

Hormone-therapy mediated interactions between platelets and breast cancer cells: *an in vitro* study



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Declaration

I, Kyrta Pather declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Kyrta Pather

4 day of November 20 20 in Parktown

Presentations and Publications Arising from this Thesis

Local Conference – Oral Presentation

Hormone-therapy induced platelet CD62P expression alterations. Kyrtania Pather, Therese Dix-Peek, Raquel Duarte, Tanya Augustine. *The 45th Annual Conference of the Anatomical Society of Southern Africa*, 23 – 26 April 2017, Cape Town, Western Cape, South Africa.

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Abstract

Breast cancer-associated thrombosis is a growing concern. Reciprocal interactions between platelets and tumour cells facilitate tumour progression, ultimately leading to thromboembolic complications. Treatment strategies including hormone-therapies are associated with increased thrombotic risk. However, *in vitro* studies found that Tamoxifen reduces thrombotic risk, contradictory to clinical evidence; with little evidence on the mechanistic effects of Anastrozole. Furthermore, while hormone-dependent tumours are treated with hormone therapy based on the presentation of the oestrogen receptor (ER), ER α , little is understood regarding ER β . This study developed an *in vitro* system to mimic the effects of cumulative hormone-therapy (Anastrozole or Tamoxifen) by pre-treating MCF7 and T47D cells with hormone-therapy (Model 1) prior to exposure to whole blood (WB).

Early stages of platelet activation described using CD62P expression and ultrastructure, and associated cancer cell ER (*ESR1* and *ESR2*) gene expression were assessed. The study was then expanded to include an additional model to recapitulate circulatory effects of hormone-therapy (Model 2) where blood constituents (WB, platelet-rich plasma and platelet-poor plasma) were pre-treated prior to exposure to cancer cells. Hypercoagulation was assessed as a function of thrombin activity, CD62P and CD63 expression defined by an index of platelet activation, and corresponding ultrastructure. Furthermore, ER protein expression was determined and correlated with hypercoagulation markers to assess whether tumour profiles and hypercoagulation were associated.

Breast cancer cells induced hypercoagulation, compounded by hormone-therapy regardless of Model employed, but dependent on cellular subphenotype. Furthermore, ER expression altered, highlighting subphenotypic differences; with T47D cells, having greater ER β expression. We postulate this profile is associated with the induction of a hypercoagulatory environment under Anastrozole treatment. Additionally, it was determined that Tamoxifen treatment induces a hypercoagulatory environment. This not only compares with clinical studies indicating a greater thrombotic risk for patients undergoing hormone-therapy treatment but highlights that subphenotypes, which clinically would be treated similarly, may be associated with differing thromboembolic profiles.

The present study provides a framework and *ex vivo* models in which to undertake further research, to complement our findings regarding breast tumour subphenotype heterogeneity and to determine other biomarkers that may be predictive of hypercoagulatory risk.

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Nomenclature: List of Abbreviations

DCIS	Ductal Carcinoma <i>in situ</i>
ER	Oestrogen Receptor
PR	progesterone Receptor
HER2	Human Epidermal Growth Factor Receptor 2
ER α	Oestrogen Receptor Alpha
E1	Oestrone
E2	17- β Oestradiol
E3	Oestriol
ERE	Oestrogen Response Elements
ER β	oestrogen receptor beta
EMT	Epithelial-to-Mesenchymal Transition
TGF- β	Transforming Growth Factor Beta
PTEN	Phosphatase/Tensin Homolog
IV	Intravenous
SERM	Selective Oestrogen Receptor Modulator
AI	Aromatase Inhibitor
CVS	Cardiovascular System
DVT	Deep Vein Thrombosis
PAR	Protease Activated Receptors
OCS	Open Canalicular System
TF	Tissue Factor
VEGF	Vascular Endothelial Growth Factor
ADP	Adenosine Diphosphate
TCIPA	Tumour Cell-Induced Platelet Aggregation
MMP	Matrix Metalloproteinase
VTE	Venous Thromboembolisms
IPA	Index of Platelet Activation
WB	Whole Blood
PRP	Platelet-Rich Plasma

PPP	Platelet-Poor Plasma
SEM	Scanning Electron Microscopy
LDH	Lactate Dehydrogenase
NR	Neutral Red
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagles Medium
FBS	Foetal Bovine Serum
P/S	Penicillin/Streptomycin
RPMI	Roswell Park Memorial Institute
DMSO	Dimethyl Sulfoxide
PDGF	Platelet Derived Growth Factor
EGFR	Epidermal Growth Factor Receptor
PAF	Platelet Activating Factor
CD62P	P-selectin

1 CHAPTER 1: General Introduction

Breast cancer is one of the most commonly diagnosed cancers in women worldwide (Tao *et al.*, 2015; Walker *et al.*, 2017; Bray *et al.*, 2018); with not only the tumour microenvironment, but the haemostatic system, identified as regulators in the progression of the disease (Lal, Dittus and Holmes, 2013; Franco, Corken and Ware, 2015; Lambert, Pattabiraman and Weinberg, 2017). Platelets and coagulation factors have been shown to facilitate breast cancer progression by affecting hallmarks of the disease including selective sustained proliferation, vascularisation, immune modulation, tumour invasion and metastasis (Lal, Dittus and Holmes, 2013; Franco, Corken and Ware, 2015; Fouad and Aanei, 2017; Lambert, Pattabiraman and Weinberg, 2017). Furthermore, breast cancer cells are able to induce platelet responses through manipulation of the haemostatic system itself (Nash *et al.*, 2002; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2014). This is exacerbated by hormone-therapy treatment strategies, further increasing the risk of thrombotic complications in patients (Carter, 2015; Walker *et al.*, 2017).

1.1 The Incidence and Prevalence of Breast Cancer

Cancer is one of the leading causes of death worldwide (Nagai and Kim, 2017; Mahase, 2019), with its incidence rate steadily increasing in many developing countries due to adoption of cancer-associated lifestyle choices and other factors (Jemal *et al.*, 2011). Breast cancer is one of the most commonly diagnosed cancers in women worldwide (Tao *et al.*, 2015; Walker *et al.*, 2017; Bray *et al.*, 2018) and is the second leading cause of cancer deaths in females, following lung cancer (Sharma *et al.*, 2010; Azamjah, Soltan-Zadeh and Zayeri, 2019). Conversely, males account for less than 1% of diagnosed breast cancer cases worldwide (Giordano, 2005; Yalaza, Inan and Bozer, 2016), and have not been extensively studied. Due to its rarity, men primarily present with late stage breast cancer (Yalaza, Inan and Bozer, 2016), and treatment options at this stage are based on that from female breast cancer studies (Giordano, 2005). Furthermore, due to the low incidence rates of breast cancer in males, most studies on breast cancer remain conducted on female-derived breast cancer cells *in vitro* or on female breast cancer patients (Yalaza, Inan and Bozer, 2016).

South Africa has one of the highest incidence rates of female breast cancer (Schlebusch *et al.*, 2010; Trupe *et al.*, 2017; Adeloye *et al.*, 2018; Joffe *et al.*, 2018), and the lifetime risk of developing breast cancer varies, depending on many factors including ethnicity. For example, African women experience the highest risk while Caucasian women have a greater risk for developing breast cancer at an earlier age (Vorobiof, Sitas and Vorobiof, 2001; Singh *et al.*, 2015, 2017). This is similar to risk rates in the United States of America; however, their incidence rate of lower stage breast cancers is at 82%, regardless of ethnicity and is higher than that of South Africa (Iqbal *et al.*, 2015). As reported by the National Cancer Registry of South Africa, between 1993 and 1995, there was a yearly average over 3000 new breast cancer diagnoses in women with an incidence rate of 18.5 per 100 000 (Vorobiof, Sitas and Vorobiof, 2001; Singh *et al.*, 2015, 2017). Breast cancer is predominant in women over the age of 40, and more so in postmenopausal women (Sun *et al.*, 2017). Furthermore, 5% of breast cancers diagnosed in women under the age of 40 are at an advanced stage and are of a more aggressive nature (Sun *et al.*, 2017).

1.2 Breast Cancer Heterogeneity

Breast cancer is not a single disease, but a complex of subtypes with their own molecular profiles which influence diagnosis and treatment (Carey *et al.*, 2006; Dai *et al.*, 2017c). Most breast cancers are defined as adenocarcinomas, which begin in the cells of the ducts or lobules of the breast, with the earliest form of the disease being ductal carcinoma *in situ* (DCIS) (Polyak, 2011; Toss *et al.*, 2017). DCIS defines proliferation of malignant cells within the ducts and lobules of the breast, surrounded by epithelial cells and an intact basement membrane, with progression giving rise to invasive ductal carcinoma (Pang, Gorringer and Fox, 2016; Toss *et al.*, 2017).

Breast tumour samples are typically classified based on their molecular heterogeneity, the presence or absence of certain biomarkers, specifically oestrogen receptor alpha (ER α) and progesterone receptor (PR); the presence or absence of Human Epidermal Growth Factor Receptor 2 (HER2) (Dai *et al.*, 2016; Bertozzi *et al.*, 2018; Søkilde *et al.*, 2019), and the presence of the cell proliferation marker Ki67 (Bertozzi *et al.*, 2018). This subdivides breast cancer into distinct subtypes: Luminal A tumours (ER α positive and/or PR high, HER2 negative

and Ki67 low); Luminal B tumours (ER α positive and/or PR low, HER2 negative and Ki67 high); Luminal B-like (ER α positive and/or PR positive, HER2 overexpressed and Ki67 positive); HER2 positive tumours (HER2 overexpressed, ER α and PR negative); basal-like tumours, which are triple-negative tumours (ER α , PR and HER2 negative), claudin-low tumours (ER α , PR and HER2 negative; downregulation of claudin-3 and claudin-4, protein components of tight junctions, and Ki67 low) and normal-like tumours (ER α positive and/or PR positive, HER2 negative and Ki67 negative) (Carey *et al.*, 2006; Holli-Helenius *et al.*, 2017; Fragomeni, Sciallis and Jeruss, 2018). Luminal A and B tumours are hormone-dependent and account for the majority of diagnosed breast cancers, having low proliferation potential compared to other subtypes, and responding well to tailored/ targeted therapeutic approaches (Carey *et al.*, 2006; Masoud and Pagès, 2017). Conversely, HER-2, basal-like subtypes and triple-negative breast cancers, which account for approximately 10 to 20% of invasive breast cancers (Perou, 2010; O'Toole *et al.*, 2013), are more aggressive, do not respond to targeted approaches and indicate poor prognosis (Carey *et al.*, 2006; Narrandes *et al.*, 2018).

Hormone-dependent breast cancers are associated with lower tumour grades and with lower rates of recurrence; however, a loss of the tumour suppressor gene *p53* is also noted, indicating that these tumour cells can become more aggressive during the course of the disease (Berger, Qian and Chen, 2013; Yamamoto *et al.*, 2014). While biomarkers allow for characterisation of tumours initially, it should be noted that tumour cells are able to alter their expression, including that of the ERs. This reflects the intratumoural heterogeneity of breast cancer, changing not only responses to treatment but heightening the risk of resistance to treatment (Turashvili and Brogi, 2017).

The Luminal A subphenotype, indicating hormone-dependence, is commonly modelled *in vitro* by making use of the Michigan Cancer Foundation-7 (MCF7) and T47D cells lines (Holliday and Speirs, 2011). The MCF7 cell line was the first hormone-responsive breast cancer cell line to be discovered and is one of the most commonly used lines in *in vitro* research (Burdall *et al.*, 2003; Holliday and Speirs, 2011). MCF7 cells express a range of sensitivities to oestrogen, and express both ERs and PRs, allowing for rapid proliferation in the presence of oestradiol (Hamelers *et al.*, 2003). Inhibition of production of either oestrogen or inhibition of the oestrogen receptor function is essential for successful

hormone-dependent breast cancer treatment (Hamelers *et al.*, 2003). The T47D breast cancer cell line is also commonly used *in vitro*, to model hormone-dependent breast cancers; however, this cell line is highly susceptible to progesterone, while MCF7 cells do not readily respond to progesterone in the presence of oestrogen (Yu *et al.*, 2017). Furthermore, the MCF10A human mammary epithelial cell line, which is also widely used *in vitro* is employed as a model to study normal breast tissue and cell functioning (Besette *et al.*, 2015). Cell lines also present a model in which drug metabolism can be studied further, potentially mimicking that of the clinical environment (McDonald *et al.*, 2017). Both MCF7 and T47D cells have been shown to express low levels cytochrome P450 enzymes which are involved in the oxidative metabolism of drugs (McDonald *et al.*, 2017; Rendic and Peter Guengerich, 2018; Stavropoulou, Pircalabioru and Bezirtzoglou, 2018); this expression influences the cytotoxicity of the drugs themselves (Leung *et al.*, 2013; Li *et al.*, 2017). Hormone-therapy drugs are metabolised and cleared by cytochrome P450 isoforms; specifically, Anastrozole by CYP3A4/5 and Tamoxifen by CYP1A1/2, 1B1, 2C9/19, 2D6, 2J2, 3A4/5 indicating their role in the bioavailability of hormone-therapies by cell lines (Armstrong, 2011). Furthermore, it was noted that MCF7 cells expressed cytochrome P450 enzymes higher than that in T47D cells (McDonald *et al.*, 2017), indicating further differences between these subphenotypes.

Breast cancer cell lines are considered to be representative of *in vivo* breast carcinomas (Dai *et al.*, 2017). Critiques have arisen as to whether cell lines accurately depict molecular features, due to varying evidence suggesting genetic and epigenetic alterations associated with cell line establishment and passaging, as well as whether their corresponding heterogeneity representing their respective tumours can indicate clinically relevant results (Dai *et al.*, 2017). However, cell line models still present the most effective way to study breast cancer *in vitro* due to their ability to be cultured unlimitedly, with stable biomarker expression using a standardised approach in a controlled environment (Lacroix *et al.*, 2004; Dai *et al.*, 2017).

1.3 Oestrogen Receptors (ER)

Oestrogen modulates cellular growth and differentiation in mammary breast tissue (Arendt and Kuperwasser, 2015). Oestrogens are classified as steroid hormones which are involved in

normal sexual development and function in both males and females (Thomas and Potter, 2013). Oestrogens include oestrone (E1), 17- β oestradiol (E2), and oestriol (E3); E2 is the most abundant and biologically active within the body and is known to play a role in the development of breast cancer (Williams and Lin, 2013; Tian *et al.*, 2018)). Prolonged exposure to E2 acting either through ER-dependent or ER-independent pathways could potentiate tumourigenesis (Williams and Lin, 2013; Tian *et al.*, 2018). ERs are over-expressed in 75% of hormone-dependent breast cancers (Pal, Childs and Pegram, 2011; Williams and Lin, 2013; Hashmi *et al.*, 2018), making them favourable targets for therapeutic interventions. Upon ligand activation, ERs translocate to the nucleus and bind oestrogen response elements (EREs) in DNA thereby mediating breast epithelial cell proliferation in both benign and malignant epithelium (Williams and Lin, 2013). While ERs function as transcription factors themselves, they are also able to bind DNA indirectly through other transcription factors, including NF κ B, allowing for modulation and co-regulation of responses (Marino and Ascenzi, 2008). Furthermore, ERs have been shown to exert non-genomic effects through cell membrane-associated receptors and cytoplasmic downstream signalling pathways (Singh, Su and Ng, 2013; Rainville, Pollard and Vasudevan, 2015). Therefore, targeting ERs to reduce breast cancer progression through oestrogen-dependent processes has become more widely used. ERs are members of the nuclear receptor superfamily of transcriptional regulators and consist of two ERs; ER α and oestrogen receptor beta (ER β), encoded by the *ESR1* and *ESR2* genes respectively (Jeselsohn *et al.*, 2014; Yaşar *et al.*, 2017).

1.3.1 ER α

ER α is the most commonly identified and targeted receptor in breast cancer (Iwase *et al.*, 2003; Thomas and Gustafsson, 2011; Bahreini *et al.*, 2017). It is known as the original ER and has been extensively studied (Iwase *et al.*, 2003; Thomas and Gustafsson, 2011; Bahreini *et al.*, 2017). ER α is not only found on breast cancer cells but mammary epithelial cells, cells of the endometrium, ovarian stromal cells, in the hypothalamus and in males, on the epithelium of the efferent ducts (Yaghmaie *et al.*, 2005). An abundance of ER α in benign breast epithelium indicates a higher risk for breast cancer; however, in malignant tissue ER α is known to stimulate proliferation (Ali and Coombes, 2000; Thomas and Gustafsson, 2011; Feng *et al.*, 2018). ER α has been extensively studied in relation to breast cancer and is overexpressed in more than half of hormone-dependent breast cancers (Ali and Coombes,

2000; Chen and Hei Antonio Cheung, 2019). ERs are regarded as cytoplasmic in their unliganded state, however, immunohistochemical assessment of tumours reveals liganded ERs to be primarily located in the nucleus (Welsh *et al.*, 2012). ERs in the cytosol and cell membrane exert their function linked to the non-classical or non-genomic pathway (as opposed to nuclear ER functioning via the classical or genomic pathway). Studies indicate that these pathways may converge, or function in direct conflict; either promoting or inhibiting tumour progression (Leonhardt, Boonyaratanakornkit and Edwards, 2003; Björnström and Sjöberg, 2004; Acconcia and Kumar, 2006; Marino and Ascenzi, 2008). Furthermore, ER α acts as the main transcription factor in hormone-dependent breast cancers, facilitating tumourigenesis (Oueslati *et al.*, 2017).

1.3.2 ER β

ER β was first discovered in 1996 by Gustafsson and Kuiper; resulting in the renaming the original ER to ER α to distinguish the receptors (Kuiper *et al.*, 1996; Haldosén, Zhao and Dahlman-Wright, 2014). ER α and ER β both bind E2 and share a similarity of 56% of their ligand binding domains, differences which allow for selective binding and targeting of each receptor subtype (Williams and Lin, 2013). Studies have indicated a substantial overlap in DNA binding sites of both ERs in MCF7 cells (Zhao *et al.*, 2010; Grober *et al.*, 2011). However, this does not correlate with gene regulation or with studies indicating ER α involved in regulation of a single class of genes, which is dependent on the presence of ligand and ER β able to regulate three distinct classes of genes. Class I is regulated by unliganded ER β , class II regulated by liganded ER β and class III regulated by unliganded ER β with the addition of E2, with only minimal overlap (Vivar *et al.*, 2010). For example, genes regulated by both ERs include Keratin 19, responsible for structural cell integrity and WISP-2, involved in signal transduction; however, this is notwithstanding that signalling pathways may converge (Tee *et al.*, 2004; Leitman *et al.*, 2010; Vivar *et al.*, 2010; Zhao *et al.*, 2010). Furthermore, WISP-2 plays a role in tumour cell proliferation and epithelial-to-mesenchymal transition (EMT) through the Transforming Growth Factor Beta (TGF- β) pathway (Tee *et al.*, 2004; Chai *et al.*, 2019). ER β is commonly expressed in the ovary, kidney, heart, prostate, testis, regions of the brain and is expressed more widely in mammary epithelium (Paterni *et al.*, 2014). While ER β is more widespread, ER α mediates oestrogen-dependent pubertal and menstrual functions and development of the uterus, ovaries and mammary glands (Rissman, 2008); ER β is noted to be involved in final

differentiation of the mammary gland (Mehta *et al.*, 2014; Dall *et al.*, 2018). While ER α expression indicates a propensity for growth in hormone-dependent breast cancers, ER β has not been studied extensively and thus, has no identified clear clinical links to breast cancer prognosis (Haldosen *et al.*, 2014). Studies indicate, however, that ER β which is expressed in both benign and malignant breast tissue but at lower levels in the majority of breast cancer cells, can oppose the actions of ER α (C Palmieri *et al.*, 2002; Girgert, Emons and Gründker, 2019). ER β also regulates genes such as *p53* and the Phosphatase/Tensin homolog (PTEN), which play key roles in tumour suppression pathways (Dey *et al.*, 2013). ER β binds genistein, a phytoestrogen that is known to be an anti-cancer agent due to its induction of apoptosis, cell cycle arrest, antiangiogenic and anti-inflammatory effects. This suggests that ER β may be implicated in decreased tumour progression and cancer metastasis (Lecomte *et al.*, 2017; Tuli *et al.*, 2019). Furthermore, studies have indicated a dual role for ER β ; in both proliferation and anti-proliferative roles, but its exact function has not been well defined (Platet *et al.*, 2004; Leygue and Murphy, 2013; Wei *et al.*, 2019).

1.4 Breast Cancer Treatment Strategies

The treatment options available for breast cancer patients is dependent on the tumour subtype including biomarker expression, the stage of the tumour, the patients' age, general health and menopausal status, as well as the presence of inherited breast cancer gene mutations (Akram *et al.*, 2017). In confirmed cases of breast cancer, strategies of treatment and management include chemotherapy (e.g. Doxorubicin), surgery, radiation therapy (e.g. ionising radiation), immunotherapy (e.g. Trastuzumab) and hormonal therapy (e.g. Anastrozole and Tamoxifen), with patients presenting with metastatic breast cancers receiving palliative treatment (Reeder and Vogel, 2008; Akram *et al.*, 2017).

Routes of administration for breast cancer treatments include oral and intravenous (IV) modes, which involve achieving serum concentrations high enough to affect the cancer cells without producing too many deleterious side-effects (Ishitobi *et al.*, 2013). Both oral and IV modes affect tissue and blood drug concentrations, with oral medication achieving concentrations equivalent to that of intravenous medication in a longer time frame, suggesting slight therapeutic differences when concentrations are not the same (Cyriac and

James, 2014). Oral medications must go through multiple biotransformative steps which enhance the hydrophilic nature of the drug, so it is rapidly eliminated by the body, thus taking longer to reach the equivalent bioavailability in the body (Jordan, 2007; Fernandez *et al.*, 2011). The bioavailability of IV drugs is higher than that of oral medications, indicated for faster effectiveness of the drug and relief of symptoms (Fischer *et al.*, 2003). Therapeutic drug concentrations are thus initially achieved in plasma from which the drug then enters the tissue (Lee, Azmi and Wong, 2012). Both oral and IV approaches to hormone-dependent breast cancer treatment involve targeting receptors, specifically the oestrogen receptors or reducing the bioavailability of oestrogen with hormone-therapies Tamoxifen or Anastrozole, allowing for available concentration and transport within the bloodstream itself, as well as an accumulation of the therapy at the cancer site (Fabian, 2007; Jordan, 2007; Sobczynski *et al.*, 2015; Tran *et al.*, 2017). Furthermore, the availability of the therapy within the bloodstream allows for interaction with various cells, including endothelial cells, leukocytes and platelets (Sobczynski *et al.*, 2015; Tran *et al.*, 2017).

Targeted hormone-therapy treatment has become a favoured approach for tumours of the luminal phenotype, depending on staging and menopausal status (Carey *et al.*, 2006; El Sayed *et al.*, 2019). Hormone-therapy can also be used as adjuvant therapy following surgery, to reduce recurrence and is usually taken by patients for at least 5 to 10 years (Burstein *et al.*, 2019). Hormone-therapies target oestrogen-specific proliferation, by either reducing the synthesis of oestrogen or blocking the ER itself (Bonnetterre *et al.*, 2000; Carey *et al.*, 2006; Burstein *et al.*, 2019). Luminal phenotype breast cancers are treated using Selective Oestrogen Receptor Modulators (SERM) or Aromatase Inhibitors (AI), which act to prevent pro-tumourigenic responses controlled by oestrogen (Carey *et al.*, 2006; Ogba *et al.*, 2014; Turashvili and Brogi, 2017). SERMs include Tamoxifen which is an oestrogen receptor antagonist in breast tissue and is frequently used to treat breast tumours in pre-menopausal women (Jonat *et al.*, 2006; Litton *et al.*, 2013; El Sayed *et al.*, 2019); while AIs such as Anastrozole inhibits the synthesis of oestrogen and are mainly used to treat hormone-dependent breast cancers in post-menopausal women (Mokbel, 2002; Campos, 2004; Brueggemeier, Hackett and Diaz-Cruz, 2005; Hole *et al.*, 2015).

