% paraformaldehyde at 1:1 for 10 min, washed with 1 ml Tyrode's buffer, and centrifuged at 200 ×*g* for 5 min. The resulting pellet was resuspended in 500 µl Tyrode's buffer, kept at 4 °C on ice overnight prior to data acquisition on the LSR Fortessa (BD Biosciences) at the Department of Surgery, University of the Witwatersrand. Compensation and technical controls included an unlabelled sample and single antibody labelled samples to determine auto-fluorescence and fluorescence overlap.

Data was acquired as 100 000 events per sample using FACSDiva Software (BD Biosciences). The platelet population initially gated on size and granularity (forward scatter voltage 300 V, side scatter voltage 275 V), was further gated based on expression of CD41a platelet marker (APC, 565 V), denoting the parent population. Interval gates were drawn to classify a graded level of platelet activation determined using CD62P (FITC 469 V) geometric Mean Fluorescence Intensity (gMFI) of the platelet population (Fig. 1).

2.7. Statistical analysis

Data was exported from FACSDiva software and managed in Microsoft Excel. The index of platelet activation (IPA) was calculated such that: IPA = gMFI(CD62P) X n($CD41a^+CD62P^+$ events) for each interval, and combined intervals (Fig. 1) [10,36]. IPA allows for description of P-selectin expression relative to the number of platelets expressing the marker. Statistical analyses were performed using PAST 3.04 [41]. A Shapiro-Wilk test of normality determined that data were not normally distributed thus the Kruskal-Wallis test was conducted followed by a *post-hoc* Dunn's test with significance at $p < 0.05$.

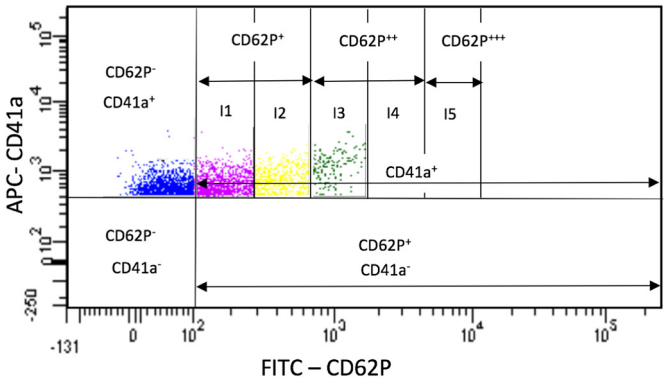

Fig. 1. Representative scatter plot showing the spread of platelet activation across the interval gates. Quadrant 1 is the inactive platelet population ($CD41a^+CD62P^-$) population, while the activated population of interest ($CD41a^+CD62P^+$) was within the 2nd quadrant. Interval gates (I1-I5) were drawn within the quadrant, indicative of increasing CD62P expression.

3. Results

The interval gating strategy employed allows mapping of the spread of P-selectin, in addition to ascertaining total P-selectin expression (exposure) with a right shift along the X-axis reflecting higher levels of P-selectin expression (Fig. 1). Interval gates beyond I5 showed no P-selectin expression for all samples and were disregarded in the IPA calculation. Interval 1 and 2 (I1 and I2) represent the bulk of $CD41a^+$ platelets with low P-selectin expression ($CD41a^+CD62P^+$), interval gates 3 and 4 (I3 and I4) represent platelets with moderate P-selectin expression ($CD4a1^+CD62P^{++}$) and interval 5 (I5) presents the smallest proportion of platelets with high P-selectin expression ($CD41a^+CD62P^{+++}$) (Fig. 1).

Baseline level of activation showing levels of IPA was determined with the negative control, whole blood unexposed to cells (Fig. 2). This was corroborated by platelet ultrastructural assessment showing that the majority were round, with a smooth membrane and multiple surface folds (Fig. 3A). This contrasted as expected, with the positive control that ensured firstly, that platelets were responsive to activation *via* agonist action and had not been rendered fully active by the platelet preparation protocol [10] and secondly, as a technical flow cytometry control. Thrombin induced a greater spread of P-selectin expression and an increase ($p > 0.05$) in overall IPA (I1–I5) (Fig. 2). This was reflected ultrastructurally (Fig. 3B), where platelets showed greater membrane folds, with filipodia extending from the platelet body.