1.4.1 Tamoxifen

Tamoxifen is considered the gold standard in adjuvant treatment for luminal phenotype tumours and displays both agonistic and antagonistic oestrogenic properties depending on the tissue types it affects (Chang *et al.*, 2011; Chang, 2012; El Sayed *et al.*, 2019). Tamoxifen can be used for the treatment of hormone-dependent breast cancers at any stage (Criscitiello *et al.*, 2011; Burstein *et al.*, 2019), and in 1998 was approved as a preventative therapy in women over the age of 35 at an increased risk for developing breast cancer (Waters *et al.*, 2012). ER expression is said to affect responses to hormone-therapy; with ER β -positive tumours responding more favourably and ER β agonists, including genistein a phytoestrogen, increasing tumour cell sensitivity to Tamoxifen (Honma *et al.*, 2008; Lattrich *et al.*, 2014); however, its exact function remains to be determined. Furthermore, Tamoxifen has shown efficacy in reducing tumour growth and potentially shrinking tumours (Cameron, Ritchie and Miller, 2001; Cortes *et al.*, 2019). Tamoxifen has also been shown to decrease tumour recurrence and mortality through its selectivity; however, it adversely increases a patient's risk of thromboembolic complications (Decensi *et al.*, 2005; Howell *et al.*, 2005; Dobryднева *et al.*, 2007; Lin *et al.*, 2018). Patients treated with Tamoxifen experience adverse cardiovascular (CVS) events including pulmonary embolism, stroke, deep vein thrombosis (DVT) and arterial thrombosis, the risk of which is 3 times higher than that of patients not on Tamoxifen treatment (Fennerty, 2006; Dobryднева *et al.*, 2007; Lin *et al.*, 2018). Tamoxifen is associated with increased risk for thrombosis and yet it is still being given to women for both treatment and prevention despite the potential for adverse CVS events (Dobryднева *et al.*, 2007). Despite the prevalent clinical data (Decensi *et al.*, 2005; Howell *et al.*, 2005; Lin *et al.*, 2018), selected *in vitro* studies have indicated inhibition of platelet activation and thus reduced risk of thrombosis by Tamoxifen, contradicting clinical studies (Chang *et al.*, 2011; Johnson *et al.*, 2017). Thus, further studies are required in this regard.

1.4.2 Anastrozole

AIs are the most commonly used hormone therapeutic drugs for the treatment of breast cancer in post-menopausal women (Mokbel, 2002; Campos, 2004; Kümler *et al.*, 2016). There are three generations of AIs, the third generation being the most effective as it is associated with fewer side effects and is more specific to the aromatase enzyme (Nabholtz *et al.*, 2000). Third generation AIs are further classified into steroidal AIs which bind irreversibly to the

aromatase enzyme and non-steroidal AIs which bind reversibly to the aromatase enzyme (Campos, 2004; Burstein *et al.*, 2019). Anastrozole, which is a third generation AI, is used as second-line therapy in postmenopausal breast cancer patients (Mokbel, 2002; Francesco *et al.*, 2005); however, when used as first-line therapy in breast cancer patients it is as effective as Tamoxifen (Bonnetterre *et al.*, 2000; Nabholz *et al.*, 2000; Mouridsen *et al.*, 2001; Smith and Dowsett, 2003). Studies have indicated that inhibition of the aromatase enzyme as opposed to treatment with SERMs improved survival rates of breast cancer patients in advanced stages (Lumachi *et al.*, 2013; Cuzick *et al.*, 2020). Studies have also shown that compared to the risk of thrombosis associated with cancer itself, AIs do not increase a patient's risk for deep vein thrombosis (Fabian, 2007), with patients being treated with Anastrozole having a reduced risk of developing venous thromboembolisms (Fennerty, 2006; Gonnelli and Petrioli, 2008). However, Anastrozole treatment is associated with a higher risk of thrombosis than that of healthy individuals (Fabian, 2007). While clinical studies have been conducted on the effects of Anastrozole and the risk for DVT, *in vitro* work to explain the mechanisms of induced thrombosis, is lacking. Since both MCF7 and T47D cells are known to express low levels of aromatase (Lisztwan *et al.*, 2008; Wong and Chen, 2012) they are ideal cell lines to investigate the effect of hormone-therapy, particularly Anastrozole, induced thrombosis.

1.5 Breast Cancer-Associated Thrombosis and Hypercoagulation

Breast tumours and associated therapies including chemotherapy, surgery and hormone-therapy are linked to an increased risk of thrombotic complications (Meier and Jick, 1998; Decensi *et al.*, 2005; Howell *et al.*, 2005; Falanga, Russo and Verzeroli, 2013). Thrombosis and cancer have a well-known relationship that has been extensively studied since 1865 when Armand Trousseau first linked symptoms such as venous thrombosis and hypercoagulability to cancer malignancy (Goubran *et al.*, 2013). These complications, known as the Trousseau sign of malignancy or Trousseau syndrome, are shown to be one of the leading causes of death in cancer patients worldwide (Ikushima *et al.*, 2016; Metharom, Falasca and Berndt, 2019). Studies have further shown this relationship by linking tumour metastases to platelet aggregation capacity; a process now well known as tumour cell-induced platelet aggregation (TCIPA) (Jurasz, Alonso-Escolano and Radomski, 2004; Yan and Jurasz, 2016).

During tumour growth, intratumoural platelets extravasate and are recruited into the tumour microenvironment, where they engage, facilitate and promote tumour growth and survival through reciprocal interactions (Li *et al.*, 2014; Plantureux *et al.*, 2020). However, during tumour metastasis, the interaction between circulating tumour cells and platelets has been shown to induce thromboembolic complications in patients with malignant metastatic tumours (Karimi and Cohan, 2010). While tumour cell metastasis has been identified as the main cause of cancer-associated mortality in patients, thromboembolic complications follow closely behind (Francis, Biggerstaff and Amirkhosravi, 1998; Sierko and Wojtukiewicz, 2004; Hanahan and Weinberg, 2011). The formation of tumour-associated thrombi is dependent on the activation and aggregation of platelets (Jurasz, Alonso-Escolano and Radomski, 2004) and the formation of a fibrin-rich network (Menter *et al.*, 2014). Tumour cells can facilitate this formation through prothrombotic mechanisms such as the secretion of thrombin, tissue factor, procoagulants and inflammatory cytokines (Nash *et al.*, 2002; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2014). Tissue factor is a platelet activator, whereas the secretion of procoagulants and inflammatory cytokines increase the adhesion properties of platelets (Falanga, Russo and Verzeroli, 2013; Fuentes, Palomo and Rojas, 2016). Cancer cells also induce platelet activation, forming a heterotypic aggregate with platelets which serves as a mechanism for avoiding immune surveillance *in vivo* (Bambace and Holmes, 2011; Li *et al.*, 2014). Furthermore, these secreted agonists promote the process of metastasis, which involves cells separating from their primary tumour mass breaking through basement membrane (Jurasz, Alonso-Escolano and Radomski, 2004; Bambace and Holmes, 2011). Matrix metalloproteinases (MMPs) are required for cells to invade into a pro-tumourigenic microenvironment, entering the vascular or lymphatic system through degradation of the extracellular matrix (ECM) and decreased expression of E-cadherins, circulating within the system recruiting and relying on platelets, extravasation and colonisation (Jurasz, Alonso-Escolano and Radomski, 2004; Bambace and Holmes, 2011).

1.6 Platelet Activation and their role in Cancer-associated Hypercoagulation

Platelets can be activated and recruited by breast cancer cells through the secretion of agonists; including ADP and thromboxane A₂, and cytokines (Falanga, Russo and Verzeroli,

2013; Kedzierska *et al.*, 2013). Platelets are also able to extravasate into the tumour microenvironment, allowing for tumour cell engagement. This results in platelet activation, regulating primary tumour growth (Li *et al.*, 2014; Plantureux *et al.*, 2020). Moreover, platelets also interact with other cells of the tumour microenvironment, including immune cells via cytokine secretion (Plantureux *et al.*, 2018). These interactions reflect a local inflammation, which could progress to systemic inflammation. This includes, firstly, where platelet signalling prepares the pre-metastatic niche, and secondly through interactions with white blood cells and tumour cells within the circulation (Li *et al.*, 2010). Furthermore, platelets in the tumour microenvironment are able to facilitate tumour progression to that of a more invasive phenotype through delivery of tumour-associated factors contained within platelet granules (Huong *et al.*, 2019). Studies have shown a relationship between heightened platelet activation and upregulation of *Snail 1* and downregulation of *E-cadherin*, indicating an EMT (Miyashita *et al.*, 2015; Huong *et al.*, 2019). Additionally, and more well-defined, is the crucial role that platelets play in the metastatic process, where activated platelets release growth factors including vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF β), is proposed to facilitate EMT, degradation of the ECM and extravasation (Jurasz, Alonso-Escolano and Radomski, 2004; Labelle, Begum and Hynes, 2011; Huong *et al.*, 2019). In the vascular system, platelets act as a physical shield, protecting tumour cells from immunosurveillance and shear forces, and further allowing for adherence to the vascular wall and extravasation for establishment of secondary malignant sites (Jurasz, Alonso-Escolano and Radomski, 2004; Labelle, Begum and Hynes, 2011).

Platelets are small, anucleate blood cells which are formed from the cytoplasm of megakaryocytes (Deutsch and Tomer, 2006). Platelets are primarily involved in haemostasis and contain growth factors within their granules; once activated these growth factors are secreted and allow for adhesion to, as well as stimulation of, the endothelium, in addition to the recruitment of more platelets, leukocytes and inflammatory cells (Wagner and Burger, 2003; Sylman *et al.*, 2017). Platelets, which maintain a discoid shape in their inactive state, are activated by physiological agonists including adenosine diphosphate (ADP), tissue factor and thrombin that can induce structural alterations including shape changes, shedding of microparticles and fibrinogen receptor expression (Jurasz, Alonso-Escolano and Radomski, 2004; Meikle *et al.*, 2017; Deutsch and Tomer, 2006).

Early platelet activation presents the formation of projections or blebs on the platelet membrane, which then form pseudopodia, extending outward from the platelet, initiating the dendritic phase of activation (Wagner and Burger, 2003; Gresele and Page, 2002; Kuwahara *et al.*, 2002; Tomaiuolo *et al.*, 2018). This phase is followed by further extension of pseudopodia and final release of granular contents from the merging with the plasma membrane or via the open canalicular system (OCS) thereby allowing for further adhesion and platelet aggregation facilitated by the binding of fibrinogen, demonstrating an irreversible phase prior to the final or spread phase (Kuwahara *et al.*, 2002; Augustine *et al.*, 2016; Tomaiuolo *et al.*, 2018). In the final phase of platelet activation, platelet membranes are fully spread; this phase involves clot stabilization also facilitated by thrombin, in which fibrinogen is converted to fibrin allowing for the formation of fibrin networks ultimately stabilising the platelet plug (Pretorius *et al.*, 2011; Weisel and Litvinov, 2017). Furthermore, the extracellular vesicles or microparticles are released through the process of plasma membrane budding into the extracellular milieu (Rijkers *et al.*, 2017). Platelet-derived microparticles associated with the process and progression of platelet activation (Senzel, Gnatenko and Bahou, 2009; Rijkers *et al.*, 2017; Sabrkhany *et al.*, 2018). Calcium ions also play a pivotal role in the initiation of platelet activation and the formation of platelet aggregates, and without sufficient intracellular free calcium ion, platelets are unable to aggregate (Varga-Szabo, Braun and Nieswandt, 2009). The concentration of intracellular free calcium is dependent not only on physiological components, but agents introduced into the body as well. Hormone-therapies, particularly Tamoxifen, influence the increase of intracellular calcium into platelets thereby increasing their aggregatory capacity (Dobrydneva *et al.*, 2007).

Platelets can be identified by surface markers such as CD41a, with platelet activation being identified by markers such as; CD62P and CD63 (Nishibori *et al.*, 1993; Marquardt *et al.*, 2002; Yun *et al.*, 2016). CD41a forms part of the GPIIb/III α and is the most prominent cell surface antigen expressed on platelets and is associated with many proteins, as well as a receptor for thrombin (Rivière *et al.*, 1999; Yun *et al.*, 2016). CD41a has been identified as one of the most abundant platelet markers, it acts as a receptor for fibrinogen and is bound during the activation process facilitating adhesion (Yun *et al.*, 2016). CD62P (P-selectin) is a surface antigen expressed on the cell membrane of α -granules and also facilitates platelet adhesion

(Chen and Geng, 2006; McNicol and Israels, 2008; Yun *et al.*, 2016). Once platelets are activated, CD62P translocates from the α -granule to the platelet membrane allowing it to be used as an identifying marker for activated platelets (Chen and Geng, 2006; McNicol and Israels, 2008; Yun *et al.*, 2016). α -granular contents include several membrane proteins (e.g. GPIb complex, GPIIb/IIIa, P-selectin), coagulation factors (e.g. Factor's V, XI, XIII; fibrinogen and von Willebrand factor), growth factors involved in inflammation and angiogenesis, and chemokines (McNicol and Israels, 2008; Yun *et al.*, 2016). A marker associated with later stages of activation, CD63, is a component of platelet lysosomes and plays a role in protecting the plasma membrane from being degraded by lysosomal enzymes (Nishibori *et al.*, 1993; Marquardt *et al.*, 2002). It is also found to be released from platelet dense granules following activation and plays a role in mediating later events including fibrinolysis (Nishibori *et al.*, 1993; Marquardt *et al.*, 2002). Both growth factor and coagulation factor secretion from platelet α -granules occur concurrently, with greater release of granular contents from the OCS following further adhesion and aggregation (Kuwahara *et al.*, 2002; Whiteheart, 2011; Augustine *et al.*, 2016). The release of these granular components not only further facilitates coagulation processes but plays a role in tumour progression (Wagner and Burger, 2003).

1.7 Rationale: Breast Cancer Hormone-therapy and Platelet Activation contributing to Hypercoagulation

The binding of oestrogen predominantly E2, to ERs, specifically the more traditionally targeted ER α , activating the receptor and promotes breast epithelial cell proliferation in both benign and malignant epithelium (Palmieri *et al.*, 2002; Thomas and Gustafsson, 2011; Dall *et al.*, 2018). SERMs and AIs are commonly used in the treatment of breast cancers as they affect the binding or biosynthesis of oestrogen, therefore disrupting the pathway and inhibiting the proliferation of breast cancer cells (Mokbel, 2002; Kisanga, Gjerde, Guerrieri-Gonzaga, *et al.*, 2004; Chen *et al.*, 2015).

Tamoxifen, a SERM prescribed for the prevention and treatment of oestrogen-receptor positive breast tumours (Onitilo *et al.*, 2012), increases survival time and reduces the risk of recurrence of invasive and non-invasive breast cancer in women who have used the drug for a minimum of 5 years (Kisanga, Gjerde, Guerrieri-Gonzaga, *et al.*, 2004; Decensi *et al.*, 2005;

Jonat *et al.*, 2006; Kümler *et al.*, 2016). However, it has been associated with many adverse effects including increasing a patients' risk of developing venous thromboembolism (Decensi *et al.*, 2005; Hernandez *et al.*, 2009; Onitilo *et al.*, 2012). The precise mechanism by which Tamoxifen exerts its' procoagulant effect remains to be discovered (Onitilo *et al.*, 2012; Kovac *et al.*, 2015); although recent studies have implicated Factor VIII in promoting thrombotic events via its effect on platelet activation (Kovac *et al.*, 2015). Furthermore, Tamoxifen is also prescribed as a chemopreventive agent in both pre- and post-menopausal women at a greater risk for developing breast cancer (Chang, 2012; Waters *et al.*, 2012).

While Tamoxifen usage remains the predominant gold standard adjuvant treatment strategy for hormone-responsive breast cancer, Anastrozole, an AI aimed at reducing the bioavailability of oestradiol has been shown to reduce the risk of developing venous thromboembolisms with a lower toxicity profile, and still able to reduce breast cancer recurrence (Hillner, 2004; Howell *et al.*, 2005). However, the risk for thrombotic complications remains elevated compared to that of tumour-free individuals, with AI use coupled with chemotherapy indicative of a higher risk of venous thromboembolism (Walker *et al.*, 2017). Additionally, patients undergoing Tamoxifen or Anastrozole treatment have presented with high endogenous thrombin potential as well as greater numbers of CD62P positive microparticles indicating a state of hypercoagulation (Trappenburg *et al.*, 2009).

Hormone therapies are known to downregulate oestrogen-mediated transcription of target genes associated with pro-tumourigenic processes; however, platelets themselves can secrete pro-tumourigenic growth factors (Viedma-Rodriguez *et al.*, 2014), indicating a role for platelet-induced activation of pro-tumorigenic gene transcription despite treatment options (Quail and Joyce, 2013). Furthermore, tumour cells are able to develop resistance to treatment options, particularly Tamoxifen, indicating not only continued manipulation of the tumour microenvironment but the haemostatic system as well furthering tumour progression (Martin *et al.*, 2000).

While targeted hormone-therapy treatment has shown promise in hormone-dependent breast cancer patients, its long-term effectiveness and increased risk of thromboembolic complications due to induced hypercoagulation are not encouraging. Further research is

required into the reciprocal interactions between platelet activation and hormone-therapy, which may affect future treatment strategies. As such this study focuses on the reciprocal interactions between platelets and hormone-dependent breast cancer cell lines (MCF7 and T47D) as shown by markers of coagulation, particularly platelet activation and thrombin release, and cell invasion profiles as evidenced by ER expression, mediated by hormone-therapies Tamoxifen and Anastrozole. This study thus presents the importance of considering the subphenotypes of Luminal A breast cancer, which are not individually categorised clinically. This study further assesses the genetic and protein marker expression of ER's, particularly of ER β , which is not well defined both clinically and *in vitro* in response to hormone therapy. Furthermore, while light transmission platelet aggregometry remains the gold standard for platelet function assessment (Ling *et al.*, 2017) as previously described by our laboratory, we present a calculation; the index of platelet activation (IPA) accounting for CD62P and CD63 expression in direct relation to the number of platelets expressing the marker (Augustine *et al.*, 2016), to identify platelet activation as an earlier event, preceding aggregation, which may impact tumour processes. This study also provides information on the pro-thrombotic effects of Anastrozole, the research on which is lacking.

1.7.1 Null Hypothesis

Reciprocal interactions between platelets and hormone-therapy treated breast cancer cells show no relationship to both platelet activation and alterations in the subphenotype of breast cancer cells.

1.7.2 Aim

The aim of this study was to investigate hormone-therapy, specifically Tamoxifen- and Anastrozole-, mediated interactions between platelets and hormone-dependent breast cancer cells in an *in vitro* environment.

1.7.3 Objectives

The objectives of this study assessed the effects of common hormone therapies on platelet activation and ER expression. These are presented in the forthcoming Chapters as follows;

1.7.3.1 To determine the effect of cumulative Tamoxifen and Anastrozole drugs on ER gene expression and their relationship to platelet activation. (Chapter 2).

This objective formed part of the MSc.Med portion of the study, which was converted to a PhD in 2018 with the addition of the objectives below.

- 1.7.3.2 To determine the effect of cumulative and circulating Tamoxifen on ER protein expression in hormone-dependent breast cancer cells and their relationship to hypercoagulation. (Chapter 3).
- 1.7.3.3 To determine the effect of cumulative and circulating Anastrozole effects on ER expression in hormone-dependent breast cancer cells and their relationship to hypercoagulation. (Chapter 4).

2 CHAPTER 2: Tamoxifen and Anastrozole compounds breast cancer induced early stage platelet activation and alter *ESR1* and *ESR2* expression *in vitro*.

2.1 Introduction

Breast cancer is of the most commonly diagnosed cancers, with associated therapies including hormone-therapy linked to an increased risk of thrombotic complications identified as the second main cause of cancer-associated mortality in patients (Meier and Jick, 1998; Decensi *et al.*, 2005; Howell *et al.*, 2005; Falanga, Russo and Verzeroli, 2013). Tamoxifen and Anastrozole are favoured hormone-therapies which target oestrogen induced proliferation, through varying mechanisms; either targeting the ER directly or the biosynthesis of E2 itself (Bonnetterre *et al.*, 2000; Carey *et al.*, 2006; Burstein *et al.*, 2019) and is associated with a greater risk for thromboembolic complications (Caine *et al.*, 2002; Bundred, 2005; Carter, 2015). Furthermore, interactions between tumour cells and platelets have been shown to induce thromboembolic complications in patients with malignant metastatic tumours (Karimi and Cohan, 2010). Studies indicate an increase in platelet number in cancer patients (Carpenter *et al.*, 2006) and hormone-therapy treatment associated with greater numbers of CD62P positive microparticles (Trappenburg *et al.*, 2009).

2.1.1 Breast Cancers and Cell Lines of the Luminal Phenotype

Breast cancers of the luminal phenotype are subdivided into Luminal A and Luminal B molecular subtypes, based on the presence of biomarkers and receptors (Tamimi *et al.*, 2008). The luminal A molecular subtype accounts for the 59% of diagnosed breast cancers (Fallahpour *et al.*, 2017). Due to their biomarker expression (ER-positive, PR high, HER2 negative and Ki67 low), Luminal A breast cancers are indicated as hormone-dependent and tend to have a better prognosis with chemotherapy and hormone-therapy treatment options, with lower recurrence rates (Carey *et al.*, 2006; Hashmi *et al.*, 2018).

Breast cancer is modelled using established cell lines, however, due to the immortalization of cancer cells and their ability to change their characteristics *in vitro*, continual re-

characterisation is proposed to ensure correct matching clinically (Mota *et al.*, 2017). Luminal A breast cancer cell lines commonly used in laboratory research include MCF7 and T47D breast cancer cell lines, with normal breast tissue represented by cell lines, including MCF10A (Mota *et al.*, 2017). The MCF7 breast cancer cell line is one of the most commonly used luminal A breast cancer cell lines in research (Burdall *et al.*, 2003). MCF7 cells express a range of sensitivities to oestrogen which allow for rapid proliferation when the cells are exposed to oestradiol (Hamelers *et al.*, 2003). The T47D breast cancer cell line is also a widely used cell line in laboratory studies, accounting for two-thirds of cell lines used in associated studies (Lacroix *et al.*, 2004). Furthermore, while both cell lines are derived from mammary cells, differences have been found in the receptor profile of the T47D cell line, with studies indicating the responsiveness of T47Ds to progesterone, while the MCF7 cell line did not elicit a response to progesterone in the presence of oestrogen (Yu *et al.*, 2017). While strong associations are made between cell line studies and the clinical context, their predictive value may be skewed due to the differential responses they may elicit in response to hormone-therapy treatment strategies, as well as their potential to activate platelets.