Breast cancer cells (diluent and matched media controls) induced significantly higher overall IPA levels compared to untreated whole blood, comparable with the positive control. While not significantly greater to the IPA induced by cells treated with normal media, the diluent elicited a slightly higher IPA and a greater spread of data (Fig. 2A, B). Ultrastructurally, platelets in the diluent control for both cell lines (Fig. 4A, 5A), displayed a relatively smooth but folded platelet membrane with lamellipodia extending and attaching to the substratum. This was more apparent in media controls, which also showed microvesicle presence (Fig. 4B, 5B). Microvesicles were characterised as small rounded particles of varying sizes with a clear border, adhered to the coverslip. However, some debris was interspersed throughout the sample making the distinction between the specific particles difficult [42].

Cells treated with hormone-therapy prior to incubation with whole blood induced a greater spread and overall IPA than the negative control (Fig. 2A, B). Compared to matched media and diluent controls, Anastrozole treatment reduced, albeit not significantly, breast cancer cell-induction of platelet activation as defined by median IPA (Fig. 2A, B). Ultrastructurally, Anastrozole-treated MCF7 cells (Fig. 4C), induced a smooth yet folded platelet membrane with extending filipodia. In stark contrast, Anastrozole-treated T47D cells although inducing a similarly reduced IPA (albeit $p > 0.05$) compared to diluent and media controls, altered morphology substantially (Fig. 5C). Platelets had a rougher, folded, membrane with pores associated with the OCS evident. Platelet aggregations were present, in addition to thick fibrin fibres forming dense plaques with fibrin pores present. The reduction in IPA induced by Anastrozole thus reflects a loss of P-selectin expression associated with later stages of coagulation.

Tamoxifen enhanced the ability of MCF7 cells to induce platelet activation, although not significantly so ($p > 0.05$) compared to the media control (Fig. 2A). Platelets were spread with lamellipodia and filipodia extension, and pores of the OCS and microvesicles evident (Fig. 4D). Tamoxifen-treated T47D cells induced a slightly lower level of IPA (Fig. 2B), compared to media and diluent controls. Platelets exhibited a smoother yet folded membrane with lamellipodia and filipodia extending outward from the platelet body towards nearby platelets suggesting early stages of platelet aggregation. Microvesicles were also visible (Fig. 5D).

Overall IPA incorporated platelet activation across intervals I1-I5 (Fig. 2A, B); however, given the morphological results we further

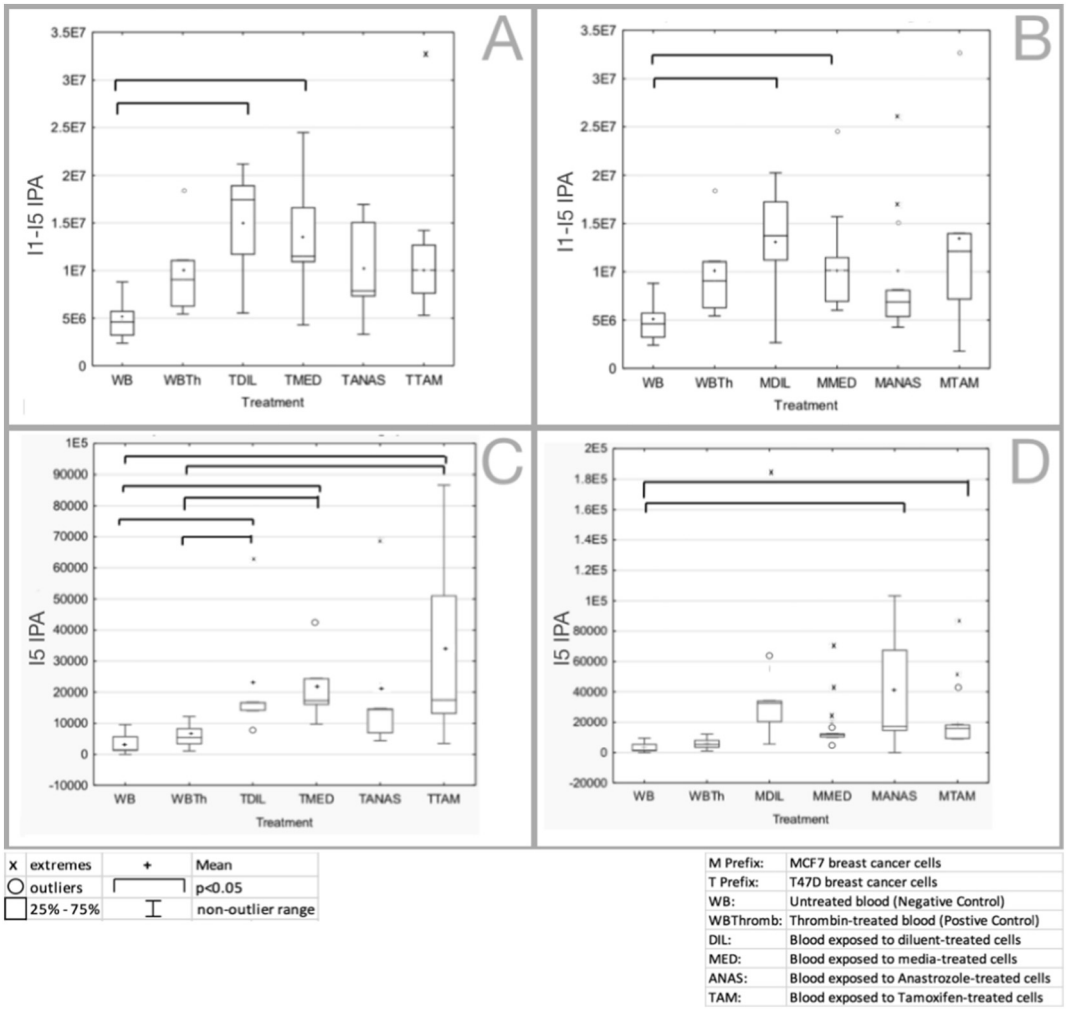

Fig. 2. Box and whisker plots indicating the Index of Platelet Activation (IPA) in lysed whole blood (WB) samples incubated with breast cancer cells and hormone-therapy treatment, as presented in intervals I1-I5. Overall IPA presented in pre-treated MCF7 (A) and T47D (B) breast cancer cell lines. Overall IPA was significantly increased following exposure to diluent and media-treated MCF7 (A) and T47D (B) breast cancer cells, compared to the untreated negative control. Neither Anastrozole nor Tamoxifen significantly altered IPA; however, compared to the diluent and media controls Anastrozole did reduce the median IPA induced by both cell lines. Tamoxifen treatment however, reduced the median IPA induced by T47D cells, while increasing the median IPA induced by MCF7 cells. Further analysis of the IPA within I5 (C and D). This interval represents a small percentage of highly active ($CD41^{+}CD62P^{+++}$) platelets. Hormone-therapy increased IPA; however, this was dependent on cell type, with MCF7 cells (C) showing significantly heightened platelet activation capacity under Anastrozole treatment and T47D cells (D), under Tamoxifen treatment.

investigated IPA within each interval to ascertain how the treatments affected the spread of P-selectin expression. The variance in median IPA observed in the P-selectin high ($CD62P^{+++}$) interval was particularly large (Fig. 2C, D). MCF7 cells induced significantly heightened IPA under Anastrozole treatment (Fig. 2C), and T47D cells, under Tamoxifen treatment (Fig. 2D) compared to the untreated control.

4. Discussion

Cancer is associated with a hypercoagulable state, which is implicated in facilitating the metastatic process [6,17]. Patients presenting with breast cancer have greater risk of developing VTE than healthy women within the same age group [20]. Our *in vitro* experiment attempted to mimic platelet-tumour cell interactions within the tumour microenvironment *via* leaky blood vessels [4], and hormone-therapy accumulation within breast tissue itself [43].