2.1.2 Oestrogen Receptors

ERs are members of the nuclear receptor superfamily and consist of two oestrogen receptors; ER α and ER β (Hartman, Ström and Gustafsson, 2009) encoded by the *ESR1* and *ESR2* genes, respectively (Lucas, Lazari and Porto, 2014). *ESR1* and *ESR2* are found on the sixth (6q25.1) and fourteenth (14q23.2) chromosomes, respectively (Menasce *et al.*, 1993; Enmark *et al.*, 1997; Ban *et al.*, 2008). Cell lines, including luminal A phenotype (MCF7 and T47D), have both *ESR1* and *ESR2* present, with an expression of ER α and ER β in both the nucleus and cytoplasmic regions (Ford *et al.*, 2011). The function of which is linked to the non-classical or non-genomic pathway (as opposed to nuclear ER functioning via the classical or genomic pathway). Studies indicate that these pathways may converge, or function in direct conflict either promoting or inhibiting tumour progression (Leonhardt *et al.*, 2003; Björnström and Sjöberg, 2004; Acconia and Kumar, 2006; Marino and Ascenzi, 2008). ERs are highly expressed by hormone-dependent breast cancers. Through progression and metastasis of the disease, ER expression profiles may alter, as a compensatory response to therapy (Thomas and Gustafsson, 2011). While information exists on the process of metastasis, there is minimal and contradictory literature highlighting the molecular alterations associated with breast

cancer progression and the role of ER signalling (Sgroi, 2010; Thomas and Gustafsson, 2011; Velloso *et al.*, 2017; Plava *et al.*, 2019).

ESR1 and ER α have been extensively studied in relation to breast cancer and have been shown to be overexpressed in the majority of hormone-dependent breast cancers (Ali and Coombes, 2000). Studies on *ESR1* amplification in metastatic breast cancers indicates its use as a prognostic marker of poor survival, which can be detected in blood samples (Clatot, Augusto and Di Fiore, 2017; Lei *et al.*, 2019). Clinical protein expression studies indicate that the presence of ER α in malignant breast tissue stimulates proliferation of breast cancer cells, this expression of ER α has also been associated with better prognosis in patients since they can be treated with hormone-therapies which affect the efficacy of oestrogen (Ali and Coombes, 2000). *ESR1* is detected at lower copy numbers in early stages of breast cancer compared to breast cancers undergoing metastasis and/or altered by hormone-therapy treatments (Clatot, Augusto and Di Fiore, 2017; Reinert, Gonçalves and Bines, 2018). Furthermore, *ESR1* mutations in the ligand binding domain of the ER, have been identified as a potential mechanism for hormone-therapy resistance associated with a more aggressive breast cancer phenotype. This is due to the mutant receptors inducing ER-dependent transcription and proliferation in the absence of hormones, reducing the efficacy of ER therapies (Clatot, Augusto and Di Fiore, 2017; Pejerrey *et al.*, 2018; Reinert, Gonçalves and Bines, 2018; De Santo *et al.*, 2019).

ER β has not been as extensively studied and is not currently used as a clinical indicator (Palmieri *et al.*, 2002; Austin *et al.*, 2018). ER β is expressed in both benign and malignant breast tissue but is expressed at lower levels in the majority of breast cancers (Palmieri *et al.*, 2002). Studies have highlighted that ER β can oppose the actions of ER α , with its downregulation linked to poor prognosis and resistance to hormone therapy (Lazennec *et al.*, 2001; Paruthiyil *et al.*, 2004; Rayner *et al.*, 2008; Madeira *et al.*, 2013). Furthermore, the presence of ER β in all breast cancer subtypes has been correlated with an improved prognosis (Mann *et al.*, 2001; Iwase *et al.*, 2003; Esslimani-Sahla *et al.*, 2004; Hopp *et al.*, 2004; Honma *et al.*, 2008), further confirmed in studies which assessed *ESR2* expression and indicated a protective role for the gene (Ghali *et al.*, 2018); as well as in *in vitro* studies which indicated that the presence of ER β in ER-positive breast cancer modulated and decreased cellular

proliferation and invasion (Lazennec *et al.*, 2001; Roger *et al.*, 2001; Strom *et al.*, 2004). Platelets are known to express both ER α and ER β , with ER β being the most predominant (Jayachandran *et al.*, 2005). Additionally, the expression of ERs in platelets have been shown to be susceptible to hormone-therapy. Specifically, E2 has been shown to reduce thrombotic events by reducing platelet response and is suggested as being protective against thrombosis (Jayachandran *et al.*, 2005). To assess *ESR1* and *ESR2* alterations in breast cancer cells, we made use of digital droplet PCR (ddPCR), a technique which employs the use of absolute quantification of gene expression, without requiring a standard curve (Day, Dear and McCaughan, 2013). Due to the robusticity and accuracy of the technique, reference genes are not commonly presented (Ståhlberg *et al.*, 2004). However, due to the possibility of error, including technical problems, the addition of stable reference genes may be included for data normalisation (Heredia *et al.*, 2013).

2.1.3 Common Hormone-Therapy Treatment Strategies

Hormone-dependent breast cancers of the Luminal A subtype are commonly treated using SERMs or AIs, which prevent oestrogen mediated proliferation and responses (Carey *et al.*, 2006; El Sayed *et al.*, 2019). The binding of oestrogen or progesterone to their respective receptors elicits pro-tumourigenic responses such as proliferation and differentiation in these tumours (Carey *et al.*, 2006; El Sayed *et al.*, 2019). Thus, the use of hormone-therapy, either SERMs or AI's, prevent the stimulation of these receptors, specifically ERs.

2.1.3.1 Tamoxifen

Tamoxifen, the most commonly used SERM, is an ER antagonist which binds to the ER itself and reduces hormone-induced proliferation (Onitilo *et al.*, 2012). Tamoxifen has been extensively investigated and has been shown to decrease tumour recurrence and mortality through its selectivity; however, it adversely increases a patient's risk of thrombotic events (Meier and Jick, 1998; Decensi *et al.*, 2005; Howell *et al.*, 2005; Jonat *et al.*, 2006; Lin *et al.*, 2018). The precise mechanism by which Tamoxifen exerts its procoagulant effect remains to be discovered (Onitilo *et al.*, 2012; Kovac *et al.*, 2015); however, recent studies implicate Factor VIII in promoting thrombotic events via its effect on platelet activation (Kovac *et al.*, 2015). The effects of Tamoxifen on ER α has also been widely studied with studies indicating better prognosis with the treatment of ER-positive breast cancers, however, evidence has

indicated a role for ER β expression as a predictive marker of Tamoxifen resistance in breast tumours (Esslimani-Sahla *et al.*, 2004; Hopp *et al.*, 2004; Fox, Davis and Shupnik, 2008; Honma *et al.*, 2008; Begam, Jubie and Nanjan, 2017). However, while clinical evidence indicates a role for Tamoxifen in thrombotic complications (Meier and Jick, 1998; Dobryднева *et al.*, 2007; Hernandez *et al.*, 2009; Onitilo *et al.*, 2012; Eroglu, 2013; Lin *et al.*, 2018), cell line studies have conversely indicated that Tamoxifen pre-treatment of platelets is able to reduce platelet-mediated breast cancer cell metastasis and further, indicated a reduction in the metastatic potential of MCF7 cells and inhibition MCF7 cell-induced platelet activation identified by P-selectin expression, at high concentrations (Johnson *et al.*, 2017).

2.1.3.2 Anastrozole

Anastrozole, one of the most commonly used AI's, inhibits the synthesis of oestrogen (Mokbel, 2002; Kümler *et al.*, 2016). *ESR1* mutations, particularly those in the ligand binding domain, has been associated with complete resistance to AI's, with only partial resistance to Tamoxifen noted (Jeselson *et al.*, 2014; Fribbens *et al.*, 2016; De Santo *et al.*, 2019). While AI effects have been studied with relation to *ESR1*, its effects on *ESR2* have not been well defined, neither has the effect of Anastrozole, specifically. Furthermore, clinical studies have shown that patients being treated with Anastrozole have a reduced risk of developing venous thromboembolisms, compared to the risk associated with cancer itself (Fennerty, 2006; Fabian, 2007; Gonnelli and Petrioli, 2008), however, the risk remains higher than that of healthy individuals; and studies on its' effect on platelet function and responses lacking.

2.1.4 Tumour Cells and Platelet Engagement

During the metastatic process mesenchymal tumour-like cells enter the vasculature, initiating intravasation, then once in the bloodstream tumour cells are able to engage with platelets activating and recruiting them (Li *et al.*, 2014). The induction of platelet activation by tumour cells in the vasculature allows for the formation of a heterotypic aggregate between the cells and serves as a mechanism for avoiding immune surveillance (Jurasz, Alonso-Escolano and Radomski, 2004; Bambace and Holmes, 2011; Elaskalani *et al.*, 2017). Tumour cells facilitate this engagement through secretion of prothrombotic mechanisms such as thrombin, TF, procoagulant and inflammatory cytokines (Nash *et al.*, 2002; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2014). Tissue factor is a platelet activator, whereas the secretion of

procoagulant and inflammatory cytokines increases the adhesion properties of platelets (Falanga, Russo and Verzeroli, 2013). These secreted agonists enable tumour cell arrest in the circulation and facilitate extravasation at secondary sites, promoting metastasis (Jurasz, Alonso-Escolano and Radomski, 2004; Bambace and Holmes, 2011).

Activated platelets secrete cytokines and growth factors, such as TGF- β , which are involved in tumour progression (Sierko and Wojtukiewicz, 2004; Goubran *et al.*, 2013). Upon activation, platelets undergo rapid ultrastructural alterations and secretion of growth factors allowing for adhesion to, as well as stimulation of, the endothelium (Kuwahara *et al.*, 2002; Wagner and Burger, 2003; Augustine *et al.*, 2016). Early platelet activation begins with the folding and budding of the platelet membrane, these folds begin to elongate forming lamellipodia, filipodia and pseudopodia, extending outward from the platelet, initiating the dendritic phase of activation (Gresele and Page, 2002; Kuwahara *et al.*, 2002). This phase is followed by further extension and final release of granular contents, including membrane proteins (e.g. GPIb complex, GPIIb/IIIa, P-selectin), coagulation factors (e.g. Factor's V, XI, XIII; fibrinogen and von Willebrand factor), growth factors involved in inflammation and angiogenesis, and chemokines; from the OCS facilitating further adhesion and aggregation, prior to the final or spread phase (Kuwahara *et al.*, 2002; Augustine *et al.*, 2016). The release of these granular components not only further facilitates platelet aggregation but play a role in tumour cell proliferation (Wagner and Burger, 2003). Previous studies on platelets in cancer patients have shown that an increase in platelet number compared to those of healthy people (Carpenter *et al.*, 2006).

2.1.4.1 Platelet Markers

Platelets are studied and identified by surface markers. CD41a is the most prominent cell surface antigen expressed on platelets (Rivière *et al.*, 1999; Saboor, Moinuddin and Ilyas, 2013; Riedl *et al.*, 2016). CD41a is a marker for the GPII β / α III glycoprotein complex, associated with platelet-leukocyte adhesion and fibrinogen mediated adhesion (Betz *et al.*, 1992; Spangenberg *et al.*, 1993). CD62P is a surface antigen expressed on the cell membrane of α -granules, as well as the surface of activated endothelial cells and facilitates platelet adhesion and initial recruitment of leukocytes (McNicol and Israels, 2008). Early in the activation process CD62P translocates from the membrane of the α -granule to that of the platelet body

allowing it to be used as an identifying marker for activated platelets (McNicol and Israels, 2008; Augustine *et al.*, 2016). Agonists such as thrombin, type II collagen and ADP (Adenosine Diphosphate) induce the translocation of CD62P, with studies also indicating a role for a calcium independent pathway in its release (Chen and Geng, 2006; Wang *et al.*, 2016). Due to its expression on both endothelial cells and platelets, CD62P plays a functional role in cancer metastasis by facilitating invasion and recruiting and forming aggregates with tumour cells and leukocytes in the vasculature, preventing immunological recognition (Chen and Geng, 2006).

2.1.4.2 Platelet-derived Microparticles

Platelets further aid cancer cell migration and metastasis with the release of microparticles (Zarà *et al.*, 2018). During the early phase of platelet activation, the membrane begins to bud producing extracellular vesicles referred to as microparticles when derived from platelets and play a role in inflammatory processes (Buzas *et al.*, 2014). Platelet-derived microparticles primarily occur when the lamellipodia, filipodia and pseudopodia become adherent indicative of the final phase of platelet activation, in which a loss of markers associated with adhesion is noted, including that of actin and CD62P (Boilard, Ducheze and Brisson, 2015). These markers, now expressed by microparticles, influence coagulatory processes and further facilitate platelet activation and recruitment in disease states (Magnus *et al.*, 2014; Boilard, Ducheze and Brisson, 2015; Melki *et al.*, 2017). In cancer, platelet-derived microparticles act as pro-inflammatory molecule carriers and contribute to tumourigenesis (Varon and Shai, 2009; Ponomareva *et al.*, 2017). Studies have found that breast cancer cell lines with different metastatic potential are able to stimulate platelets to release microparticles, which could affect the metastatic aggressiveness of cancer cells by regulating migration and invasion (Zarà *et al.*, 2018). Additionally, Tamoxifen and Anastrozole treatment have been associated clinically with high endogenous thrombin potential as well as greater numbers of CD62P positive microparticles (Trappenburg *et al.*, 2009).

2.1.5 Hormone-therapy in breast cancer and thrombosis

Breast cancer itself is associated with hypercoagulability, with therapies including chemotherapy, surgery and hormone-therapy linked to an increased risk of thrombotic complications (Muthuvel Jayachandran and Miller, 2003; Kedzierska *et al.*, 2013; Paulus and

Rosenberg, 2016; Walker *et al.*, 2017). Furthermore, changes in ERs in response to hormone-therapies, may relate to the metastatic potential of breast cancer cells, in which prothrombotic cytokines and thrombin would be released, activating platelets to facilitate tumour progression (Falanga *et al.*, 2013).

Tumour cells can induce thrombosis through prothrombotic mechanisms; tumour cell induction of platelet activation precedes and as such, facilitates tumour cell-induced platelet aggregation (TCIPA) (Nash *et al.*, 2002; Jurasz, Alonso-Escolano and Radomski, 2004; Bambace and Holmes, 2011; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2014). TCIPA indicates the crucial role platelets play in the metastatic process, where they act as a physical shield, protecting tumour cells from immunosurveillance and shear forces, and further allowing for adherence to the vascular wall and extravasation, indicating a more complex role for platelets in metastasis (Jurasz, Alonso-Escolano and Radomski, 2004; Labelle, Begum and Hynes, 2011). Clinically, elevated platelet counts, platelet aggregation, and heightened levels of P-selectin have been proposed as risk factors for venous thromboembolism in cancer patients (Weber *et al.*, 2002; Mezouar *et al.*, 2016). These interactions between breast cancer cells and platelets have been highlighted by experimental animal models showing that inhibition of platelet aggregation results in significantly reduced metastases (Mezouar *et al.* 2016; Wenzel *et al.* 2010; Gebremeskel *et al.* 2015).

Hormone therapies, such as Tamoxifen and Anastrozole, are also known to downregulate oestrogen mediated transcription of target genes associated with pro-tumourigenic processes; however, platelets themselves are able to secrete pro-tumourigenic growth factors, such as VEGF and TGF β (Viedma-Rodriguez *et al.*, 2014). As such, tumour cell-induced activation of platelets could potentially allow for non-classical methods of gene transcription opposing downregulation elicited by hormone-therapy (Quail and Joyce, 2013). Since ER-positive tumours have been shown to downregulate ER during the metastatic process, understanding this regulation by using targeted approaches including that of modulating the receptor directly (Tamoxifen), or reducing the bioavailability of oestradiol (Anastrozole), is thus essential to successful management of patient treatment regimens (Aktas *et al.*, 2011). This highlights the manipulative nature of tumour cells, in which they are capable of developing resistance to treatment strategies and their recruitment of cells within the

haemostatic system, which may still allow for metastatic processes that may not be readily clinically detectable (Martin *et al.*, 2000).

Thus, this study assessed the effect of Tamoxifen and Anastrozole on ER gene expression and their relationship to platelet activation *in vitro*. We employed a model in which breast cancer cells were pre-treated with either Anastrozole or Tamoxifen and co-incubated with whole blood. Pre-treatment of breast cancer cells allows for mimicking of the cumulative effect of the drugs, as would occur *in vivo*, followed by exposure to whole blood. Furthermore, this chapter focuses on the effects of Tamoxifen or Anastrozole treatment on; the gene expression of ERs and ensuing effects on early stage platelet activation identified by CD62P expression, with corresponding platelet morphological alterations.

2.1.6 Aims and Objectives

The aim of this part of the study was to determine the effect of common hormone-therapeutic drugs on ER gene expression and their relationship to platelet activation. This, in order to assess the interactions between platelets and hormone therapy-treated breast cancer cells, the objectives were:

2.1.6.1 To assess *ESR1* and *ESR2* gene expression alterations in breast cancer cells.

2.1.6.2 To assess induced levels of platelet activation defined by CD62P (P-selectin) expression interpreted by the Index of Platelet Activation (IPA) and confirmed ultrastructurally.

2.1.6.3 To assess the correlation between *ESR1* and *ESR2* gene alterations and platelet activation, to determine whether ER gene profiles may hold predictive value in determining associated platelet activation.

2.2 Materials and Methods

Human Research Ethics Clearance was obtained from the Human Ethics Research Committee (Medical), University of the Witwatersrand, Clearance Certificate Number M160826 (Refer to Appendix for Clearance Certificate).

2.2.1 Methodology Framework – in brief

To conduct the methodology for this aim, the optimal cell number of each cell line (MCF7 and T47D) were determined. This was done by testing plated concentrations of each cell line and analysing the release of lactate dehydrogenase (LDH) by making use of an LDH Cytotoxicity assay. The optimal cell number for both MCF7 and T47D cell lines was 1×10^5 cells/well. Following this, drug concentrations of Anastrozole and Tamoxifen were tested at ranges equivalent to the literature on 1×10^5 cells/well to determine the optimal drug concentrations for ensuing experimentation; optimal drug concentrations were 1 μ M for Anastrozole and 2 μ M for Tamoxifen (Figure 2.2A). MCF7 and T47D cells were plated and treated with either Anastrozole and Tamoxifen and co-incubated with whole blood for 2.5 minutes. Following co-incubation breast cancer cells and whole blood were isolated and prepared for their ensuing techniques (Figure 2.2B).

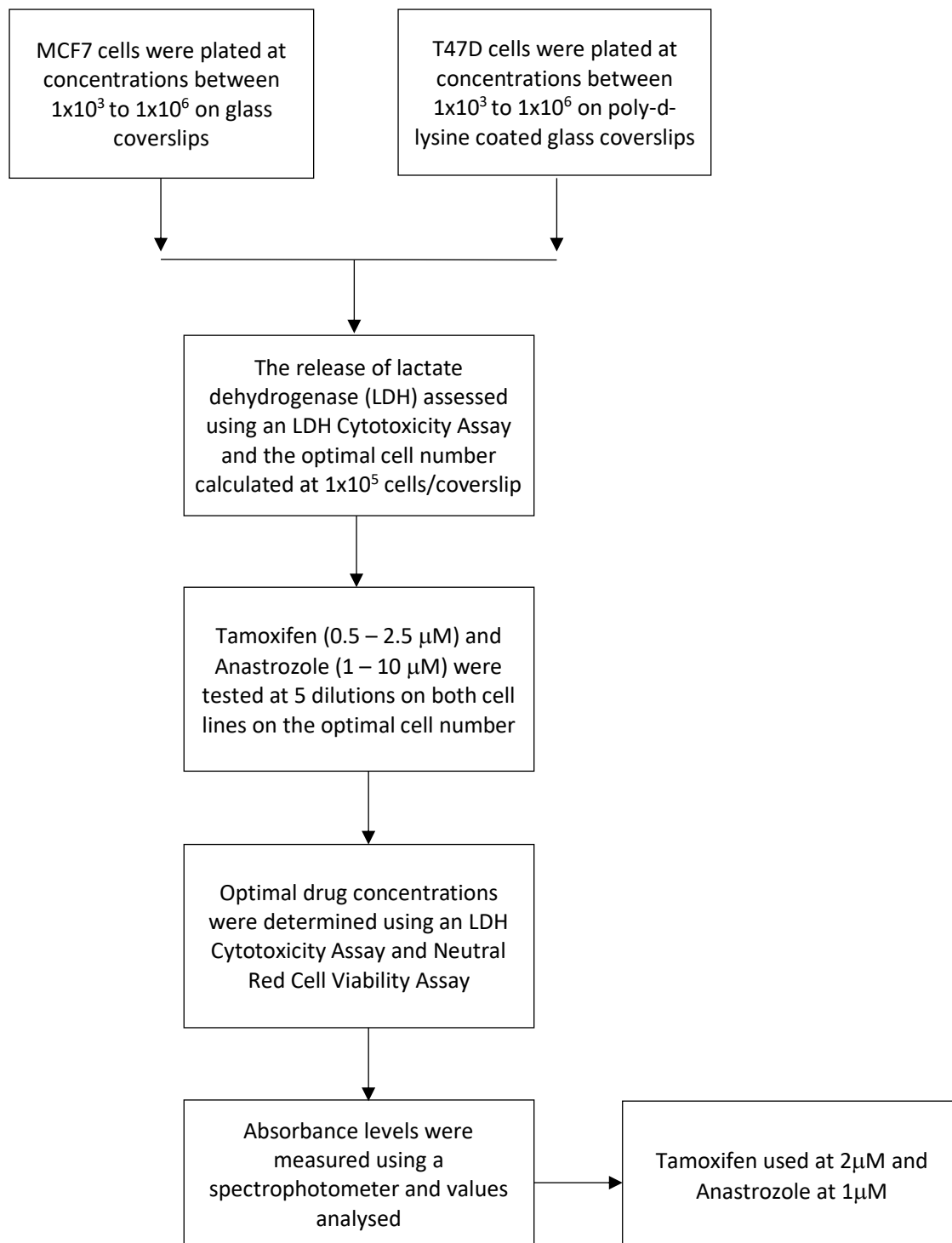


Figure 2.1: Flow diagram indicating the stepwise methodology followed to obtain the optimal cell numbers of MCF7 and T47D breast cancer cells for experimentation by making use of a Lactate Dehydrogenase (LDH) Cytotoxicity Assay; followed by the assessment of drug concentrations of Anastrozole and Tamoxifen by making use of both the LDH Cytotoxicity Assay and Neutral Red Cell Viability Assay. Anastrozole was indicated for use at 1 μM and Tamoxifen at 2 μM .

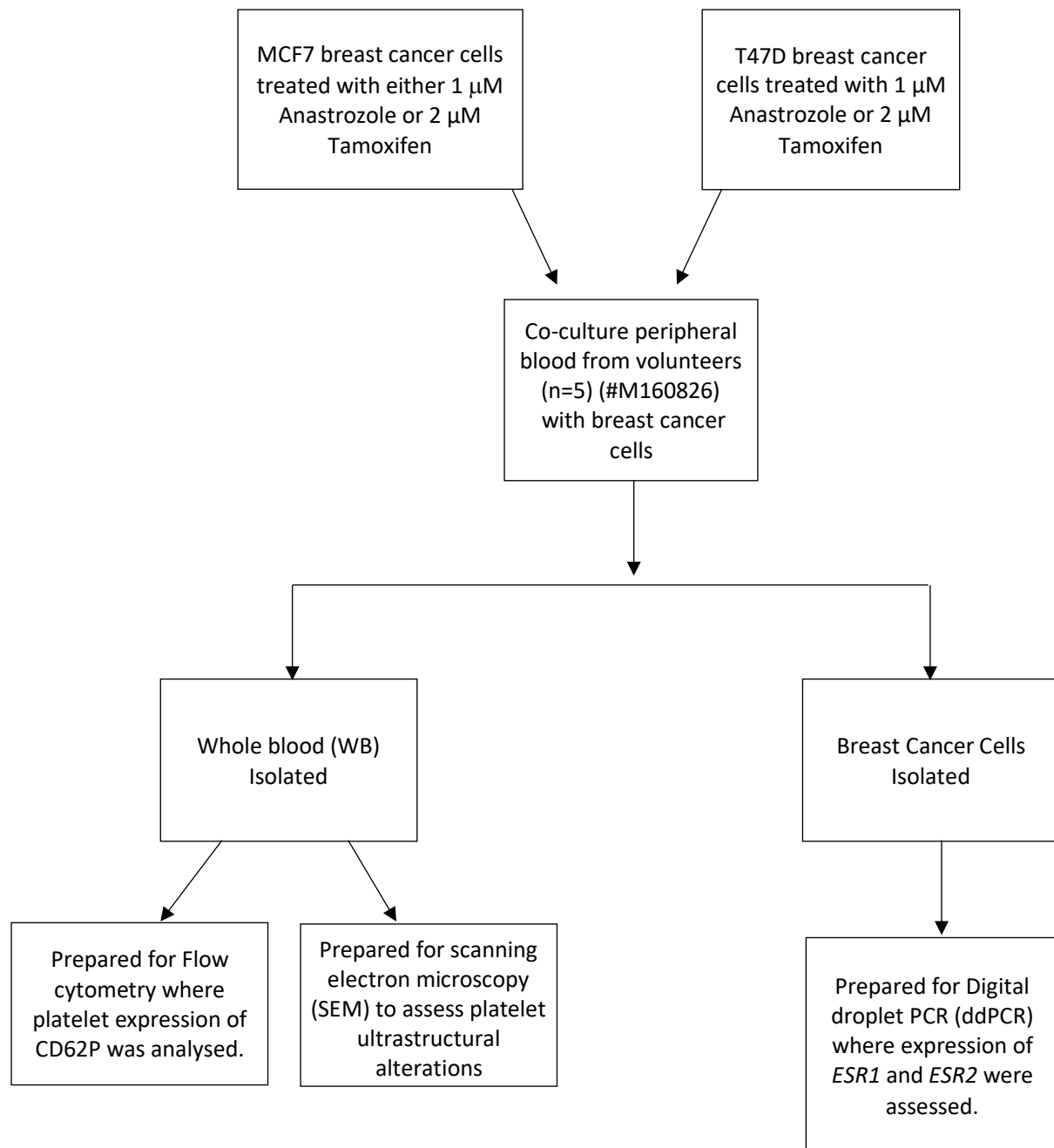


Figure 2.2: Flow diagram indicating the methodology followed for experimentation. Optimal numbers of MCF7 and T47D breast cancer cells were treated with corresponding concentrations of Tamoxifen (2 μ M) or Anastrozole (1 μ M). Whole blood obtained from seemingly healthy female volunteers was exposed to pre-treated breast cancer cells (MCF7 or T47D) for 2.5 minutes; following which whole blood and breast cancer cells were isolated and prepared for ensuing techniques. Whole blood was prepared for flow cytometry and scanning electron microscopy, to assess levels of platelet activation. Breast cancer cells were prepared for digital droplet PCR, where oestrogen receptor gene expression was assessed.

2.2.2 Cell Culture and Establishment of Stock Populations

Luminal phenotype MCF7 and T47D breast cancer cells were obtained from American Type Culture Collection (ATCC) (Virginia, USA) were cultured in 75 cm² Nunc (Greiner Bio-one, Kremsmünster, Austria; 658170) culture flasks to passage numbers of 36 (MCF7) and 29

(T47D). MCF7 cells were propagated in DMEM (Dulbecco's Modified Eagles Medium; Lonza, Basel, Switzerland; BE12-604F), supplemented with 10% FBS (Foetal Bovine Serum, Thermo Fisher Scientific, Waltham, USA; 10499044) and 0.1% P/S (Penicillin/Streptomycin, Lonza, Basel, Switzerland; OMB 206). T47D cells were propagated in RPMI (Roswell Park Memorial Institute medium, Lonza, Basel, Switzerland; BE12-115F) supplemented with 0.2 U/ml bovine insulin, 10% FBS and 0.1% P/S. Cells were incubated at 37°C and 5% CO₂.

Once cells reached ~70-90% confluency, cells were washed with 1 M Phosphate Buffered Saline (PBS), pH 7.4. For subculture, cells were incubated in 3 ml 0.25% Trypsin/EDTA (Thermo Fisher Scientific, Waltham, USA) for 5 minutes. Thereafter, 3 ml complete respective medium was added to each flask to stop enzymatic effects. Cells were then transferred to 15 ml centrifuge tube (Nunc, Roskilde, Denmark) and centrifuged for 5 minutes at 400xg using a Rotafix 32A centrifuge (Hettich, Germany).

Following the establishment of stock populations, MCF7 cells were cryopreserved in 10% DMSO (Dimethyl sulfoxide, Merck, Johannesburg, South Africa), with 60% FBS and 30% DMEM; and T47D cells cryopreserved in 10% DMSO, with 60% FBS and 30% RPMI, at -80°C for subsequent experimentation.

2.2.3 Optimal Cell Number Determination

In order to determine the optimal cell number of each cancer cell line (MCF7 and T47D), a lactate dehydrogenase (LDH) cytotoxicity assay (ScienCell Research Laboratories, Carlsbad, USA) was conducted. Cryopreserved MCF7 and T47D breast cancer cells were thawed and washed once in 1 ml 1 M PBS, centrifuged at 400xg, followed by resuspension in 1 ml of their respective cell culture media. Ten microliters (10 µl) of the cell suspension was mixed with 0.4% trypan blue at a ratio of 1:1 and counted using a TC20 Automated Cell Counter (Biorad, Hercules, CA, USA) to determine the total number of cells, live cells and percentage cell viability. Cells with a percentage viability greater than 85% were used for subsequent experimentation. Cell numbers of: 1×10^3 , 3×10^3 , 7×10^3 , 15×10^3 , $3,1 \times 10^4$, $6,25 \times 10^4$, 1×10^5 , $2,5 \times 10^5$, 5×10^5 , and 1×10^6 per well, was tested for each cell line.

Both MCF7 and T47D cells were seeded in duplicate, directly into the 24 well plate (Lasec, Cape Town, South Africa; 662160). Cells were incubated in their respective media overnight. The plate was thereafter centrifuged at 400xg for 5 minutes and 150 µl supernatant transferred into a corresponding 96 well plate (AEC Amersham, Kyalami, South Africa, 167008). The working reaction mix, which contains sodium lactate, INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride), substrate mix and 1 M PBS, was added to each well and incubated for 20 minutes at room temperature (RT) in the dark; following which sodium oxymate solution was added to stop enzymatic activity. Controls included: media control, background control, serum-free media control and a positive control for cell death (cells exposed to 1% Triton X-100) for 24 hours. The LDH released from the cells was analysed using a spectrophotometer (Multiskan Ascent Plate Reader, MTX Lab Systems, Virginia, USA) at the detection wavelength of 492nm and the reference wavelength of 680nm. The results were exported into Microsoft Excel and further analysed. The following formula was used to calculate cytotoxicity (Smith *et al.*, 2011) (Refer to Appendix for calculations and result):

Cytotoxicity (%)

$$= \frac{(\text{compound} - \text{treated LDH activity}) - (\text{media control LDH activity})}{(\text{high control LDH activity}) - (\text{media control LDH activity})} \times 100$$

The optimal cell number for both cell lines was determined to be 1×10^5 cells per well, which was used for ensuing drug (Anastrozole and Tamoxifen) concentration assessment, and throughout further experimentation.

2.2.4 Drug Concentration Assessment of Anastrozole and Tamoxifen

Concentrations of Anastrozole (Sigma Aldrich, Saint Louis, USA; A273) and Tamoxifen (Sigma Aldrich, Saint Louis, USA; T5648), were tested using an LDH cytotoxicity assay and a Neutral Red (NR) cell viability assay. Tamoxifen and Anastrozole were tested at ranges from 0.5 – 2.5µM and 1 – 10µM, respectively, which correlates with other cellular studies conducted in which concentrations mimicked that of treatment regimens given to women with breast cancer (Jeng, Langan-Fahey and Jordan, 1993; Karami-Tehrani and Salami, 2003; Kijima, Itoh and Chen, 2005; Pawlak and Wiebe, 2007; Hassan *et al.*, 2017). Stock solutions of both Anastrozole and Tamoxifen were prepared at 1.0 mM diluted in DMSO, further diluted to

0.1% in respective normal media for each cell line, in order to obtain the following final concentrations; 0.5 µM, 1 µM, 1.5 µM, 2 µM and 2.5 µM for Tamoxifen and 1 µM, 2 µM, 4 µM, 8 µM and 10 µM for Anastrozole.

2.2.4.1 LDH Cytotoxicity Assay

The LDH cytotoxicity assay was conducted according to manufacturer's instructions. MCF7 and T47D cells seeded at a concentration of 1×10^5 cells per well in a 24 well plate, were incubated with their respective media for 24 hours and then treated with 0.5 µM, 1 µM, 1.5 µM, 2 µM and 2.5 µM of Tamoxifen and 1 µM, 2 µM, 4 µM, 8 µM, 10 µM of Anastrozole, diluted in their respective media, for 24 hours. The plate was thereafter centrifuged at 400xg for 5 minutes and the methodology and analysis conducted as that for 2.2.3 (Refer to Appendix for stock concentrations).

2.2.4.2 Neutral Red Cell Viability Assay

The initial cell seeding, and treatment protocol was followed as per 2.2.4.1. Cells were subsequently rinsed with 1 M PBS three times and incubated with 100 µg/ml neutral red dye (Gurr BDH Chemicals, Poole, England, P34056) in appropriate culture media for 3 hours at 37°C and 5% CO₂. Culture media was removed, and cells were rinsed twice with 1 M PBS. Neutral red dye diluted in 1% acetic acid in 50% ethanol, was thereafter added to the wells. The plate was then placed on a microplate shaker at RT for 1 hour. The supernatant was then transferred into corresponding wells in a 96 well plate and absorbance levels measured using a spectrophotometer at the detection wavelength of 540nm and the reference wavelength of 690nm. The serum-free media control was subtracted from all absorbance values (corrected absorbance) and cell viability was calculated (Borenfreund and Puerner, 1985; Fautz, Husein and Hechenberger, 1991; Ates *et al.*, 2017). The following formula was applied (Refer to Appendix);

$$\text{Viability (\%)} = \frac{\text{corrected absorbance}}{\text{media control}} \times 100$$

To determine the repeatability of the experimentation of both LDH and NR assays, the coefficient of variance (CV) was calculated. The CV between concentrations for each run was

found to be less than 0.5%; indicative of the precision and repeatability of the experimentation (Bartlett and Frost, 2008).

2.2.5 Whole Blood Acquisition

Healthy female volunteers between the ages of 19 – 30 years old and who were between days 1 – 10 of their menstrual cycle were asked to participate in this study. The volunteer exclusion criteria included: pregnancy, contraceptive use, previously identified autoimmune diseases or immunodeficiency, previous history of cancer, cancer, smoking, and consumption of anti-platelet and/or anti-coagulation medication (e.g. aspirin and warfarin) in the previous 72 hours. Blood was collected between days 1 – 10 of the menstrual cycle due to the lower levels of oestrogen and progesterone present within the circulating blood (Reed and Carr, 2000), therefore lessening these hormonal effects on the breast cancer cells (Refer to Appendix for participant information sheet and signed volunteer participation forms).

Peripheral whole blood (WB) (n=5) was collected in three 3.2% sodium citrate vacuette™ coagulation tubes (Lasec, South Africa). The first 2ml of blood drawn was discarded to exclude the effect of mechanically activated platelets (Leytin *et al.*, 2000).

2.2.6 Establishment of a co-culture system

Cells (MCF7 and T47D) were plated at 1×10^5 cells/well in 200 μ l of their respective media for 24 hours at 37°C and 5% CO₂ to facilitate cellular adherence. Coverslips were thereafter rinsed with 1 M PBS and pre-treated with 200 μ l 1 μ M Anastrozole or 200 μ l 2 μ M Tamoxifen for 24 hours at 37°C and 5% CO₂. Cells were rinsed twice with 1 M PBS and incubated with 200 μ l WB for 2.5 minutes. For generation of a positive control for accurate assessment of platelet activation, lysed WB was incubated with 0.1 U/ml of human thrombin- α (SANBS, South Africa), a platelet agonist, for 5 minutes to induce platelet activation (Augustine *et al.*, 2016). All samples, experimental and control, were prepared in duplicate for each subsequent assay.

Table 2.1: Control and experimental samples, with the abbreviation indicating the sample key used in ensuing analysis. Prefix: M: MCF7 breast cancer cells. T: T47D breast cancer cells. Suffix: treatment options employed

Blood Constituent Controls	
Abbreviation	Explanation
WB	Whole Blood
WBThr	Positive control: Whole blood incubated with 0.1 U/mL human thrombin
Cell Line Controls	
Abbreviation	Explanation
MMED	MCF7 breast cancer cell media control
MMEDWB	MCF7 breast cancer cells exposed to whole blood
MDIL	MCF7 breast cancer cells treated with 0.1% DMSO
MDILWB	MCF7 breast cancer cells treated with 0.1% DMSO exposed to whole blood
MANAS	MCF7 breast cancer cells treated with 1 μ M Anastrozole
MTAM	MCF7 breast cancer cells treated with 2 μ M Tamoxifen
TMED	T47D breast cancer cell media control
TMEDWB	T47D breast cancer cells exposed to whole blood
TDIL	T47D breast cancer cells treated with 0.1% DMSO
TDILWB	T47D breast cancer cells treated with 0.1% DMSO exposed to whole blood
TANAS	T47D breast cancer cells treated with 1 μ M Anastrozole
TTAM	T47D breast cancer cells treated with 2 μ M Tamoxifen
Experimental	
Abbreviation	Explanation
MANASWB	MCF7 breast cancer cells pre-treated with 1 μ M Anastrozole exposed to whole blood
MTAMWB	MCF7 breast cancer cells pre-treated with 2 μ M Tamoxifen exposed to whole blood
TANASWB	T47D breast cancer cells pre-treated with 1 μ M Anastrozole exposed to whole blood
TTAMWB	T47D breast cancer cells pre-treated with 2 μ M Tamoxifen exposed to whole blood

2.2.7 Assessment of *ESR1* and *ESR2* expression

2.2.7.1 RNA Extraction

Following co-incubation with WB, cells were rinsed 3 times with 1 M PBS. Total cellular RNA was extracted using the RNeasy Mini-Kit (Qiagen), as per manufacturers recommendation (Refer to Appendix for optimisation). Cells were incubated with beta-mercaptoethanol diluted in RLT (Lysis) buffer and homogenised with a 21-gauge needle fitted into an RNase-free syringe. Duplicate samples were pooled. The reaction was halted by the addition of 70%

ethanol and lysed cells transferred to a RNeasy spin column. Samples were centrifuged at 14534 x *g* for 15 seconds and the flow-through discarded. On column DNase digestion was conducted for 15 minutes, following which samples were centrifuged briefly and the flow-through discarded. Thereafter, 500 µl of washing buffer (RPE) was added to the columns, centrifuged at 14534 x *g* for 15 seconds and the flow-through discarded, and repeated once more. 30 µl of RNase free water was added directly to the spin column filter and samples were centrifuged at 14534 x *g* for 1 minute to elute RNA. The RNA elute was placed in a microfuge tube and stored at -80°C.

RNA concentration and quality were determined by a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The Nanodrop was first “blanked” using 1 µl RNase-free water, following which 1 µl of sample of RNA was analysed. The ratios of sample absorbance at 260nm and 280nm, and 260nm and 230nm were measured. Both ratios are indicative of RNA purity; a ratio of 2 indicates higher RNA purity, while anything lower indicates sample contamination (Desjardins and Conklin, 2011). RNA integrity (RINs) was analysed using an RNA 600 Pico Kit (Agilent, Johannesburg; 5067-1513) and Bioanalyzer 2100 (Agilent Technologies). All RINs values obtained were between 8 – 10 (Refer to Appendix for values obtained), indicating a high integrity value for RNA samples used in the ensuing experimentation

2.2.7.2 cDNA synthesis

Reverse transcription was conducted using the High Capacity cDNA Archive Kit (Applied Biosystems, USA, #4368813), as per manufacturer’s recommendations. 100 ng of total RNA in 10 µl of RNase free water was combined with 10 µl of the reverse transcription master mix; which consisted of 2 µl buffer, 0.8 µl dNTP mix (100 mM), 2 µl RT Random Primers, 4.2 µl nuclease free water and 1 µl Multiscribe reverse transcriptase; to a final volume of 20 µl per microtube. Microtubes were centrifuged briefly to eliminate air bubbles and placed in an MJ Mini Personal Thermocycler (Applied Biosystems, CA, USA) (Refer to Appendix for parameters used). cDNA was stored in microtubes at -20°C.

2.2.7.3 Digital Droplet PCR (ddPCR)

Reference genes (Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Ribosomal Protein Lateral Stalk Subunit P0 (*RPLP0*) and β -Actin (*ACTB*)) were optimised on both cell lines using ddPCR (Refer to Appendix for Reference Gene optimisation). *ACTB* was selected as a “reference gene” for our study as it was amplified in both cell lines, while *GAPDH* was only amplified within the T47D cell line and *RPLP0* in MCF7 cells. ddPCR allows for the absolute quantification of target genes without reference to a standard curve, minimizing the effects of matrix differences between calibrator and sample resulting in greater accuracy of the technique itself (Ståhlberg *et al.*, 2004). *ESR1* and *ESR2* primers were designed and optimised on both cell lines using the NCBI Primer-BLAST method, tested on the Applied Biosystems 7500 qPCR machine and subsequently confirmed using ddPCR as per MIQE guidelines (Bustin *et al.*, 2009; Whale *et al.*, 2020) (Table 2.3).

cDNA at a final concentration of 5 ng/ 20 μ l, was combined with optimised *ACTB*, *ESR1* and *ESR2* primers at 100 nM (Table 2.3) and Bio-Rad ddPCR supermix. Droplets were generated by the QX200 Droplet Generator to create an emulsion of monodispersed droplets which were transferred to a 96-well plate for PCR amplification. Thereafter the plate was loaded into the QX200 droplet reader which analysed each droplet individually using a two-colour detection system, allowing for an absolute count of the positive and negative droplets per sample. Controls: a no template control (NTC) was included on all plates to rule out any cross-contamination and primer-dimer formations which would result in false positives, as well as a non-reverse transcriptase control (NRT) to account for any DNA contamination which may have occurred during RNA preparation.

Table 2.2: Primer designs for each gene of interest; *ESR1*, *ESR2* and *ACTB*. Primers were designed using Primer-BLAST.

Gene	Name	Primers (5'→3')	GenBank Accession Number	Amplicon Lenth
ESR1	ER α	F: TATGTGTCCAGCCACCAACC R: TCGGTCTTTTCGTATCCCACC	NG_011535.1	121 bp
ESR1	ER β	F: CGGCAGACCACAAGCCCAAATG R: CGATCTTGCTTCACACCAGGGAC	NG_008493.2	114 bp
ACTB	β -actin	F: CTTCCAGCCTTCCTTCCTGG R: CTGTGTTGGCGTACAGGTCT	NM_001101.3	110 bp

2.2.7.4 Data Analysis

The QX200 Quantasoft software generated absolute quantification of *ACTB*, *ESR1* and *ESR2*. Data from experiments (n=5) was exported into Microsoft Excel for data management and exported into Statistica v23 for further analysis. A Shapiro-Wilk test of normality determined that the data was not normally distributed. The non-parametric Friedman's test was conducted to determine whether gene expression altered in response to hormone-therapy, following which a *post-hoc* Wilcoxon Signed Ranks test was conducted to determine whether these responses were significant ($p < 0.05$).

2.2.8 Assessment of platelet activation

This technique follows on from [2.2.6](#) Establishment of a co-culture system.

2.2.8.1 Whole Blood Sample Preparation for flow cytometry

Following co-incubation of pre-treated cells with WB, WB was removed and samples prepared according to Augustine et al. 2016. To analyse platelets within WB, erythrocytes were lysed with 3 ml ammonium chloride (NH₄Cl) buffer for 10 minutes at RT. Samples were centrifuged at 200xg for 5 minutes and the supernatant discarded. The resulting pellet was resuspended in 150 μ l Tyrode's buffer (Refer to Appendix for composition) and samples double-labelled with allophycocyanin (APC)-conjugated mouse anti-human CD41a (BD Pharmingen, CA, USA; 559777) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD62P (BD Pharmingen, CA, USA; 555523) at a concentration of 1:15, for each antibody, as determined as the optimal antibody concentration via titration. Samples were then fixed in 1% paraformaldehyde (ACE, Southdale, South Africa, 26276) at a ratio of 1:1 for 10 minutes.

Samples were subsequently washed with 1 ml Tyrode's buffer and centrifuged at 200xg for 5 minutes. The supernatant was discarded, and the resulting pellet resuspended in 500 µl Tyrode's buffer, which was kept at 4°C on ice overnight prior to data acquisition on the LSR Fortessa (BD Biosciences, CA, USA) in the Department of Surgery, University of the Witwatersrand.

2.2.8.2 Gating Strategy and Data Acquisition

Prior to flow cytometry experimentation, compensation for spectral overlap was first conducted on the LSR Fortessa (BD Biosciences, CA, USA) using FACSDiva software. Following which, antibody titration was conducted to ensure accurate measurement of fluorescence and to minimize noise due to non-specific binding of the antibodies (Refer to Appendix for optimisation).

Events were recorded at 100 000 events per sample using FACSDiva Software (BD Biosciences). The platelet population was initially gated on size and granularity, using a forward scatter voltage of 300V and a side scatter voltage of 275V. This was followed by a second gate placed around the singlet population identified in the forward scatter height and forward scatter area, both at a voltage of 300V. Platelets within the gated sample were identified and further gated based on the expression of the CD41a a marker for the GPIIb/αIII glycoprotein complex present on all platelets, denoting the parent population, and separated using the APC voltage at 565V. CD62P, a platelet activation marker, was discriminated by making use of the FITC voltage at 469V. Interval gates (Augustine *et al.*, 2016) were drawn to classify a graded level of platelet activation determined using Geometric Mean Fluorescence Intensity (gMFI) of the platelet population (Figure 2.2).

The interval gating strategy employed allowed mapping of the spread of CD62P, in addition to ascertaining total CD62P expression with a right shift along the X-axis reflecting higher levels of CD62P expression (Figure 2.2). Quadrants: Q1 represents the inactive platelet population (CD41a⁺CD62P⁻) population, while the activated population of interest (CD41a⁺CD62P⁺) was within the Q2 quadrant. Q3 represents other cell types within the sample (CD41a⁻CD62P⁻), while Q4 is postulated to present CD62P⁺ microparticles within the

sample. Interval gates (I1-I5) were drawn within the Q2 quadrant, indicative of increasing CD62P expression. No expression was noted in interval gates beyond I5 for all samples, and these gates were thus disregarded in the calculation of the IPA. Interval 1 and 2 (I1 and I2) represents the bulk of CD41a⁺ platelets with low CD62P expression (CD41a⁺CD62P⁺), while interval gates 3 and 4 (I3 and I4), represent platelets with moderate CD62P expression (CD41a⁺CD62P⁺⁺), with interval 5 (I5) presenting the smallest proportion of platelets with the greatest expression of CD62P (CD41a⁺CD62P⁺⁺⁺) (Figure 2.3).