The results obtained indicate that hormone-dependent breast cancer cells induce significant platelet activation comparable to clinical studies [13, 19, 22, 23]. However, we further show that this effect may vary between cell lines, potentially linked to phenotypic differences. T47D breast cancer cells present low oestrogen receptor expression, with exceptionally high progesterone receptor expression and are regarded as more aggressive than MCF7 breast cancer cells [44]. This indicates that variation in hormone-receptor profiles and tumour subphenotypes may elicit a differential thrombotic response, not normally considered in clinical management. Ultrastructural analysis supports these results by showing heightened levels of platelet activation compared to the negative control, platelets expressed greater membrane folds with extension of pseudopodia, which was more visible in samples incubated with the T47D cell line.

Platelet activation is associated with the release of pro-tumorigenic growth factors and chemokines in addition to providing a physical shield for circulating tumour cells [5,15,16]. Activation of platelets is dependent on the synthesis of agonists such as tissue factor, platelet activation factor (PAF) and thrombin facilitating a cascade of events including growth factor and coagulation factor secretion from platelet α-granules [45,46]. The release of granular contents from the OCS facilitates further adhesion and aggregation [45,46]. The heightened level of platelet activation induced by T47D breast cancer cells could be related to the expression of Platelet Activating Factor Receptor (PAF-R),

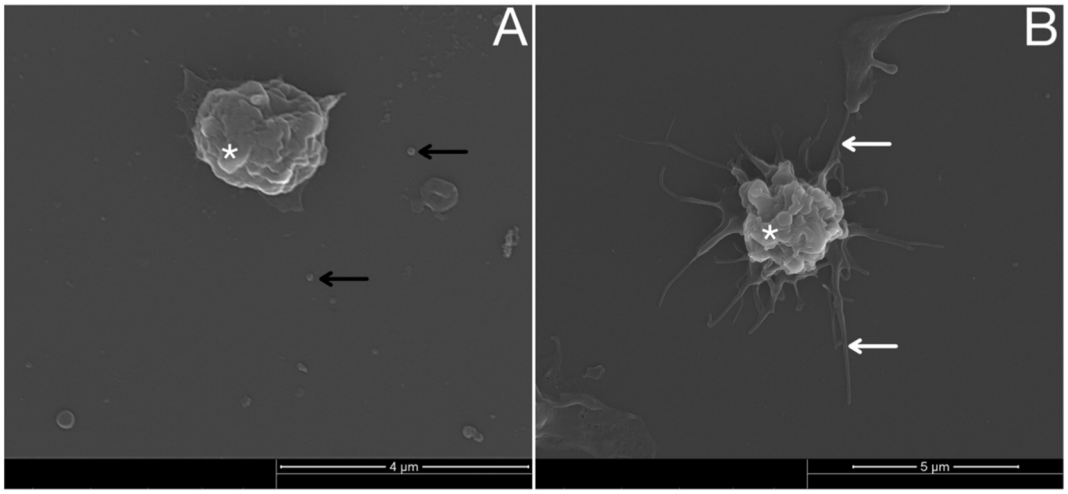

Fig. 3. Scanning electron microscopy images of platelets in lysed whole blood (WB) samples. A: Untreated, negative control, showing an inactive platelet with a smooth membrane and membrane folds (*), as well as the presence of microvesicles (black arrows). B: Platelet exposed to 0.1 U/ml thrombin displaying multiple membrane folds (*), with many filipodia (white arrows) extending outward from the platelet body.