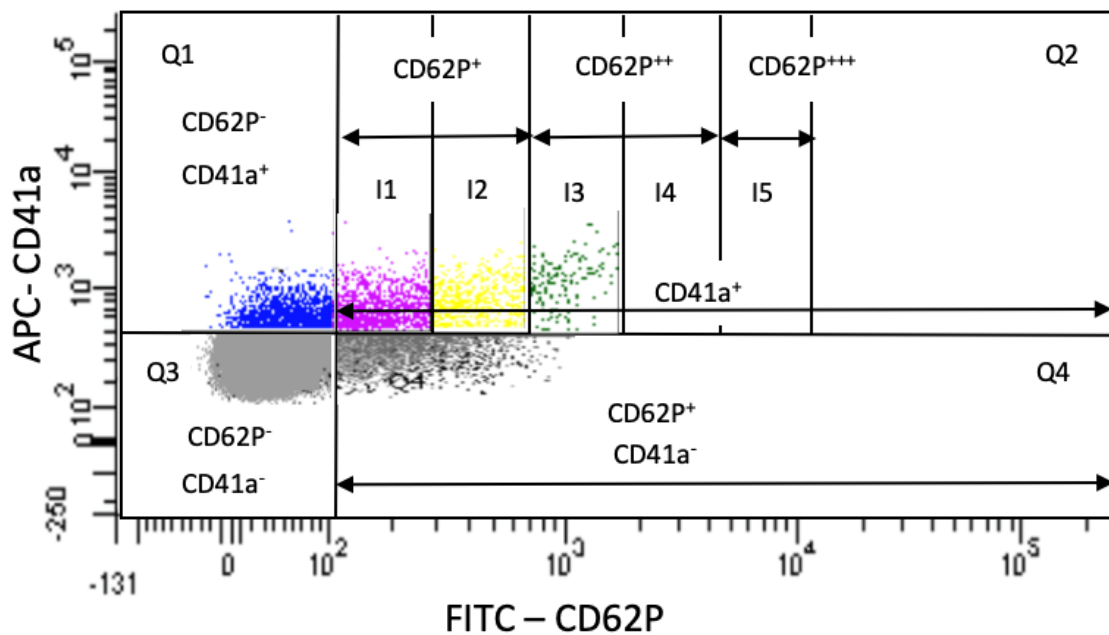


Figure 2.3: Representative scatter plot showing the spread of platelet activation across the interval gates. Quadrant 1 is the inactive platelet population (CD41a⁺CD62P⁻) population, while the activated population of interest (CD41a⁺CD62P⁺) was within the 2nd quadrant. Interval gates (I1-I5) were drawn within the quadrant, indicative of increasing CD62P expression. The 3rd quadrant depicts the negative cell population, while the 4th quadrant is postulated to show CD62P⁺ microparticles.

2.2.8.3 Statistical Analysis

Data acquired using FACSDiva software was exported into Microsoft Excel for data processing.

The index of platelet activation (IPA) was calculated such that:

$$IPA = \text{gMFI (CD62P)} \times n(\text{CD41aCD62P events})$$

for each interval, and combined intervals (Leytin *et al.*, 2000; Augustine *et al.*, 2016). The IPA allows for derivation of a parameter that describes CD62P fluorescent expression (gMFI CD62P) in direct relation to the number of platelets expressing the marker (CD41a⁺CD62P⁺).

Statistical analyses were performed using PAST 3.04 (Ryan *et al.*, 2001). A Shapiro-Wilk test of normality determined that the data was not normally distributed. The non-parametric Kruskal-Wallis test was conducted to determine whether IPA altered in response to hormone-therapy, following which a *post-hoc* Dunn's test was conducted to determine whether these differences were significant ($p < 0.05$).

2.2.8.4 Scanning Electron Microscopy (SEM)

Following erythrocyte lysis, 20 μ l of sample was placed on glass coverslips (10mm round) (Lasec, Johannesburg, South Africa) in labelled 24 well plates (Nunc, Roskilde, Denmark, 662160), and incubated at 37°C with 5% CO₂ for 5 minutes to allow for adhesion. Samples were washed in 0.1 M PBS on a microplate shaker for 20 minutes and fixed in 2.5% formaldehyde (ACE, Southdale, South Africa, 7181)/glutaraldehyde (Merck, Darmstadt, Germany, UN2922) for 15 minutes. This was followed by rinsing thrice with 0.1 M PBS and secondary fixation in 1% osmium tetroxide. Following further rinsing with 0.1 M PBS, cells were dehydrated through an increasing series of ethanol (30%, 50%, 70%, 90%, and three times absolute ethanol), and dried using hexamethyldisilazane (HMDS). Samples were then mounted onto aluminium stubs and coated by carbon evaporation. Samples were examined using an FEI Nova 600 Scanning Electron Microscope with acceleration voltage set at 30 kV, at The Microscopy and Microanalysis Unit (University of the Witwatersrand). Micrographs were assessed qualitatively.

2.2.9 Correlation between platelet activation and oestrogen receptors

To assess whether there was a direct correlation between platelet activation, identified as the IPA (CD41a⁺CD62P⁺), and oestrogen receptor encoding genes, *ESR1* and *ESR2*; a nonparametric Spearman's correlation was conducted in Statistica version 24.

2.3 Results

2.3.1 Drug Concentration Assessment of Anastrozole and Tamoxifen

2.3.1.1 LDH Cytotoxicity Assay

LDH release was determined per treatment, per cell line, as a percentage of cytotoxicity induced. Despite both MCF7s and T47Ds being luminal phenotype breast cancer cell lines, their response to the same treatment differed (Figure 2.4). In MCF7 cells, LDH release increased with increasing Tamoxifen concentration (Figure 2.4A). Specifically, MCF7 cells

under 2 μM Tamoxifen treatment showed a cytotoxicity level of 20%. A similar trend, and level of cytotoxicity was obtained in T47D cells at 2 μM Tamoxifen treatment (Figure 2.4 C). However, at 2.5 μM Tamoxifen treatment, MCF7 cells alone showed the highest level of cytotoxicity (50%), compared to T47D cells which showed a reduction in cytotoxicity (15.95%).

In both cell lines, 1 μM treatment with Anastrozole induced 4% cytotoxicity, indicating low LDH release from cells. In T47D cells, a decline in LDH release was noted coupled with increasing concentrations of Anastrozole, with a sharp increase to just 9% cytotoxicity noted at 10 μM treatment (Figure 2.4 D). MCF7 cells, conversely, showed the greatest induced cytotoxicity (8.2%) at 8 μM Anastrozole treatment, with a sharp decline at 10 μM (Figure 2.4 B).

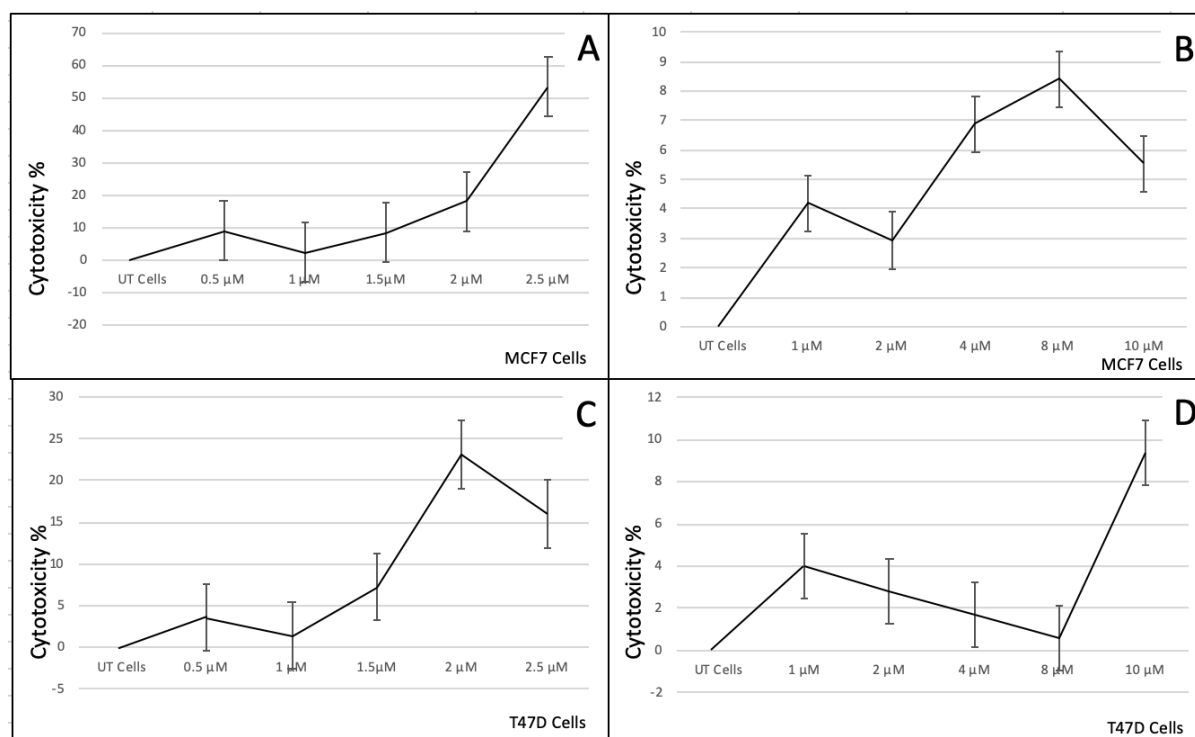


Figure 2.4: Line graphs showing the LDH released by breast cancer cells at various concentrations. A: Tamoxifen concentrations tested on MCF7 breast cancer cells. B: Anastrozole concentrations tested on MCF7 breast cancer cells. C: Tamoxifen concentrations tested on T47D breast cancer cells. D: Anastrozole concentrations tested on T47D breast cancer cells.

2.3.1.2. NR Cell Viability Assay

The NR cell viability assay assesses NR uptake through the plasma membrane of cells into lysosomes (Borenfreund and Puerner, 1985; Fautz, Husein and Hechenberger, 1991; Ates *et al.*, 2017). Results indicated high neutral red uptake in the lysosomes of T47D cells, with lower

uptake noted in MCF7 cells, overall (Figure 2.5). MCF7 cells treated with both Tamoxifen and Anastrozole (Figure 2.5 A, B) showed reduced viability with all concentrations tested; however, T47D cells predominantly showed heightened viability, over 100% (Figure 2.5 C, D). In comparing the concentrations of interest as determined by the LDH assay, 2 μ M of Tamoxifen reduced MCF7 cell viability to 47%, whereas T47D cell viability was increased to 200%. 1 μ M Anastrozole treatment reduced MCF7 cell viability to 62% cell viability, while T47D cell viability was increased to 135%. These results are further highlighted throughout all concentrations, with T47D cells consistently showing far greater levels of cell viability.

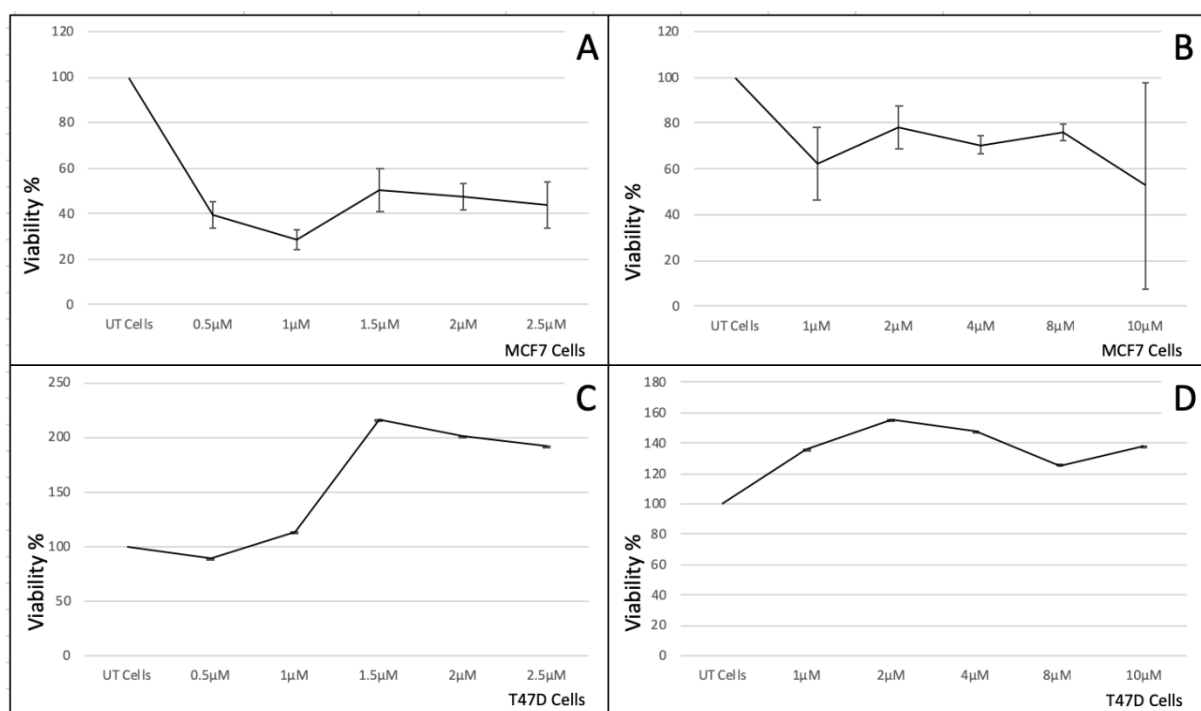


Figure 2.5: Line graphs showing the percentage viability of breast cancer cells treated with various concentrations of common hormone-therapies, as determined by the neutral red assay. A: Tamoxifen concentrations tested on MCF7 breast cancer cells. B: Anastrozole concentrations tested on MCF7 breast cancer cells. C: Tamoxifen concentrations tested on T47D cells. D: Anastrozole concentrations tested on T47D cells.

Based on these results, matched concentrations of each hormone-therapy across both cell lines, which represent the clinically diagnosed, luminal phenotype breast cancer were determined. For ensuing experimentation, we made use of 2 μ M Tamoxifen, which induced 20% cytotoxicity in both cell lines, and 1 μ M Anastrozole, which induced 4% cytotoxicity in both cell lines. These concentrations correspond to in vitro studies and correlate with clinical studies (Jeng, Langan-Fahey and Jordan, 1993; Karami-Tehrani and Salami, 2003; Kijima, Itoh

and Chen, 2005; Pawlak and Wiebe, 2007; Topcul *et al.*, 2013; Pike *et al.*, 2015; Hole *et al.*, 2015; Villegas *et al.*, 2016; Hassan *et al.*, 2017).

2.3.2 Early stage platelet activation induced by breast cancer cells heightened

2.3.2.1 Overall IPA and Corresponding Platelet Ultrastructure

The baseline level of activation was determined by the negative WB control and showed low levels of IPA ($4.61\text{E}+06$) (Figure 2.4). Lysed WB samples (Fig 2.5A) showed a smooth membrane with multiple surface folds. These folds are indicative of a non-resting but still inactive platelet. Thrombin at the suprathreshold concentration of 0.1 U/ml, induced a greater spread of CD62P expression throughout the interval gates, as well as an increase ($p>0.05$) in overall IPA (I1 – I5) at $9.08\text{E}+06$ (Figure 2.4). Platelet from samples incubated with 0.1U/ml of thrombin (Figure 2.5F), showed greater membrane folds, with extending filipodia from the platelet body.

Breast cancer cells, both diluent (MCF7 $1.37\text{E}+07$; T47D $1.15\text{E}+07$) and matched media (MCF7 $1.10\text{E}+07$; T47D $1.15\text{E}+07$), treated samples induced significantly higher overall IPA levels compared to untreated whole blood. These levels of induction were comparable with that of the positive control (Figure 2.4). The effects of untreated breast cancer cells on IPA, was dependent on phenotype, with T47D cells heightening platelet activation more than that of MCF7 cells. The diluent control induced a slightly higher IPA (not significant) with a greater spread of data when compared to that induced by cells alone (Figure 2.4A, B). WB incubated with 0.1% DMSO treated-MCF7 and T47D cells, respectively (Figure 2.5B, G), displayed a relatively smooth but folded platelet membrane, as well as the presence of protofibrils extending outward from the platelet body. However, incubation with breast cancer cells (Figure 2.5C, H), elicited a heightened response, with WB incubated with MCF7 breast cancer cells (Figure 2.5C) displaying a folded, yet smooth platelet membrane with extending filipodia, with the presence of microparticles, this was echoed in samples incubated with T47D cells (Figure 2.5H).

The addition of hormone-therapy further compounded these results. Pre-treatment of cells with hormone-therapies and co-incubation with whole blood induced a greater spread and a greater overall IPA than the negative control (Figure 2.4A, B). In comparison to matched

groups treated with either normal media or diluent, Anastrozole treatment (MCF7 6.90E+06; T47D 7.89E+06) appeared to reduce (not significantly ($p>0.05$)) breast cancer cell-induction of platelet activation as defined by CD62P IPA (Figure 2.4A, B). WB incubated with Anastrozole-treated MCF7 breast cancer cells (Figure 2.5D), showed a smooth yet folded platelet membrane with extending filipodia. WB incubated with Anastrozole-treated T47D cells (Figure 2.5I), induced a greater morphological response. Platelets were noted to have a rougher, folded, membrane with pores associated with the OCS evident. Platelet aggregations were evident, in addition to the presence of thick fibrin fibres, forming dense plaques or fibrin sheets with fibrin pores present.

Tamoxifen treatment, however, differentially affected the ability of breast cancer cells to induce platelet activation. Tamoxifen treatment reduced ($p>0.05$) the ability of T47D cells to induce platelet activation (1.00E+07), with this drug having an opposite effect on MCF7 cells (1.21E+07) (Figure 2.4A, B). Ultrastructurally, Tamoxifen-treated MCF7 breast cancer cells (Figure 2.5E) elicited a much greater response with platelets exhibiting hyalomere spread with further extension of pseudopodia, microparticle presence and fibrin pores. Tamoxifen-treated T47D cells (Figure 2.5J), however, induced a lower level of morphological alteration, with platelets exhibiting a smoother yet folded membrane with lamellipodia and filipodia extending outward from the platelet body, reaching towards nearby platelets suggesting early stages of platelet aggregation. Furthermore, Q4 which was postulated to represent CD62P microparticles, indicated no significant changes in response to hormone-therapy treatment when compared to Q1 (the inactive platelet population); Q2 (the positive platelet population), and Q3 (the negative cell population).

2.3.2.2 IPA in CD62P^{high} interval gate

Overall IPA incorporated platelet activation across intervals I1 –I5, as such we then further investigated IPA within each interval to ascertain how the treatments employed in this study affected the spread of CD62P expression (Figure 2.3). Interval 1 and 2 (I1 and I2) represents the bulk of CD41a⁺ platelets with low CD62P expression (CD41a⁺CD62P⁺), while interval gates 3 and 4 (I3 and I4), represent platelets with moderate CD62P expression (CD4a1⁺CD62P⁺⁺), all of which indicated no significant differences on comparison. However, it was noted in the CD62P^{medium} interval gates (I3 and I4), that IPA induced by hormone-therapy treatment of

MCF7 cells remained high, with the opposite noted for T47D cells, where hormone-therapy reduced the ability of these cells to induce platelet activation, specifically under Anastrozole treatment.

Interval 5 (I5/ CD62P^{high}) reflects the smallest proportion of platelets with the greatest expression of CD62P (CD41a⁺CD62P⁺⁺⁺). The variance observed in this interval was particularly large (Figure 2.4C, D). The ability of breast cancer cells to significantly induce platelet activation was echoed; however, MCF7 cells showed significantly heightened platelet activation capacity under Anastrozole treatment (1.72E+04), and T47D cells, under Tamoxifen treatment (1.75E+04).

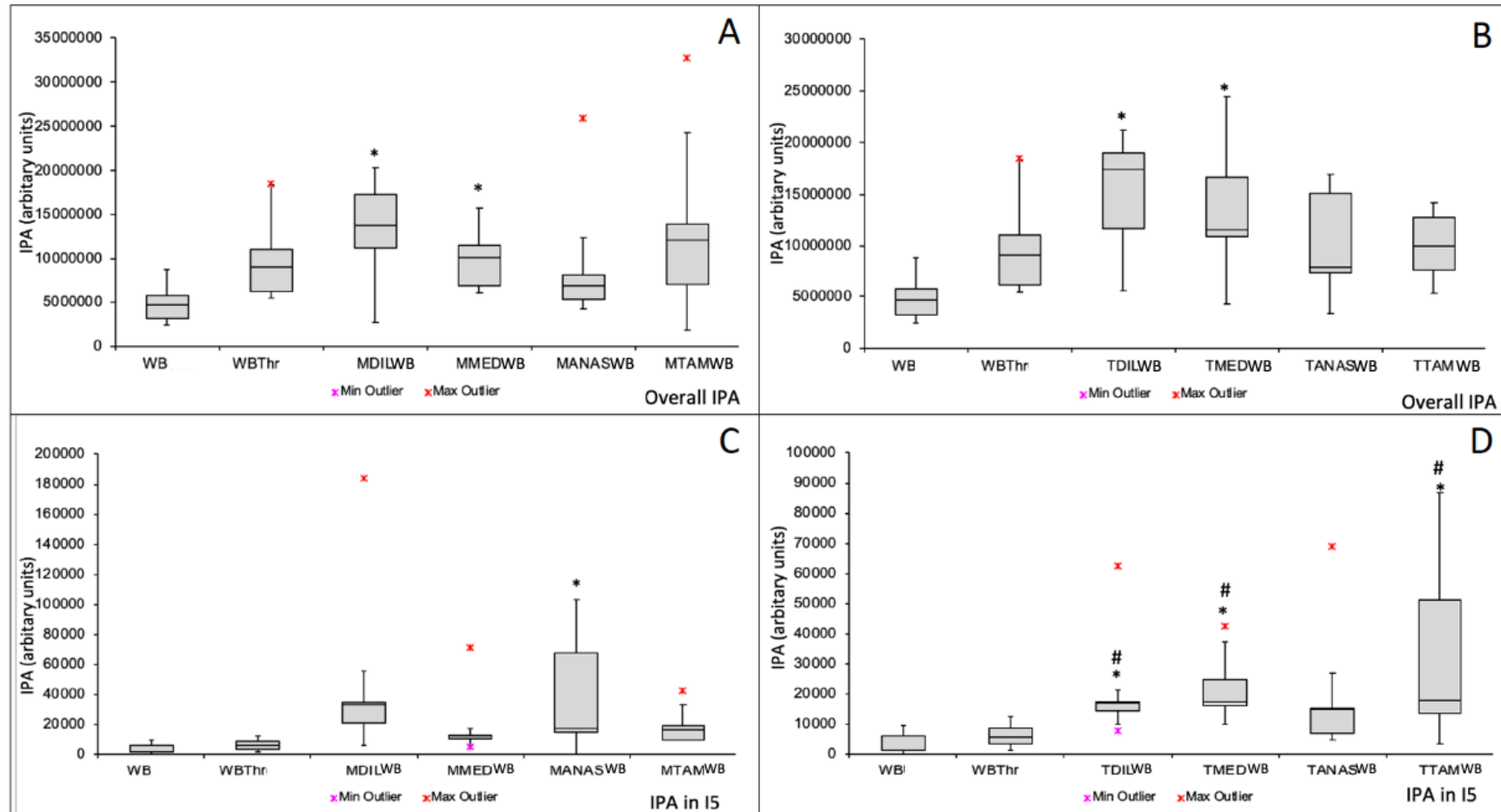


Figure 2.6: Box and whisker plots showing the Index of Platelet Activation (IPA) in lysed whole blood (WB) samples incubated with MCF7 and T47D cells pre-treated with either Anastrozole or Tamoxifen. Intervals I1–I5 (Overall IPA) in MCF7 (A) and T47D (B) cell lines incubated with WB. Overall IPA was 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, Anastrozole did reduce the median IPA induced by both cell lines. Tamoxifen treatment reduced the median IPA induced by T47D cells while increasing the median IPA induced by MCF7 cells. Very high activation of platelets (I5 interval); this interval represents a small percentage of CD41⁺CD62P⁺⁺⁺ platelets. MCF7 cells (C) showed significantly heightened platelet activation under Anastrozole treatment and T47D cells (D), under Tamoxifen treatment. * $p < 0.05$ compared to WB. # $p < 0.05$ compared to WBThr.