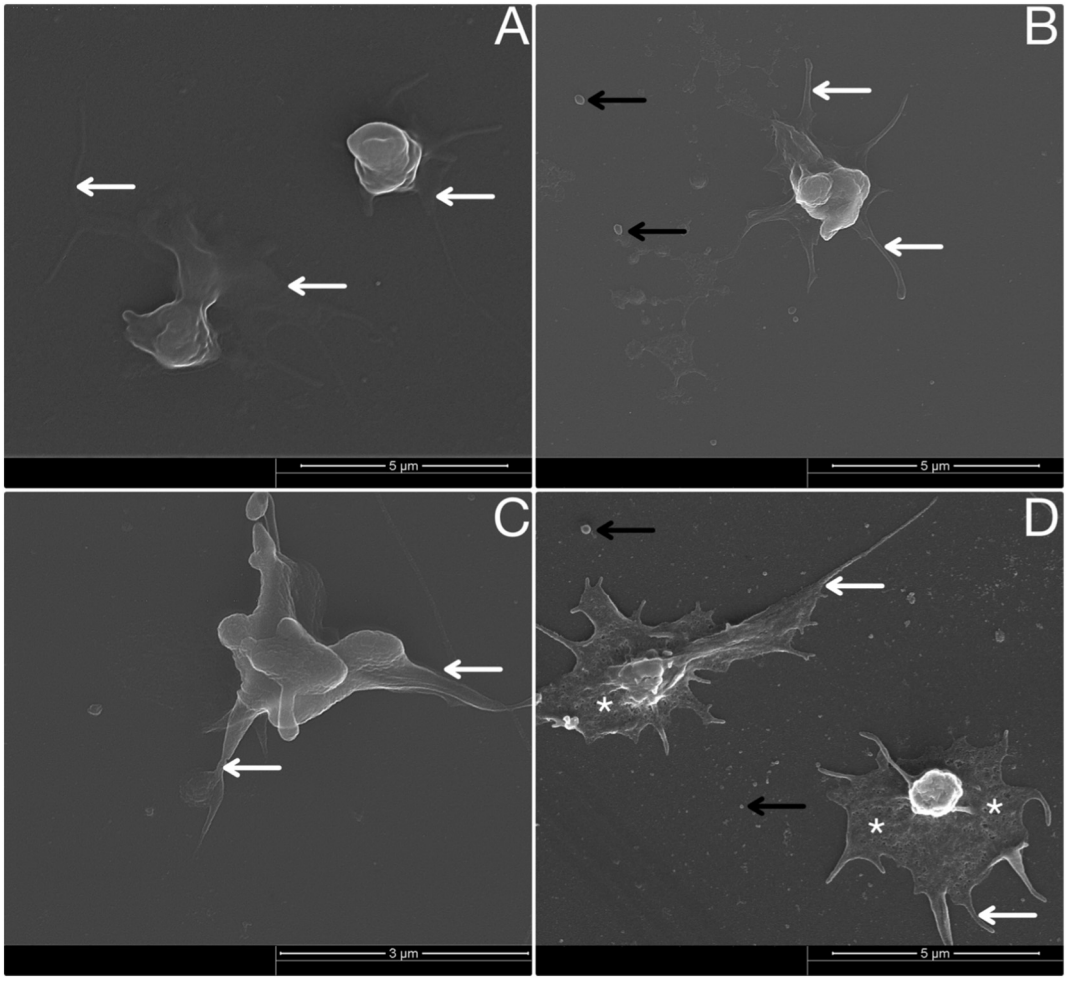

Fig. 4. Scanning electron microscopy images of platelets in lysed whole blood (WB) samples co-incubated with MCF7 cells and corresponding treatments. A: Platelets exposed to diluent-treated MCF7 cells displaying a smooth membrane with membrane folds and extending lamellipodia. B: Platelet exposed to media-treated MCF7 cells displaying membrane folds and multiple filipodia extending outward from the platelet body (white arrows), with the presence of microvesicles (black arrows). C: Platelet incubated with Anastrozole-treated MCF7 cells displaying membrane folds with extending filipodia (white arrow), with very few microvesicles. D: Platelets incubated with Tamoxifen-treated MCF7 cells displaying a spread, rough membrane and extending lamellipodia. The open canalicular system (OCS) with pores (white asterisk *) is evident, with suspected microvesicles and debris adherent to the coverslip (black arrows).

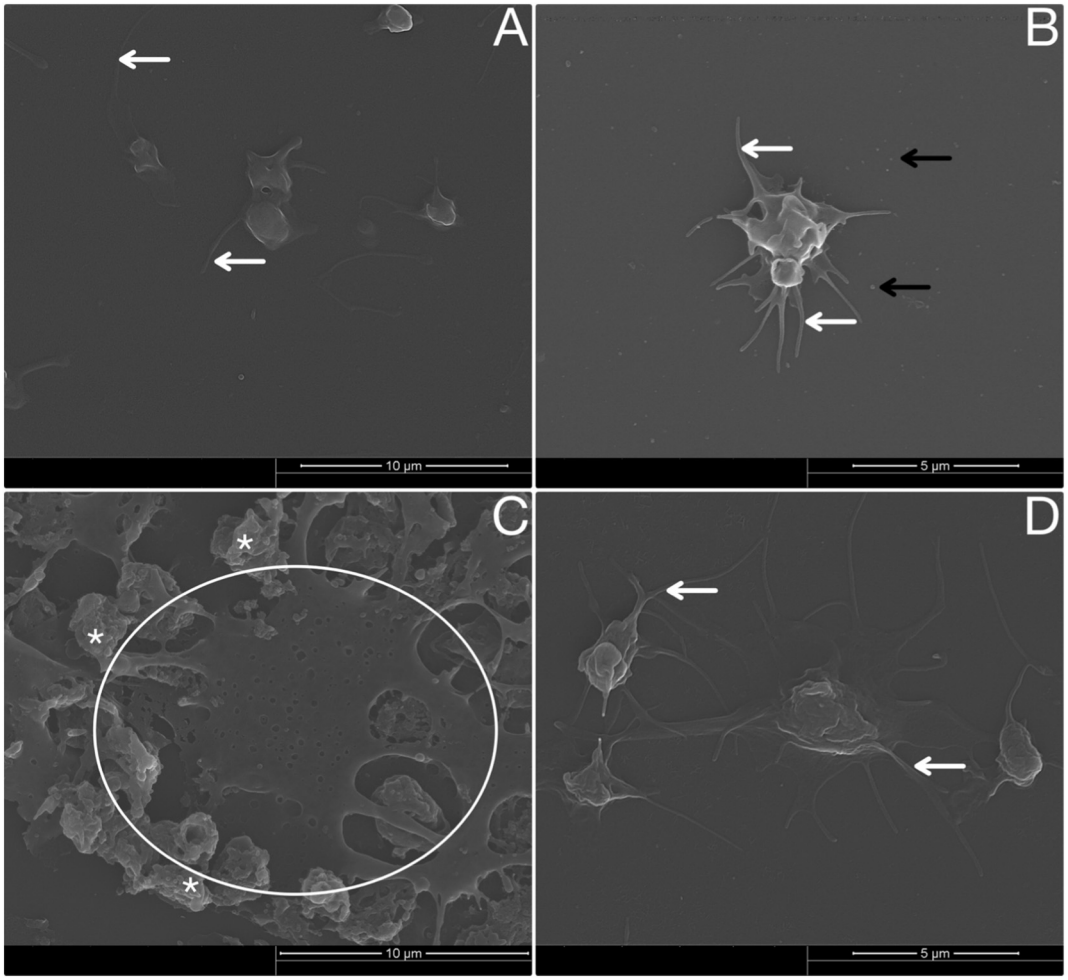

Fig. 5. Scanning electron microscopy images of platelets in lysed whole blood (WB) samples co-incubated with T47D cells and its corresponding treatments. A: Platelet exposed to diluent-treated T47D cells displaying a smooth membrane with extending filipodia. B: Platelet exposed to media-treated T47D cells displaying membrane folds (*) and multiple filipodia extending outward from the platelet body (white arrows), with the presence of microvesicles (black arrows). C: Platelets incubated with Anastrozole-treated T47D cells displaying multiple membrane folds (*) giving a rougher appearance, with thick fibrin fibres forming fibrin sheets and fibrin pores present (encircled). Platelet aggregation evident. D: Platelets incubated with Tamoxifen-treated T47D cells displaying membrane folds and lamellipodia and filipodia extending outward from the platelet body towards neighbouring platelets, showing early aggregation (white arrows).

which is not present within the MCF7 cell line [47]. PAF which can be secreted by tumour cells themselves, is an inflammatory phospholipid which mediates platelet aggregation and degranulation [48,49]. Breast cancer cells, including MCF7 and T47D cells, are able to produce thrombin, as well as synthesise inflammatory cytokines mediating platelet recruitment and activation [50]. Our laboratory had previously shown that MCF7 cells induce platelet activation, as evinced by pseudopodia extension and aggregation in as little as 5 min [30]. In this study, we thus halved the exposure time to facilitate identifying early activation [10].