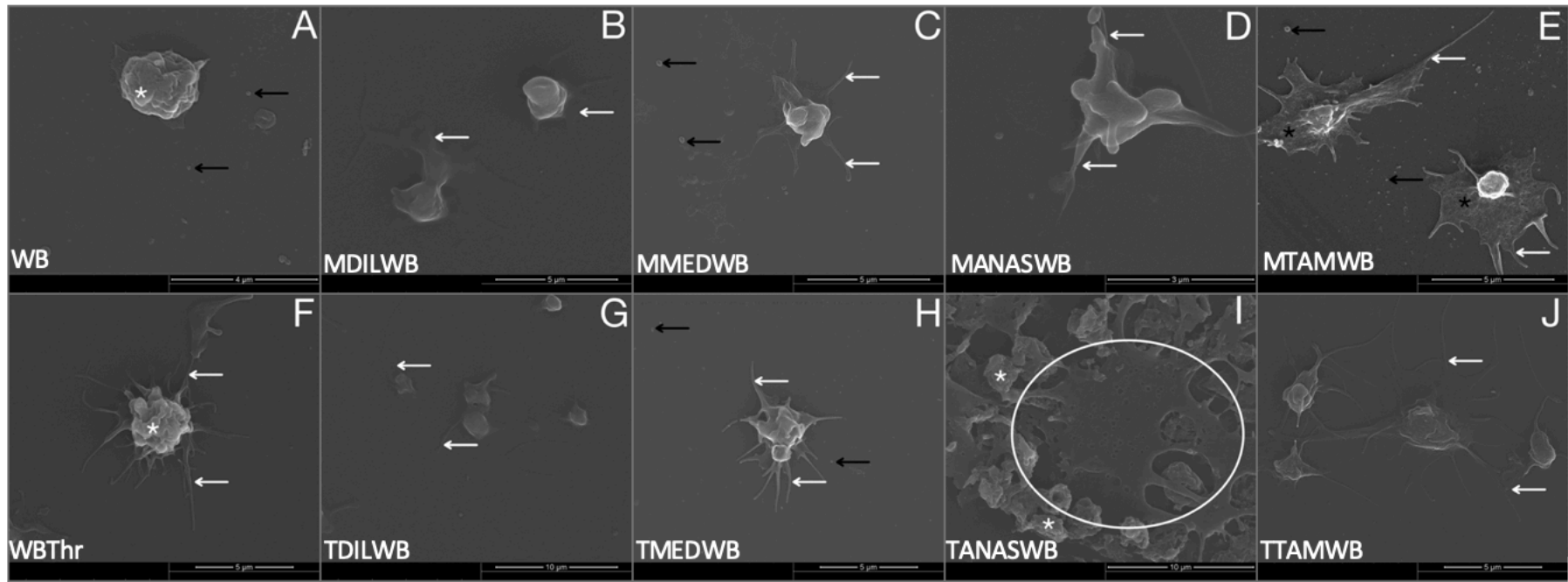


Figure 2.7: Scanning electron microscopy images of platelets in lysed whole blood (WB) samples. A: WB: inactive platelet with a smooth membrane and membrane folds (*), with microparticles (black arrows). B: MDILWB: Platelets exposed to diluent-treated MCF7 cells with a smooth membrane and folds and extending lamellipodia (white arrows). C: MMEDWB: Platelet exposed to media-treated MCF7 cells displaying membrane folds and filipodia (white arrows), with microparticles (black arrows). D: MANASWB: Platelet incubated with Anastrozole-treated MCF7 cells displaying membrane folds with extending filipodia (white arrows). E: MTAMWB: Platelets incubated with Tamoxifen-treated MCF7 cells displaying a rougher membrane appearance, with a hyalomere spread, pores (OCS) (black asterisk *) and lamellipodia and filipodia, with microparticles (black arrows). F: WBThr: Platelet exposed to 0.1U/ml thrombin displaying multiple membrane folds (*), with many filipodia extending outward from the platelet body (white arrows). G: Platelet exposed to diluent-treated T47D cells displaying a smooth membrane with extending lamellipodia (white arrows). H: Platelet exposed to T47D cells displaying membrane folds (*) and multiple filipodia extending outward from the platelet body (white arrows), with the presence of microparticles (black arrows). I: 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. J: 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).

2.3.3 ER gene alterations in response to breast cancer hormone-therapy pre-treatment and exposure to WB

Reference genes are not commonly used or presented with ddPCR data due to the robusticity and accuracy of the technique (Ståhlberg *et al.*, 2004). However, given that this technique at the time was not well established in South Africa, we chose to use and present our reference gene (*ACTB*) as significant differences were noted within treated samples and cell lines.

2.3.3.1 *ESR1* and *ESR2* Expression Alterations

The negative, NTC and NRT, controls showed no cross contamination between samples. ddPCR allows for absolute quantification of nucleic acid target sequences. The quantification of the target ribonucleic acids is dependent on the determination of the number of target RNA by Poisson statistical analysis of positive (containing amplified target) and negative (no amplified) droplets (Day, Dear and McCaughan, 2013). Thus, absolute copies per microliter will be presented.

β -Actin

β -Actin is a non-muscle cytoskeletal actin, which may be involved in cell motility, structure and integrity (Gunning *et al.*, 2015). *ACTB* is a commonly used gene in normalization (reference gene) of qPCR data, with many studies showing it is an effective reference gene for human tissue and cellular samples (Mori *et al.*, 2008). However, some controversy has surrounded its use as a reference gene, with other studies showing expressional alterations during metastasis (Le *et al.*, 1998; Nowak *et al.*, 2005; Ruan and Lai, 2007). Typically, in qPCR analysis, stable reference genes are incorporated and form part of the standard MIQE guidelines (Bustin *et al.*, 2009) for normalization of data to a standard curve. However, in ddPCR, there remains no need to include reference genes, due to the accuracy of the technique itself (Ståhlberg *et al.*, 2004). As such, β -Actin was analysed separately.

Positive *ACTB* droplets ranged between 700 – 2800 copies per microliter. *ACTB* showed the greatest variation in response to hormone-therapy treatment and WB exposure. Differences

were mediated by phenotype, with the greatest significance noted within the MCF7 cell line (Figure 2.6A, B). Both MCF7 and T47D cell lines induced significant ($p < 0.05$) *ACTB* responses between media and diluent treated samples; MCF7 cells (1723 copies/ μ L) higher than their matched diluent treatment (177 copies/ μ L) (Figure 2.6A), and T47D cells (1137 copies/ μ L) lower than their matched diluent treatment (1883 copies/ μ L) (Figure 2.6B). The treatment of MCF7 cells with both Anastrozole (920 copies/ μ L) and Tamoxifen (941 copies/ μ L) induced a significantly lower *ACTB* response, and co-incubation with WB elicited a significantly lower response (264 copies/ μ L). Comparatively, MCF7 cells co-incubated with WB and pre-treated with both Anastrozole (155 copies/ μ L) and Tamoxifen (135 copies/ μ L) significantly reduced *ACTB* expression compared to MCF7 cells co-incubated with WB (264 copies/ μ L) and the untreated control (Figure 2.6A). Conversely, T47D breast cancer cells pre-treated with Anastrozole and co-incubated with WB (1643 copies/ μ L), induced significantly higher *ACTB* expression than that of T47D cells co-incubated with WB (1034 copies/ μ L) (Figure 2.6B).

ESR1

ESR1 encodes ER α and is one of the most commonly found receptors on hormone-dependent breast cancer cells. No significant differences ($p > 0.05$) in *ESR1* was noted within the MCF7 cell line, however, slight variations were noted with the addition of WB to all samples in which a comparative decrease was seen with greater variation within the sample itself (Figure 2.6C). T47D cells, showed a significant increase in *ESR1* response under Tamoxifen pre-treatment (399 copies/ μ L), while not significant, Anastrozole pre-treatment decreased this response (235 copies/ μ L). The addition of WB to all samples elicited a differential response of *ESR1*, with WB reducing the effect of pre-treatment with Tamoxifen on T47D cells (364 copies/ μ L) and heightening the effects of pre-treatment of Anastrozole (280 copies/ μ L) (Figure 2.6D). However, the differences noted within the T47D cell line was lower than that of MCF7 cells.

ESR2

ESR2 encodes ER β , present within all breast cancer cells but at low levels. Greater variability due to pre-treatment with hormone-therapy and WB co-incubation of both cell lines was noted. MCF7 breast cancer cells pre-treated with Anastrozole (1.4 copies/ μ L) induced significantly greater *ESR2* expression when compared to untreated cells (0.6 copies/ μ L)

(Figure 2.6E). Comparatively, the addition of WB to pre-treated MCF7 cells (0.6 copies/ μL) reduced the response of *ESR2*. T47D cells, however, reduced the response of *ESR2* under Tamoxifen pre-treatment and WB co-incubation (3.3 copies/ μL). The addition of WB to T47D cells (6.3 copies/ μL) alone increasing the response of *ESR2* compared to cells alone (2.7 copies/ μL) (Figure 2.6F). However, the differences noted within the T47D cell line was higher compared to that of MCF7 cells.

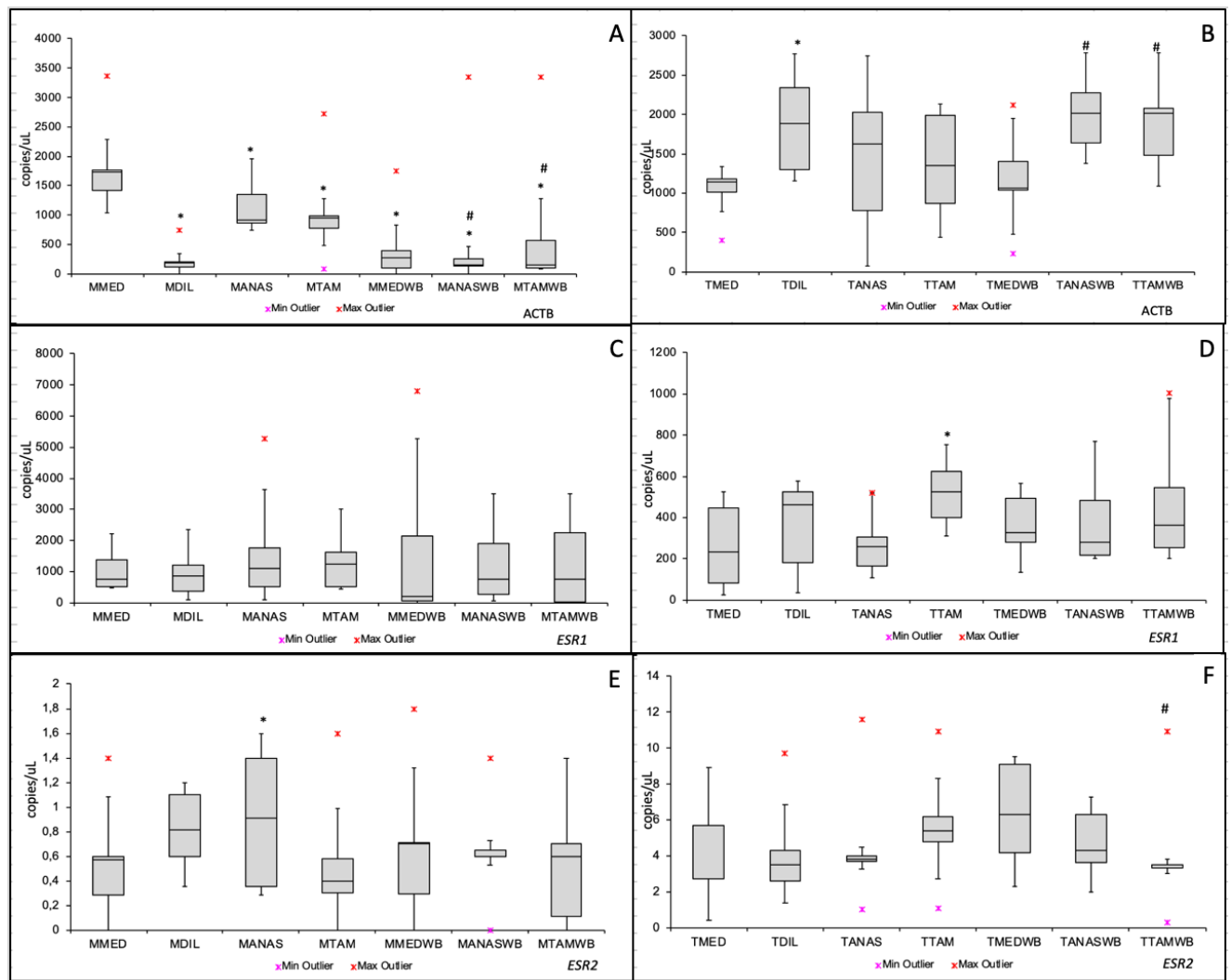


Figure 2.8: Box and whisker plots of gene expression alterations noted in hormone-therapy treated breast cancer cell present as absolute copies per microliter of sample. A: β -Actin (ACTB) expression in MCF7 cells indicating greater variation when exposed to hormone-therapy, compounded by WB addition which reduced these effects. B: β -Actin responses within T47D breast cancer cells. C: ESR1 responses in MCF7 breast cancer cells, indicating no significant differences. D: ESR1 responses in T47D breast cancer cells, indicating a significant increase in response to Tamoxifen pre-treatment of cells. E: ESR2 responses in MCF7 breast cancer cells indicating a significant increase in response to anastrozole pre-treatment compared to the untreated control. F: ESR2 responses in T47D breast cancer cells. G: Ratio of ESR1/ESR2 indicating the possible switched response of ESR1 in the presence of ESR2 in MCF7 cells. H: Ratio of ESR1/ESR2 in T47D cells. I: Ratio of ESR2/ESR1 indicating the possible switched response of ESR2 in the presence of ESR1 in MCF7 cells. J: Ratio of ESR2/ESR1 in T47D cells. * $p < 0.05$ compared to matched media sample; # $p < 0.05$ compared to matched media sample exposed to WB; \$ $p < 0.05$ compared to TANASWB.

ESR1/ESR2 Ratio

The ratio standardizes the values to ESR2, which allows for a better indication of how ESR1 responds in the presence of ESR2 (Figure 2.6A). A significantly decreased response of ESR1 is noted within the diluent treated MCF7 cells (762.72) compared to its matched media counterpart (1231.67), indicating a shift in ER profiles to that of lower ESR1 expression. The diluent is thus, reducing the levels of ESR1, possibly due to its cytotoxic nature. While not

significant, a greater *ESR1* switched response is noted within the Tamoxifen-treated MCF7 cells (1315). Whereas no significant responses were noted within the T47D cell line regardless of treatment options, greater *ESR1* expression was seen within T47D cells alone (87.03) and diluent treated cells (107.69), with the addition of WB to Tamoxifen-treated T47D cells also eliciting a greater *ESR1* response (Figure 2.6B).

ESR2/ESR1 Ratio

This ratio standardizes values to *ESR1*, which allows for a better indication of the possible switched response of *ESR2* in the presence of *ESR1*. No significant responses were noted within the MCF7 cell line (Figure 2.6C). However, a greater *ESR2* response (not significant) was seen in MCF7 cells exposed to WB (0.0033), as well as Tamoxifen-treated MCF7 cells exposed to WB (0.0025), indicating a possible shift in ER profiles to that of a more *ESR2* positive profile. A significant decrease, however, was noted within the WB co-incubated Tamoxifen-treated T47D cells (0.009) compared to the WB co-incubated Anastrozole-treated T47D cells (0.015). Indicating a switched response within the Anastrozole-treated T47D cells co-incubated with WB, to that of a more *ESR2* positive profile (Figure 2.6D). While not significant, T47D cells exposed to WB also elicited greater *ESR2* expression.

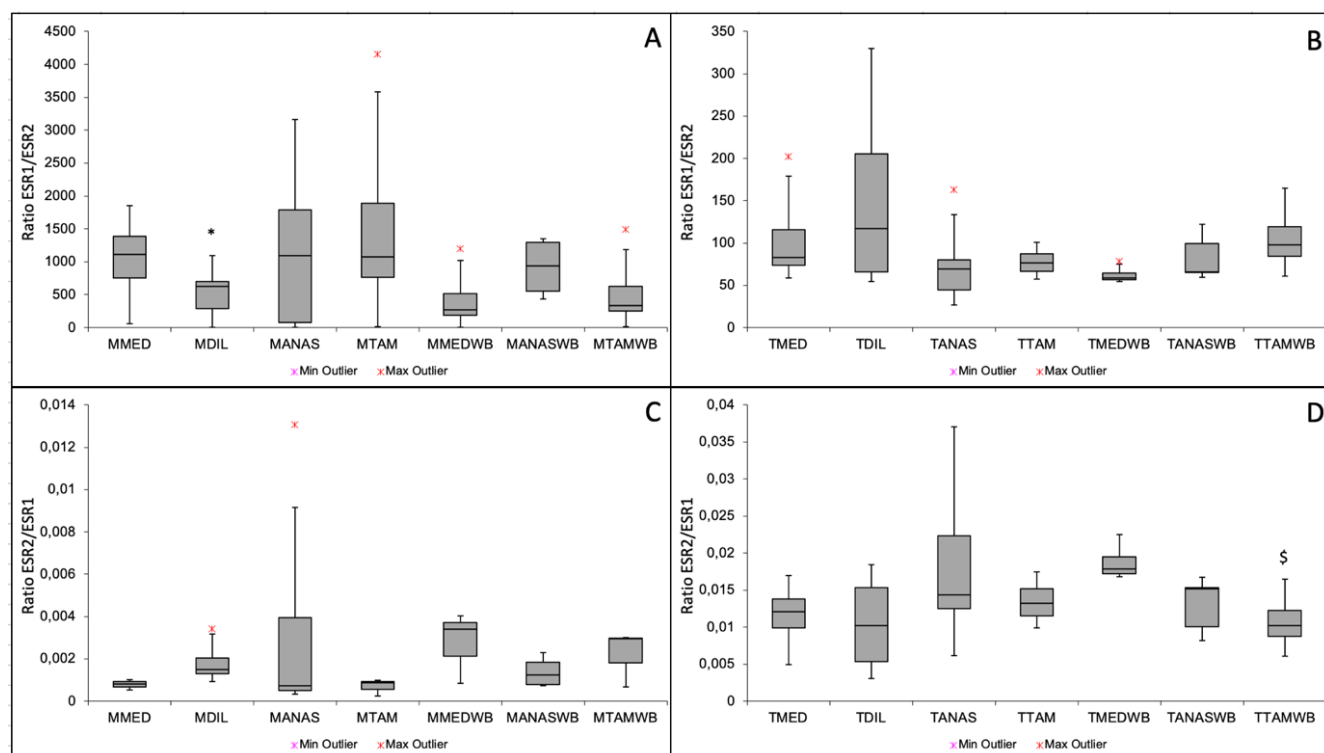


Figure 2.9: Box and whisker plots of gene expression alterations noted in hormone-therapy treated breast cancer cell present as absolute copies per microliter of sample. A: Ratio of ESR1/ESR2. B: Ratio of ESR1/ESR2 in T47D cells. C: Ratio of ESR2/ESR1. D: Ratio of ESR2/ESR1 in T47D cells. * $p < 0.05$ compared to matched media sample; # $p < 0.05$ compared to matched media sample exposed to WB; \$ $p < 0.05$ compared to TANASWB.

2.3.4 The correlation between ESR expression and platelet activation

The nonparametric spearman's correlation showed no direct functional correlation between genes (*ACTB*, *ESR1*, *ESR2*) and the index of platelet activation (IPA), overall (Table 2.3). However, on analysis per treatment group a significant positive ($p < 0.05$) correlation between *ESR1* and IPA, in T47D cells co-incubated with WB, was noted. Indicating that alteration in the *ESR1* gene in T47D cells is associated with induction of platelet activation. While not significant ($p > 0.05$), strong correlations are noted (Mukaka, 2012). Strong positive correlations are seen between *ACTB*, *ESR1* and IPA in Anastrozole-treated MCF7 cells exposed to WB; and a strong negative correlation exists between *ESR2* and the IPA of the same sample. Furthermore, while no significant direct correlations exist within other sample groups, gene alteration in *ACTB*, *ESR1* and *ESR2* may still hold predictive value for future thrombotic events.

Table 2.3: Tabulation showing *r* values obtained from the nonparametric Spearman's correlation between genes; *ACTB*, *ESR1* and *ESR2*, to the IPA.

Spearman Rank Order Correlations							
Highlighted <i>r</i> values in red (correlations) are significant at $p < 0.05$.							
	Overall	MMEDWB	MANASWB	MTAMWB	TMEDWB	TANASWB	TTAMWB
	IPA	IPA	IPA	IPA	IPA	IPA	IPA
<i>ACTB</i>	0,113695	-0,200000	0,700000	0,300000	0,100000	0,000000	0,600000
<i>ESR1</i>	0,186450	-0,500000	0,700000	0,100000	0,900000	0,500000	0,200000
<i>ESR2</i>	0,022227	-0,800000	0,316228	-0,200000	0,400000	0,200000	-0,400000

2.4 Discussion

The interaction between circulating tumour cells and platelets have been associated with both metastasis facilitation and possible induction of thromboembolic complications, which is the second leading cause of death in breast cancer patients (Karimi and Cohan, 2010; Bambace and Holmes, 2011; Mezouar *et al.*, 2014). Furthermore, sustained signalling processes within the circulatory system, including sustained biomarker signalling of platelets associated with activation and aggregation, which is possibly induced by cancer cells themselves increasing the tumour metastatic profile (Labelle, Begum and Hynes, 2011; Menter *et al.*, 2014). Thus, this study assessed the effects of Tamoxifen and Anastrozole on ER gene expression and their relationship to platelet activation *in vitro*.

ERs have traditionally been looked at holistically in breast cancer, with most studies investigating their presence, effects and targeting them for treatment strategies. However, studies have focused on ER α , the conventionally studied ER, encoded by the *ESR1* gene, which responds to hormone-therapies and has positive prognostic outcomes in breast cancer patients treated with Tamoxifen or Anastrozole (Altundag and Ibrahim, 2006; Giacinti *et al.*, 2006; Bahreini *et al.*, 2017). While the ER β , encoded by the *ESR2* gene, has a probable opposing effect to that of ER α and is associated with a poorer outcome in patients treated with hormone-therapies, but remains poorly assessed (Strom *et al.*, 2004). It should be noted that breast cancers of the luminal phenotype are treated holistically as ER-positive, therefore the expression of specific ERs are not assessed clinically. Our results present data on ER gene expression in luminal A breast cancer cell lines, indicating greater *ESR1* expression in both MCF7 and T47D cells, compared to *ESR2*. However, we also note that the T47D line expressed

ESR1 at a lower level than that of MCF7 cells, but its' *ESR2* expression was markedly higher than that of untreated MCF7 cells. These results support clinical studies which indicate that luminal A breast cancers benefit from hormone-therapy treatment strategies (Ali and Coombes, 2000; Clatot, Augusto and Di Fiore, 2017); however, these strategies may have slower responses in clinical subphenotypes due to ER profile differences.

On assessment of *ESR1* and *ESR2* gene expression alterations in MCF7 cells treated with either Tamoxifen or Anastrozole and exposed to WB, we note a switch in ESR expression to that of a more *ESR2* positive profile when cells are exposed to WB, as well as pre-treated with Tamoxifen and exposed to WB. The MCF7 cell line, altered its receptor profile in response to WB and Tamoxifen pre-treatment, indicating greater *ESR2* presence, which has been associated with a decrease in ER associated proliferation and invasion (Lazennec *et al.*, 2001; Roger *et al.*, 2001; Strom *et al.*, 2004; Ghali *et al.*, 2018). This switched response, to that of a more *ESR2* positive profile, was again noted in T47D cells exposed to WB, and pre-treated with Anastrozole exposed to WB. This result indicates, not only a cell line response to WB but how these cell lines respond to either an AI or SERM. Anastrozole does not directly target ERs but rather targets the availability of oestrogen (Fabian, 2007), the T47D cell line is known to have a greater PR presence, with fewer ERs compared to the MCF7 cell line, making them less oestrogen dependent and more aggressive (Ogba *et al.*, 2014; Mota *et al.*, 2017), thus indicating its potential susceptibility to Anastrozole and its altered ER profile in response. Additionally, Anastrozole has been shown in clinical studies to upregulate the expression of ER β with a significant decrease noted in the expression of ER α (Torrise *et al.*, 2007), correlating with our results. Studies have indicated that Tamoxifen would be expected to decrease the expression of both ERs in the breast cancer cell lines (Jonat *et al.*, 2006), however, our results indicate a greater *ESR1* response in both cell lines to Tamoxifen treated cells, which is compounded by exposure to WB. This may be due to the breast cancer cells altering and upregulating their ER profile as a compensatory mechanism in response to the SERM, which directly targets the receptor. Breast cancers are able to downregulate their receptor in an attempt to reduce ER binding, a characteristic associated with the transition to a more aggressive phenotype that does not respond to therapy (Begam, Jubie and Nanjan, 2017). This downregulation has also been noted in breast cancer cells during the metastatic process

(Aktas *et al.*, 2011). The ability of breast cancer cells to alter their receptor profiles in response to treatment strategies highlights their metastatic potential and invasive capacity.

We further assessed ACTB expression within our samples, as significant expressional changes were noted in response to hormone-therapy pre-treatment. ACTB is an actin isoform involved in cell motility, structure and integrity, which may be manipulated by cancer cells for invasion and metastasis (Le *et al.*, 1998; Nowak *et al.*, 2005; Guo *et al.*, 2013). Studies have associated higher ACTB expression in metastatic tumours, as well as with metastasis of cancer cells and their inherent cytoskeletal changes (Mori *et al.*, 2008; Guo *et al.*, 2013). Furthermore, ACTB expression has been shown to positively correlate with tumour invasiveness and metastatic potential (Le *et al.*, 1998; Nowak *et al.*, 2010; Sikand *et al.*, 2012; Guo *et al.*, 2013). Our results indicate significantly lower ACTB expression in response to hormone-therapy (Anastrozole and Tamoxifen) pre-treatment and WB exposure of MCF7 cells compared to the untreated control, correlating with studies indicating positive outcomes in metastatic breast cancers treated with hormone-therapies (Cuzick *et al.*, 2015; Kümler *et al.*, 2016).

The role of platelets are firstly, *in situ* as intratumoral platelets interacting with tumour cells in the tumour microenvironment, potentially facilitating tumour growth (Plantureux *et al.*, 2018) and secondly, in the metastatic process is to act as a physical shield, protecting disseminated tumour cells from immunosurveillance and shear forces, and allowing for adherence to the endothelium for extravasation (Meikle *et al.*, 2017). However, it is the early events of platelet activation that remain of interest to this study. The results obtained indicate that hormone-dependent breast cancer cells induce a significant increase in platelet activation, as defined by IPA in this study, comparable to clinical studies (Meier and Jick, 1998; Onitilo *et al.*, 2012; Kedzierska *et al.*, 2013; Zarà *et al.*, 2018); however, this increase is phenotype based, with more aggressive breast tumours, represented by the T47D cell line, due to its receptor profile differences (Adjo Aka and Lin, 2012) that induce a greater level of activation. Thus, the variation in hormone-receptor profiles and hormone-dependent breast tumour subphenotypes may elicit a differential thrombotic response, which is not usually considered in clinical management. Ultrastructural analysis further confirmed these results by showing heightened levels of activation compared to that of the negative control, platelets

expressed greater membrane folds with extension of pseudopodia, which was more visible in samples incubated with the T47D cell line.

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, with the release of granular contents facilitating further adhesion and aggregation (Escolar and White, 1991; Selvadurai and Hamilton, 2018). Clinical studies indicate that hormone-therapies, specifically Tamoxifen, are associated with a 4% higher risk of venous thromboembolism development (Walker *et al.*, 2017), with a lower but significant risk noted for patients under Anastrozole treatment (Fennerty, 2006). In this study, hormone-therapy differentially affected the ability of either cell line to induce platelet activation. In addition to investigating the overall index of platelet activation, we further assessed the spread of platelets corresponding with heightened CD62P expression by using an interval gating strategy (Augustine *et al.*, 2016). The capacity of both cell lines to induce platelet activation was enhanced by pre-treatment of the cells with Tamoxifen, being at levels higher than that of the untreated blood control. While our results support other studies assessing Tamoxifen induction of platelet activation (Vitseva *et al.*, 2005), our results are contradictory to recent laboratory-based studies which show that Tamoxifen is able to inhibit platelet activation, and consequently, platelet aggregation (Chang *et al.*, 2011; Johnson *et al.*, 2017). This may be due to differences in dosages, 2 μ M Tamoxifen used in our study, corresponding with other studies and reflecting its' antagonistic behaviour physiologically (Karami-Tehrani and Salami, 2003; Salami and Karami-Tehrani, 2003), compared to a range of 3 μ M-20 μ M (Chang *et al.*, 2011; Johnson *et al.*, 2017), but also in experimental methodology.

To determine the appropriate concentrations of hormone-therapies used in this study, the LDH cytotoxicity and NR cell viability assays were conducted and the results informed by corresponding literature. Both assays are based on lysosomal activity, with the LDH assay assessing the release of LDH from damaged cell membranes, degraded by the autophagic-lysosomal pathway (Seglan *et al.*, 2009); and the NR assay assessing the uptake of NR dye into the lysosomes of cells as a measure of cell viability (Borenfreund and Puerner, 1985; Fautz, Husein and Hechenberger, 1991; Ates *et al.*, 2017). LDH cytotoxicity assays particularly, while

robust and cost effective, are susceptible to background signal from exogenous sources of LDH, negatively impacting the overall signal window (Niles, Moravec and Riss, 2009; Aslantürk, 2018). The NR cell viability assay is limited by factors including the loss of membrane integrity in damaged cells which may take several days, sustained proliferation of surviving cells and the potential for unstained cells which have undergone early disintegration, which decrease the accuracy of the test itself (Weisenthal *et al.*, 1983; Son *et al.*, 2003; Lee *et al.*, 2005; Aslantürk, 2018). These assays are thus not regarded as highly specific and should be paired with more specific methods to ascertain cytotoxicity, with animal models considered the gold standard for assessment of drug-induced cytotoxicity (Niles, Moravec and Riss, 2009; Aslantürk, 2018; Xulu, Duarte and Augustine, 2020).

Moreover, since lysosomes function in cellular stress associated with pro-death or pro-survival autophagic responses (Lawrence and Zoncu, 2019), these results suggest that hormone-therapy treatment, particularly of T47D cells elicited a pro-survival response as reflected by the cell viability assay. Furthermore, studies have shown that while Anastrozole is able to induce necrosis in both MCF7 and T47D cell lines at high concentrations, cell viability was nevertheless maintained, showing that cells are able to modulate their response (Xulu, Duarte and Augustine, 2020). As such, both LDH and NR assays should be used with caution since there is further potential for autophagic cell survival (Niles, Moravec and Riss, 2009; Aslantürk, 2018; Xulu, Duarte and Augustine, 2020).

In addition, cognisance must be given to the main function of these hormone-therapies which is to prevent oestrogen-dependent proliferation of breast cancer and not necessarily, to induce cytotoxic cell death (Onitilo *et al.*, 2012; Lukong, 2017; Hassan *et al.*, 2017; Burstein *et al.*, 2019; El Sayed *et al.*, 2019). This is important clinically, with comparative *in vitro* studies highlighting the function of Tamoxifen (Jeng, Langan-Fahey and Jordan, 1993; Karami-Tehrani and Salami, 2003; Kijima, Itoh and Chen, 2005; Pawlak and Wiebe, 2007; Villegas *et al.*, 2016) and Anastrozole (Hole *et al.*, 2015; Hassan *et al.*, 2017). Tamoxifen inhibits oestrogen-induced proliferation through ER binding and is not necessarily considered to be cytotoxic in nature (Onitilo *et al.*, 2012; Lukong, 2017; El Sayed *et al.*, 2019). An induction of cytotoxicity *in vitro*, at over 50% (half maximal inhibitory concentration, IC50), as noted in the present study where MCF7 cells were treated with 2.5 μ M Tamoxifen, highlights the cytotoxic effects of this

hormone-therapy at high doses, which would then induce cell death regardless of ER presence (Majumdar, Valdellon and Brown, 2001; Cuevas and Lindeman, 2015). Thus, the highest tested concentration of Tamoxifen was not selected for further use in this study. Anastrozole conversely, induced a much lower cytotoxic effect than Tamoxifen, in both cell lines, as expected due to its indirect effect on breast cancer cells via the aromatase enzyme (Campos, 2004; Burstein *et al.*, 2019).

By assessing results from both the LDH cytotoxicity assay and NR cell viability assay, an attempt to match concentrations of each hormone-therapy across both cell lines was made. This is of particular importance, since both the MCF7 and T47D cell lines are representative of the clinically diagnosed, luminal phenotype breast cancer. We flagged concentrations 2 μ M of Tamoxifen, which induced 20% cytotoxicity in both cell lines, and 1 μ M Anastrozole, which induced 4% cytotoxicity in both cell lines. The selection of these concentrations was informed based on corresponding information from the literature reflecting both *in vitro* studies and correlating with clinical studies (Jeng, Langan-Fahey and Jordan, 1993; Karami-Tehrani and Salami, 2003; Kijima, Itoh and Chen, 2005; Pawlak and Wiebe, 2007; Topcul *et al.*, 2013; Pike *et al.*, 2015; Hole *et al.*, 2015; Villegas *et al.*, 2016; Hassan *et al.*, 2017). It should be noted that the above-mentioned concentrations were not tested directly on platelets, since this study assessed corresponding platelet activation in response to hormone-therapy treatment of breast cancer cells.

Furthermore, platelets were neither washed nor treated directly with Tamoxifen or Anastrozole, rather breast cancer cells were pre-treated with hormone-therapy prior to incubation with whole blood, to mimic the cumulative effect of the drug *in vivo*. Results from this Chapter indicating early platelet activation induction by breast cancer cells compounded by Anastrozole and Tamoxifen treatment, were published in Thrombosis Research (Pather *et al.*, 2019). While these results echoed clinical studies and selected *in vitro* work (Zara *et al.*, 2018), they were contradictory to *in vitro* studies, which showed inhibition of platelet activation by Tamoxifen (Chang *et al.*, 2011; Johnson *et al.*, 2017). The present study was thus expanded: WB was pre-treated with either Anastrozole or Tamoxifen, separated into constituents; WB, platelet-rich plasma (PRP) and platelet-poor plasma (PPP) and exposed to untreated MCF7 and T47D cells. The expansion of the study to include pre-treatment of blood

constituents allows for the for mimicking of the circulatory effects of the drugs, as would occur *in vivo*, and will be presented in the ensuing Chapters. Furthermore, the addition of blood constituents allows for the assessment of potential mechanisms associated with retaining plasma, greater and lower numbers of platelets, including higher levels of fibrinogen, in tumour-induced platelet activation and hypercoagulation (Ghassab *et al.*, 2015; Zara *et al.*, 2018; Kwaan *et al.*, 2019).

Tamoxifen and Anastrozole treatment have been associated clinically with high endogenous thrombin potential, as well as greater numbers of CD62P positive microparticles (Trappenburg *et al.*, 2009). On assessment of platelet ultrastructure, our results showed a greater number of microparticles, specifically in platelets exposed to Tamoxifen-treated MCF7 cells. In the CD62P^{high} interval gate, greater variation in IPA was noted. Tamoxifen pre-treatment resulted in T47D induction of platelet activation greater than that of the MCF7 cell line. This was further visualized by the assessment of platelet ultrastructure, which indicated greater levels of activity by the presence of extending pseudopodia and platelet aggregation in hormone-therapy treated samples. Higher levels of activation (CD62P^{high}) was noted in Anastrozole-treated T47D samples which, additionally displayed thick fibrin fibre formation, leading to fibrin sheets and plaque formation (Michele L Nealen *et al.*, 2001; Walski *et al.*, 2002; Pretorius *et al.*, 2007, 2011; van der Spuy and Augustine, 2016). The visualization of fibrin plaque and microparticles indicates that lysing the WB did not remove the effects of plasma on the sample itself (Chen and Geng, 2006; Augustine *et al.*, 2016; Montoro-garcía *et al.*, 2016). Plasma has been shown to be essential to coagulation and platelet aggregation, and its' removal through the process of washing removes the pro-coagulatory effects of thrombin and the induction of calcium influx (Zarà *et al.*, 2018). Furthermore, the formation of plaques without the addition of activated thrombin, as observed in these results, indicates a highly inflammatory state, which is common in patients with inflammatory disorders, autoimmune diseases, HIV/AIDs and cancer (Pate and Mankowski, 2011). Plaque formation in cancer patients is extremely dangerous as due to its rigid nature, it is more likely to dislodge from the body of the thrombus creating an embolus which would then result in blockage of blood vessels. Our results further substantiate studies that indicate that patients with cancer are more likely to suffer from thromboembolic complications (Nash *et al.*, 2002; Cesarman-

Maus and Hajjar, 2005; Chapin and Hajjar, 2015), but also indicate the pro-thrombotic nature of Anastrozole itself, the data on which is lacking.

Platelets also express both ER α and ER β , predominantly ER β (Jayachandran *et al.*, 2005) making them susceptible to hormone-therapy. Hormone-therapies targeted against reducing oestradiol in the body, heighten the possibility of a thrombotic event. Oestradiol binding on the platelet ER itself reduces platelet responses and has been suggested as being protective against thrombosis (Jayachandran *et al.*, 2005). Thus, substantiating our results in which we see greater levels of platelet activation in response to Anastrozole. Activated platelets also secrete proteolytic enzymes, such as heparanase and MMPs, which aid in the process of fibrinolysis, the breakdown of the basement membrane (Huang *et al.*, 2002). Fibrinolysis is a key component in metastasis and is essential for the release of tumour cells from their primary site and functions in promoting cell motility, and facilitating tumour angiogenesis (Cesarman-Maus and Hajjar, 2005; Mosesson, 2005; Chapin and Hajjar, 2015). This contribution of platelets is a critical step in cancer metastasis facilitating tumour cell migration and invasion by the degradation of the ECM, and cellular epithelial to mesenchymal transitions (EMT). Our results which indicate an increase in levels of platelet activation in response to hormone-therapy substantiate clinical studies indicating an increase in thromboembolic complications in patients diagnosed with cancer, further heightened by treatment options (Nash *et al.*, 2002; Reinert *et al.*, 2017). Furthermore, by investigating the intricacies of the index of platelet activation using interval gates, we have identified a switched response of Tamoxifen-treated MCF7 and T47D cells on CD62P^{high} expression, further confirmed ultrastructurally. This reflects the heterogeneous nature of platelet α -granule content, with specific release related to the effects of various agonists of activation (Italiano *et al.*, 2007; Maynard *et al.*, 2007), as such we expanded the scope of this study to include assessment of detectable levels of thrombin and CD63, a marker released from dense and lysosomal platelet granules associated with potentially later stages of activation, which will be presented with CD62P, to identify hypercoagulation in the following Chapters.

Additionally, by investigating the altered responses of *ESR1* and *ESR2*, we postulate that the induced responses differentiated by cell line receptor profiles and hormone-therapy pre-treatment may indicate a role for *ESR2* (ER β) in tumour progression (Esslimani-Sahla *et al.*,

2004; Hopp *et al.*, 2004; Honma *et al.*, 2008). Studies have indicated a role for heightened *ESR2* expression with decreased ER associated proliferation and invasion in breast cancers (Ghali *et al.* 2018; Strom *et al.* 2004; Roger *et al.* 2001; Lazennec *et al.* 2001). Our results indicated greater *ESR2* expression in the T47D cell line, regardless of treatment with either hormone therapy. Furthermore, samples with altered *ESR2* expression, to that of a more *ESR2* positive profile, particularly T47D cells under Anastrozole pre-treatment, was associated with greater platelet activation as evidenced through the loss of CD62P and the formation of fibrin networks; which may indicate a role for *ESR2* in the formation of a hypercoagulatory environment. Our results, further indicate a correlation between *ESR1* expression and platelet activation in T47D cells exposed to WB, indicating a potential role for *ESR1* associated with platelet activation. While these results indicate an association between ER gene expression and early platelet activation, with a single correlation indicating the prothrombotic association of *ESR1* in T47D cells; further investigation is required into the relationship between ERs and platelet activity, as such this study was expanded and ER protein expression alterations investigated and presented in the forthcoming Chapters.

2.5 Upgrade

On assessment of results obtained for this Chapter, the project was expanded to assess the intricacies and controversies identified. This Chapter formed part of the MSc portion of the project, which was converted to PhD in 2018.

Aspects of this Chapter, namely; platelet activation has been published:

Pather K., Dix-Peek T., Duarte R., Augustine T. (2019). Breast cancer cell-induced platelet activation is compounded by Tamoxifen and Anastrozole *in vitro*. Thrombosis Research.

3 CHAPTER 3: Induction of Hypercoagulation and ER alterations by Tamoxifen-treatment of breast subtypes.

3.1 Introduction

Breast cancer hormone-therapy comprises one of the most commonly used targeted treatment strategies against hormone-dependent breast cancers (Reeder and Vogel, 2008; Walker *et al.*, 2017; Akram *et al.*, 2017), which overexpress ERs in 75% of breast cancers making them a particularly favourable target for such therapies (Pal, Childs and Pegram, 2011; Williams and Lin, 2013; Hashmi *et al.*, 2018). Tamoxifen, a SERM, which can be taken orally or intravenously, depending on the severity, staging and patient preference (Ishitobi *et al.*, 2013), is known to increase a patient's risk for thrombosis (Onitilo *et al.*, 2012; Lin *et al.*, 2018). While Tamoxifen has been extensively studied in relation to oestrogen receptor-specific effects, as well as thrombotic effects (Karimi and Cohan, 2010; Onitilo *et al.*, 2012; Lin *et al.*, 2018; Pather *et al.*, 2019; Cuzick *et al.*, 2020), recent *in vitro* studies have indicated opposing effects of the drug in comparison to clinical findings (Chang *et al.*, 2011; Johnson *et al.*, 2017), indicating an urgent need to conduct further research in defining the role of Tamoxifen in hypercoagulation and ER expression.

3.1.1 Breast Cancer Metastasis and Intertumoral Heterogeneity

Biomarker assessment of breast cancers is useful in the prediction of responses to adjuvant treatment options, in place of molecular subtyping (Schmidt *et al.*, 2012; Bownes *et al.*, 2019). ER α biomarker expression is well studied; however, patients with heterogeneous tumours indicate a heightened risk for resistance to therapy and progression (Welsh *et al.*, 2012; Reinert *et al.*, 2017). The progression of breast cancer is dependent on oestradiol, a pivotal hormone which binds to ERs that are highly expressed by hormone-dependent breast cancers (Thomas and Gustafsson, 2011). It should be noted that the majority of breast cancers may present initially as oestrogen dependent, only to alter their ER expression through progression and metastasis of the disease (Thomas and Gustafsson, 2011). While information exists on the process of metastasis, there is minimal and contradictory literature highlighting the molecular alterations associated with breast cancer progression and the role of ER signalling (Sgroi, 2010; Thomas and Gustafsson, 2011; Velloso *et al.*, 2017; Plava *et al.*, 2019).

Breast cancer metastasis is the process that involves breast cancer cells separating from their primary tumour mass, entering the vascular or lymphatic system, circulating within the system until it reaches their secondary site; where cells exit into the tissue and colonize the microenvironment (Berny-Lang *et al.*, 2011). Malignant invasive tumour cells are also able to enter the circulation even before the tumour presents clinically (Massagué and Obenauf, 2016), with ER α signalling implicated in cellular motility in breast cancer progression and metastasis (Zheng *et al.*, 2011; Ganapathy *et al.*, 2012). Within the tumour microenvironment, breast cancer cells come into direct contact with not only non-tumorigenic epithelial cells, fibroblasts and immune cells, but platelets as well (Li *et al.*, 2014). Platelets are known to facilitate tumour invasion processes, a key factor for metastatic spread; however, recent studies have detected extravascular platelets within the tumour bed indicating a role for platelets in regulation of primary tumour growth and initiation of metastasis (Labelle, Begum and Hynes, 2011; Li *et al.*, 2014; Haemmerle *et al.*, 2018).

Breast cancer is a heterogeneous disease and this heterogeneity of breast cancer cells themselves stems from their origin (Keller *et al.*, 2010). Due to their heterogeneity, proper cell line models of breast cancer subtypes are required for *in vitro* studies. Hormone-dependent breast cancers, modelled by the MCF7 and T47D cell lines *in vitro*, are commonly treated using SERMs, including Tamoxifen, which prevents oestrogen-mediated proliferation (Carey *et al.*, 2006; Kümler *et al.*, 2016; Lukong, 2017). The MCF7 breast cancer cell line is one of the most commonly used luminal A breast cancer cell lines in research (Burdall *et al.*, 2003; Radde *et al.*, 2015; Dai *et al.*, 2017). MCF7 cells express a range of sensitivities to oestrogen which allow for rapid proliferation when the cells are exposed to oestradiol (Hamelers *et al.*, 2003; Dai *et al.*, 2017). The T47D breast cancer cell line is also a widely used cell line in laboratory studies, accounting for two-thirds of cell lines used in associated studies (Lacroix *et al.*, 2004). While both cell lines are derived from mammary epithelial tumour cells, T47D cells are more responsive to progesterone, while the MCF7 cell line does not elicit a response to progesterone in the presence of oestrogen (Yu *et al.*, 2017). Furthermore, MCF7 and T47D breast cancer cells are known to express Platelet Activating Factor Receptor (PAF-R) (Youlyouz and Magnoux, 2002; Mezouar *et al.*, 2016; da Silva Junior *et al.*, 2018). PAF-R's cognate ligand PAF which can be secreted by both tumour cell lines is an inflammatory phospholipid

mediating platelet aggregation and degranulation (Youlyouz *et al.*, 2002; Zimmerman *et al.*, 2002; da Silva-Junior *et al.*, 2018), and is also implicated in enhancing tumour progression (Bussolati *et al.*, 2000; da Silva Junior *et al.*, 2018). While strong associations are made between cell line studies and the clinical context, their predictive value may be skewed due to the differential responses they may elicit in response to hormone-therapy treatment strategies, as well as their potential to activate platelets and induce hypercoagulation.

3.1.2 Thrombin Generation and Platelet Activation

Breast cancer cells are able to facilitate platelet activation and aggregation and the formation of a fibrin-rich network to form thrombi by either direct contact with platelets or by the release of mediators including ADP, thrombin, thromboxane A₂ (TXA₂) or tumour associated proteinases (Nash *et al.*, 2002; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2017; Reddel, Tan and Chen, 2019). Of these agonists, thrombin is proposed to be the most significant and potent contributor to platelet activation (Nash *et al.*, 2002; Reddel, Tan and Chen, 2019).