Clinical studies indicate Tamoxifen is associated with a 4% higher risk of VTE development [20], while Anastrozole is associated with a lower but significant risk [51]. In this study, hormone-therapy differentially affected the ability of the cell lines to induce platelet activation. While our results support other studies assessing Tamoxifen induction of platelet activation [35], our results contradict recent laboratory-based studies which show that Tamoxifen is able to inhibit platelet activation and consequently platelet aggregation [26,27]. This reflects not only differences in dosage, 2 µM Tamoxifen used in our study compared to a range of 3 µM–20 µM [26,27], but also in experimental methodology. In this study platelets were neither washed nor treated directly with Tamoxifen (or Anastrozole); rather cells were pre-treated with hormone-therapy prior to incubation with whole blood reflecting the tumour microenvironment and complementing clinical studies. Notably by not washing platelets, plasma was retained, a component which has recently been shown to be essential to platelet activation [13].

Tamoxifen pre-treatment caused T47D cells to induce platelet activation greater than that of the MCF7 cell line, in the P-selectin high ($CD62P^{+++}$) interval gate. Anastrozole pre-treatment of both cell lines reduced overall IPA. This reduction in IPA does not however, reflect a protective effect against platelet activation. Qualitative assessment indicated platelets in a highly active state with pseudopodia extension and the OCS present. This reduction in IPA is thus due to the subsequent loss of P-selectin, which is released rapidly initially, allowing for high amounts to be detected, with levels diminishing as platelet activation progresses [52]. This reduction could also be attributed to a lack of sensitivity of our flow cytometry protocol and thus non-detection of P-selectin positive microvesicles, which were evident under scanning electron microscopy. This agrees with clinical studies showing that Tamoxifen and Anastrozole are associated clinically with greater numbers of P-selectin positive microparticles [53].

Additionally, Anastrozole-treated T47D samples displayed thick fibrin fibre and plaque formation, indicative of a hypercoagulatory state [30,54,55]. The visualisation of both fibrin plaque and microvesicles further indicates that lysing whole blood maintained the effects of

plasma in the coagulatory process [10,50,56]. Plaque formation is indicative of potentially impaired fibrinolytic activity, which while essential for the release of tumour cells from their primary site, also reflects the potential for thromboembolic complications [57].

Our study highlights the importance of the potential effects of different cancer subphenotypes on platelet activation. Limitations of this study include small sample size and would benefit from additional exploration of platelet activation. In addition, our study shows the effects of DMSO pre-treatment was not significantly different from respective media controls. Previous studies indicate a converse inhibitory effect of DMSO on platelet aggregation [58,59]. However, the effects of DMSO on platelet activation, has not been well characterised in the literature [58,59].

By investigating the intricacies of the index of platelet activation, we have identified a switched response of hormone-therapy-treated MCF7 and T47D cells on P-selectin high expression (exposure), where MCF7 cells induced heightened IPA under Anastrozole treatment and T47D cells, under Tamoxifen treatment. This was further confirmed ultrastructurally. These results reflect the heterogeneous nature of platelet α-granule content, with specific release related to the effects of various agonists of activation [60,61]. Tumour cells are able to secrete tumour-associated tissue factor, thrombin or ADP [7,8], thus while the levels of activation seen in our study correspond to that of the positive control (thrombin), further research is required to determine the tumour-secreted factors that are responsible for platelet activation in our system. Our results, which indicate Tamoxifen and Anastrozole pre-treatment increase platelet activation, substantiate clinical studies indicating patients are more likely to suffer from thromboembolic events [7], supporting the utilisation of targeted platelet therapies to prevent thromboembolic complications. We further highlight the difference between methodological approaches, which mirror different aspects of the effects of hormone-therapy on the tumour environment – *in situ* and circulatory. By using whole blood as opposed to washed platelets, we provide further evidence for the pro-thrombotic potential of Tamoxifen. In addition, this is one of the first studies investigating the effects of Anastrozole pre-treatment *in vitro*. This emphasises the importance of investigating platelet -cancer cell interaction using different approaches to mimic the tumour microenvironment more holistically, including a detailed assessment of the tumour phenotype on hypercoagulation.

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Declaration of interest

The researchers report no conflict of interest.

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