Thrombin binds to and activates the Protease Activated Receptors (PAR); specifically, PAR₁, and PAR₄ (Kreutz *et al.*, 2012; Nag and Bar-Shavit, 2018). Thrombin binding induces the coagulation cascade and is said to be the main effector in its induction (Lisman, Weeterings and de Groot, 2005; Chapin and Hajjar, 2015). During the coagulation cascade, tissue factor (TF) interacts and forms a complex with circulating factor VIIa. This complex, in turn, activates factor X, producing factor Xa. Factor Xa then converts prothrombin to active thrombin (Lal, Dittus and Holmes, 2013; Reddel, Tan and Chen, 2019). Thrombin activation cleaves soluble fibrinogen to fibrin, an insoluble protein, and increases its presence within the microenvironment (Lisman, Weeterings and de Groot, 2005; Pretorius *et al.*, 2007, 2011; Weisel and Litvinov, 2017; Zarà *et al.*, 2018). This cleavage allows for the formation of fibrin strands which bind platelets at the GPIIa/IIIb site, ultimately forming a meshwork over the platelet aggregate/plug (Lisman, Weeterings and de Groot, 2005; Pretorius *et al.*, 2007, 2011; Weisel and Litvinov, 2017). Cellular responses, including that of platelets, to thrombin desensitises PAR-1. Additionally, activation of PAR-1 is irreversible (Herkert *et al.*, 2001), thus constant replenishment of receptors and delivery to the plasma membrane is required (Nag and Bar-Shavit, 2018). Thrombin promotes coagulation through the production of fibrin and

the activation of platelets, which eventually leads to platelet aggregation (Brass, 2003; Reddel, Tan and Chen, 2019).

The primary function of platelets is haemostasis (Ghoshal and Bhattacharyya, 2014). Agonists, including thrombin, activate and induce platelet shape changes, as well as microparticle shedding (Brass, 2003; Reddel, Tan and Chen, 2019). During this process, platelets synthesise and release thromboxane (TXA₂), which further facilitates platelet activation and aggregation, and by also acting as a vasoconstrictor mediator, plays a role in the formation and stabilisation of thrombi (Lisman, Weeterings and de Groot, 2005; Weisel and Litvinov, 2017; Zarà *et al.*, 2018). During platelet activation; platelets also release granular content to facilitate the formation of a thrombus as well as to recruit additional cells (Nash *et al.*, 2002; Zheng *et al.*, 2017). P-selectin (CD62P) is released from the α -granules and is translocated to the membrane of the platelet body allowing it to be used as an early marker for activation (McNicol and Israels, 2008; Augustine *et al.*, 2016); CD63, however, is released from dense and lysosomal granules and is associated with platelet spreading and platelet tyrosine phosphorylation on immobilized fibrinogen (Saboor, Moinuddin and Ilyas, 2013; Yun *et al.*, 2016).

Platelets are thus further able to influence tumour growth and metastasis *in situ* through non-classical haemostatic mechanisms allowing for intravasation, the formation of a platelet shield, arrest within the vasculature and extravasation at secondary sites (McNicol and Israels, 2008; Schlesinger, 2018). Additionally, thrombocytosis, an increase in platelet number, is common in several cancer types including in breast cancer patients and is linked with a poorer prognosis (Stravodimou and Voutsadakis, 2013; Sylman *et al.*, 2017). Breast cancer cells can induce platelet activation via various mechanisms; they are able to release platelet-activating mediators, including ADP and thrombin, TF as well as Factor V, VIII, IX and XI; leading to thrombin generation and hypercoagulation (Nash *et al.*, 2002; Schlesinger, 2018). Clinically this is evident in cancer patients who present with elevated plasma levels of markers of platelet activation, with these heightened levels being indicative of tumour progression towards that of a more invasive tumour (Caine *et al.*, 2002; Chaari *et al.*, 2012; Kyriazi, 2012; Schlesinger, 2018; Bauer, 2019).

3.1.3 Tamoxifen-treatment of Hormone-dependent breast cancers

Tamoxifen is the most widely prescribed breast cancer hormone-therapy (Criscitiello *et al.*, 2011; Heery, Corbett and Zolkowitz, 2018), and is also used as a chemopreventive drug by pre- and post-menopausal women, who have potential risk for breast cancer (Chang, 2012; Waters *et al.*, 2012). Tamoxifen functions by blocking the transcriptional activity of oestrogen receptors by binding them directly, reducing hormone-induced proliferation (Onitilo *et al.*, 2012; Lukong, 2017; El Sayed *et al.*, 2019). Tamoxifen can be taken either orally or intravenously, depending on the severity of cancer (Jonat *et al.*, 2006). Tamoxifen is reported to decrease tumour recurrence by 40%-50% in postmenopausal women and by 30%-50% in premenopausal women (Bakkum-Gamez *et al.*, 2011). As part of the prevention strategy, its usage is associated with reducing the risk of new cancer development by almost 50% (Wickerham, 2010; Sauter, 2018).

Tamoxifen induces apoptosis of cells, regardless of ER presence, due to its cytostatic and cytotoxic effects (Majumdar, Valdellon and Brown, 2001; Cuevas and Lindeman, 2015). Studies conducted on the effects of Tamoxifen as an adjuvant treatment strategy for breast cancers in post-menopausal women showed positive disease-free state; however, a greater risk for development of venous thromboembolisms (VTE) was identified (Meier & Jick 1998; Onitilo *et al.* 2012; Walker *et al.* 2016; MacLellan *et al.*, 2012). Additionally, despite initial positive responses to Tamoxifen-treatment, at least 1/3 of patients will develop resistance to treatment, though their ER α status may not change (Chang, 2012). Notably, lower ER β expression is noted within Tamoxifen-resistant tumours, while higher levels of ER β expression is associated with a better clinical outcome in ER α positive breast tumours (Fox, Davis and Shupnik, 2008), with contradictory studies implicating low ER β expression in Tamoxifen-resistant tumours (Begam, Jubie and Nanjan, 2017). While some studies indicate a role for ER β expression in predicting a better disease-free survival in patients treated with Tamoxifen (Tan *et al.*, 2016); others suggest that ER β positivity is associated with low cellular differentiation indicating a lower survival rate (Leung *et al.*, 2010).

While Tamoxifen has shown efficacy in reducing mortality and increasing survival in patients (Kmieciak *et al.*, 2011; Mittendorf *et al.*, 2016; Rugo, 2016; Masoud and Pagès, 2017); associated and resulting hypercoagulation and thrombosis remain the second main cause of

associated mortality (Meier and Jick, 1998; Decensi *et al.*, 2005; Howell *et al.*, 2005; Jonat *et al.*, 2006; Walker *et al.*, 2017; Bray *et al.*, 2018). This induction of hypercoagulation indicates an interplay between oestrogen-induced platelet activation as well as activation due to the cytotoxic nature of Tamoxifen itself (Eroglu, 2013; Kim, Kim and Kim, 2015; Elaskalani *et al.*, 2017). The precise mechanism by which Tamoxifen exerts its procoagulant effect remains to be discovered (Onitilo *et al.*, 2012; Kovac *et al.*, 2015). However, studies implicate the anti-oestrogenic activity of the drug on ER α on the platelets further increasing the risk of VTE (M Jayachandran and Miller, 2003; Dupuis *et al.*, 2019), and Factor VIII in promoting thrombotic events via its effect on platelet activation (Kovac *et al.*, 2015). Furthermore, high Factor VIII is noted in women undergoing Tamoxifen adjuvant therapy and is associated with an increased risk of VTE (Kovac *et al.*, 2015).

Tamoxifen is also able to directly induce rapid increases in intracellular free calcium in human platelets and is further able to act together with other agonists, including thrombin and ADP to achieve the same (Dobrydneva *et al.*, 2007). Tamoxifen is able to cause this rapid increase in calcium by inducing the release of stored calcium within the endoplasmic reticulum and by inducing calcium entry from the extracellular space (Dobrydneva *et al.*, 2007). An increase in intracellular free calcium leads to platelet activation and aggregation and contributes to the development of Tamoxifen-induced thrombosis (Dobrydneva *et al.*, 2007). Moreover, studies have shown an increase in basal calcium ions in MCF7 breast cancer cells and cytotoxicity induced by Tamoxifen (Azimi, Roberts-Thomson and Monteith, 2014); this translates clinically where noted side effects, however rare, of Tamoxifen use is the development of high calcium levels within the blood which cannot be controlled further inducing a thrombotic state (Arumugam *et al.*, 2006).

In Chapter 2 we showed that Tamoxifen pre-treatment of breast cancer cells, particularly the T47D cell line, compounds their ability to induce platelet activation. However, recent *in vitro* studies making use of Tamoxifen in the pre-treatment of washed platelets indicated direct inhibition of tumour cell-induced platelet activation and the reduction of the pro-angiogenic and pro-metastatic effects (Johnson *et al.*, 2017); as well as inhibition of collagen-induced platelet aggregation, greater than that of other agonists, in healthy human subjects (Chang *et al.*, 2011). Both studies contrast with our previously presented *in vitro* results as well as

contradict clinical evidence (Meier and Jick, 1998; Hernandez *et al.*, 2009; Eroglu, 2013); we postulate these differences to be attributed to methodological differences. The removal of plasma, and subsequently the effects of thrombin and the induction of calcium influx, during the washing process, inhibits tumour-cell induced platelet activation and subsequent aggregation (Zarà *et al.*, 2018), favouring a methodological approach which complements the physiological environment so as not to negate such effects. Clinically, Tamoxifen is administered orally or intravenously, depending on the severity, staging and patient preference; allowing for availability within the circulation as well as ultimate accumulation of the therapy at the tumour site (Ishitobi *et al.*, 2013; Sobczynski *et al.*, 2015). Thus, this Chapter presented cumulative and circulating effects of Tamoxifen on ER expression and the relationship to hypercoagulation *in vitro*. This was done by employing two models to mimic the physiological environment: Model 1 where breast cancer cells are pre-treated with Tamoxifen to mimic accumulation of the drug in breast tissue; Model 2 where blood is pre-treated with Tamoxifen followed by exposure to (untreated) breast cancer cells to reflect circulatory effects of the drug and subsequent association of platelets with tumour cells. Both models made use of blood constituents; including whole blood, platelet-rich plasma and platelet-poor plasma exposed to MCF7 and T47D breast cancer cells to assess associated hypercoagulation, defined by thrombin release and generation by breast cancer cells with ensuing effects on platelet activation identified by CD62P and CD63 expression, with corresponding platelet morphological alterations. We further assessed whether ERs, ER α , the most commonly studied (Ali and Coombes, 2000; Clatot, Augusto and Di Fiore, 2017) and ER β , could potentially be used as a predictive marker of Tamoxifen resistance in tumours and could potentially be associated with markers of hypercoagulation, further holding a predictive value of thrombotic risk dependent on tumour phenotype.

3.1.4 Aims and Objectives

The aim of this study was to determine the effect of cumulative (Model 1) and circulating (Model 2) Tamoxifen on ER expression and their relationship to hypercoagulation *in vitro*. Thus, the objectives were:

- 3.1.4.1 To assess whether approximated normal MCF10A breast epithelial cells alter ER expression and induce platelet activation as a proxy for 'normal' mammary epithelium.
- 3.1.4.2 To assess the interactions between platelets and MCF7 and T47D breast cancer cells pre-treated with Tamoxifen, indicating the cumulative effects of the drugs (Model 1).
- 3.1.4.3 To assess the interactions between platelets and MCF7 and T47D breast cancer cells exposed to Tamoxifen-treated blood constituents, indicative of freely available drugs within the bloodstream (Model 2).

This was achieved by analysing:

- 3.1.4.4 Spontaneous thrombin release from breast cancer cells
- 3.1.4.5 Induced levels of platelet activation defined by CD62P and CD63 expression interpreted by the Index of Platelet Activation (IPA) and confirmed ultrastructurally.
- 3.1.4.6 ER α and ER β protein expression profiles in breast cancer cells.
- 3.1.4.7 The correlation between ER protein alterations and spontaneous thrombin release from breast cancer cells, to determine whether ER expression profiles may predict endogenous thrombin availability.
- 3.1.4.8 The correlation between spontaneous thrombin release from breast cancer cells and the Index of Platelet Activation (IPA), to determine whether changes in endogenous thrombin associated with ER expressional alterations may hold predictive value in determining associated platelet activation.

3.2 Materials and Methods

Human Research Ethics Clearance was obtained from the Human Ethics Research Committee (Medical), University of the Witwatersrand, Clearance Certificate Number M160826 (Refer to Appendix for Clearance Certificate). This Chapter presents results for Tamoxifen-treatment, with Anastrozole-treatment presented in Chapter 4. Therapies were presented separately due to them not being given concurrently clinically.

3.2.1 Methodology Framework – in brief

To conduct the methodology for this aim MCF10A, MCF7 and T47D breast cancer cells were seeded at 1×10^5 cells/well onto glass coverslips. T47D cells were seeded onto glass coverslips coated with poly-d-lysine (see Appendix) to facilitate adhesion. For Model 1: MCF7 and T47D cells were pre-treated with $2 \mu\text{M}$ Tamoxifen for 24 hours. For Model 2: 18 ml of peripheral WB was obtained from volunteers and 4.5 mL treated with $2 \mu\text{M}$ Tamoxifen for 1 hour at RT. WB was then centrifuged and separated into its constituents: WB, platelet-rich plasma (PRP) and platelet-poor plasma (PPP). For Model 1: untreated blood constituents were exposed to Tamoxifen-treated cells, and for Model 2: Tamoxifen-treated blood constituents were concurrently exposed to untreated cells for 2.5 minutes.

Following this co-incubation breast cancer cells and blood constituents were isolated and prepared for their ensuing techniques (Figure 3.1).

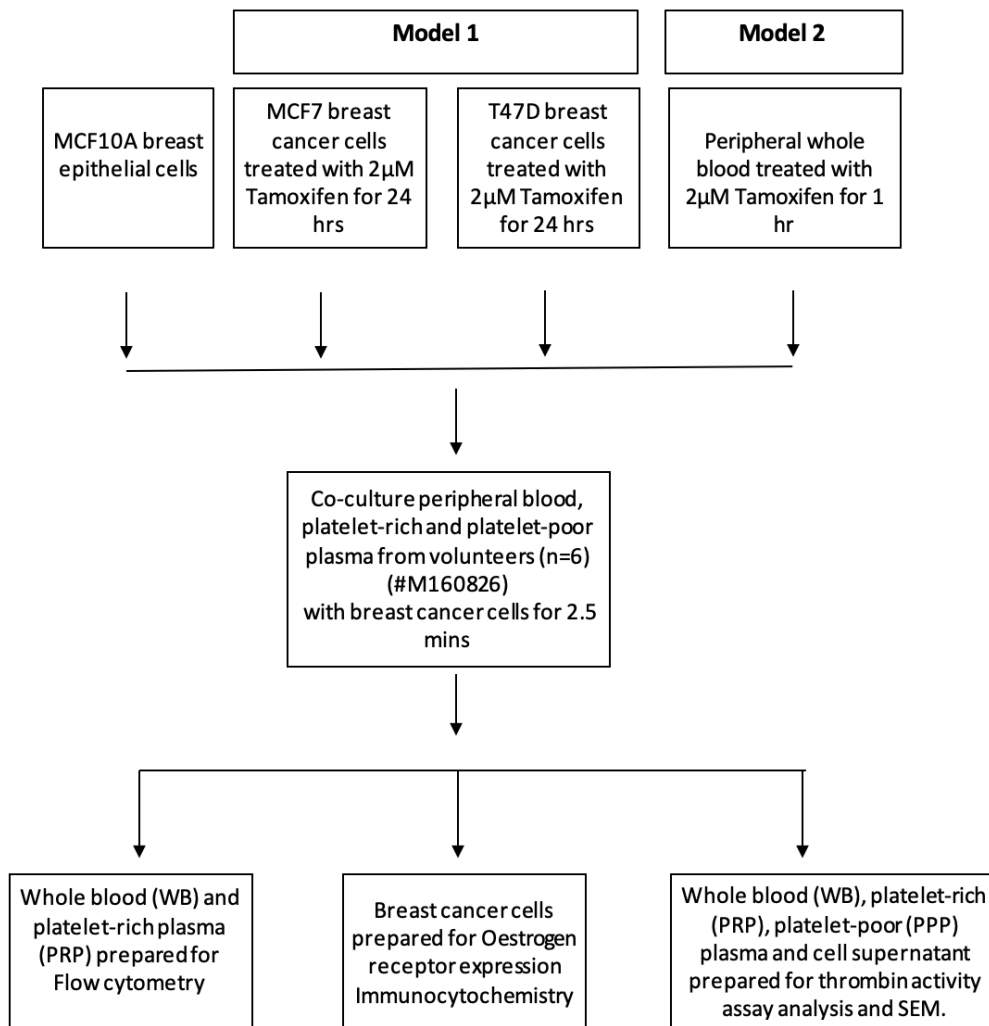


Figure 3.1: Flow diagram indicating the stepwise methodology followed for experimentation. MCF10A, MCF7 and T47D breast cancer cells were seeded at 1×10^5 cells/well onto glass coverslips. MCF7 and T47D cells were pre-treated with $2 \mu\text{M}$ Tamoxifen for 24hr. Peripheral WB was obtained from volunteers, 4.5 ml was treated with $2 \mu\text{M}$ Tamoxifen, for 1 hour at RT. WB was separated into its constituents: WB, PRP and PPP. Untreated blood constituents were exposed to Tamoxifen-treated cells (Model 1), Tamoxifen-treated blood constituents were, concurrently exposed to untreated cells (Model 2), for 2.5 minutes. Following co-incubation breast cancer cells and blood constituents were isolated and prepared for their ensuing techniques. Blood constituents were prepared for flow cytometry and scanning electron microscopy to assess levels of platelet activation. Breast cancer cells were prepared for ICC for assessment of ERs, and supernatant including; WB, PRP, PPP and cell supernatant were prepared for assessment of thrombin detection.

3.2.2 Cell Culture and Establishment of Stock Populations

MCF7 and T47D breast cancer cells were cultured with stock populations established as per [2.2.2](#). MCF10A, non-tumorigenic breast epithelial cells were obtained from ATCC (Virginia, USA) and propagated in MEGM (Mammary epithelial cell growth medium BulletKit, Lonza, Walkersville, MD, USA; CC-3150), supplemented with 100 ng/mL cholera toxin (Merck, Johannesburg, South Africa; C8052). Once cells reached ~70-90% confluency, cells were washed with 1 M PBS, pH 7.4. Cells were subcultured as per 2.2.2. Following the establishment of the stock population, cells were cryopreserved in 10% DMSO (Dimethyl

sulfoxide, Saarchem, Johannesburg, South Africa; 186500), with 60% FCS and 30% respective cell culture media at -80°C.

3.2.3 Tamoxifen-treatment of Breast Cancer Cells

Cryopreserved cells (MCF7, T47D and MCF10A) were thawed and washed once in 1ml 1 M PBS followed by resuspension in 1ml of their respective cell culture media, with cell seeding following as per Chapter 2, section [2.2.3](#) paragraph 2. MCF10A and MCF7 cells were seeded directly onto glass coverslips (Nunc, Roskilde, Denmark; 111500) in 24 well plates (Nunc, Roskilde, Denmark; 662160), while T47D cells were seeded onto glass coverslips coated with 5 mg/ml poly-d-lysine (Appendix) (Sigma-Aldrich, St. Louis, Missouri, USA; P6407) to facilitate attachment.

The maximum effective concentration of Tamoxifen was determined as per Chapter 2, section [2.2.4](#). For Model 1 plated cells were rinsed with 1 M PBS and pre-treated 200 µl 2 µM Tamoxifen for 24 hours at 37°C and 5% CO₂ prior to exposure to blood constituents.

3.2.4 Whole Blood Acquisition, Preparation and Co-culture

Peripheral WB (n=6) was extracted under the same conditions as Chapter 2, section [2.2.5](#). First, for establishment of Model 2, 4 mL of WB was removed from the sodium citrate vacuette coagulation tubes and treated with 2 µM Tamoxifen in a separate centrifuge tube for 1 hour at RT. The remaining WB was retained untreated for use in Model 1. Subsequently, WB was separated into its component parts; platelet-rich plasma (PRP) and platelet-poor plasma (PPP). To obtain PRP, WB samples were centrifuged at 200 x *g* and the supernatant removed and used. To obtain PPP, WB samples were centrifuged at 400 x *g* and the supernatant removed and used.

3.2.5 Establishment of a co-culture systems Model 1 and Model 2

Breast cancer cells were rinsed twice with 1 M PBS and incubated with 200 µl WB, PRP or PPP for 2.5 minutes. For Model 1: Tamoxifen-treated breast cancer cells were incubated with 200 µl of WB, PRP and PPP for 2.5 minutes. For Model 2: Untreated breast cancer cells were incubated with 200 µl Tamoxifen-treated WB, PRP or PPP for 2.5 minutes (Table 3.1). All

samples, experimental and control, were prepared in singlet for each subsequent assay (SEM, flow cytometry, ICC and thrombin assessment) with 6 biological replicates ($n = 6$).

Table 3.1: Control and experimental samples, with the abbreviation indicating the sample key used in ensuing analysis. Samples are further grouped into; Model 1: assessment of cumulative effects of Tamoxifen. Prefix: M: MCF7 breast cancer cells. T: T47D breast cancer cells. Suffix: treatment options employed; Model 2: assessment of circulating effects of Tamoxifen. Prefix: pre-treated blood constituent Suffix: M - MCF7 breast cancer cells. T- T47D breast cancer cells.

Blood Constituent Controls	
Abbreviation	Explanation
WB	Whole Blood
WBThr	Positive control: Whole blood incubated with 0.1 U/mL human thrombin
PRP	Platelet-rich Plasma
PRPThr	Positive control: platelet-rich plasma incubated with 0.1 U/mL human thrombin
PPP	Platelet-poor Plasma
Cell Line Controls	
Abbreviation	Explanation
M10MED	MCF10A breast epithelial cell media control
M10WB	MCF10A breast epithelial cells exposed to whole blood
M10PRP	MCF10A breast epithelial cells exposed to platelet-rich plasma
M10PPP	MCF10A breast epithelial cells exposed to platelet-poor plasma
MMED	MCF7 breast cancer cell media control
MMEDWB	MCF7 breast cancer cells exposed to whole blood
MMEDPRP	MCF7 breast cancer cells exposed to platelet-rich plasma
MMEDPPP	MCF7 breast cancer cells exposed to platelet-poor plasma
MDIL	MCF7 breast cancer cells treated with 0.1% DMSO
MDILWB	MCF7 breast cancer cells treated with 0.1% DMSO exposed to whole blood
MDILPRP	MCF7 breast cancer cells treated with 0.1% DMSO exposed to platelet-rich plasma
MDILPPP	MCF7 breast cancer cells treated with 0.1% DMSO exposed to platelet-poor plasma
MTAM	MCF7 breast cancer cells treated with 2uM Tamoxifen
TMED	T47D breast cancer cell media control
TMEDWB	T47D breast cancer cells exposed to whole blood
TMEDPRP	T47D breast cancer cells exposed to platelet-rich plasma
TMEDPPP	T47D breast cancer cells exposed to platelet-poor plasma
TDIL	T47D breast cancer cells treated with 0.1% DMSO
TDILWB	T47D breast cancer cells treated with 0.1% DMSO exposed to whole blood
TDILPRP	T47D breast cancer cells treated with 0.1% DMSO exposed to platelet-rich plasma
TDILPPP	T47D breast cancer cells treated with 0.1% DMSO exposed to platelet-poor plasma
TTAM	T47D breast cancer cells treated with 2uM Tamoxifen
Model 1	
Abbreviation	Explanation
MTAMWB	MCF7 breast cancer cells pre-treated with 2 uM Tamoxifen exposed to whole blood
MTAMPRP	MCF7 breast cancer cells pre-treated with 2 uM Tamoxifen exposed to platelet-rich plasma
MTAMPPP	MCF7 breast cancer cells pre-treated with 2 uM Tamoxifen exposed to platelet-poor plasma
TTAMWB	T47D breast cancer cells pre-treated with 2 uM Tamoxifen exposed to whole blood
TTAMPRP	T47D breast cancer cells pre-treated with 2 uM Tamoxifen exposed to platelet-rich plasma
TTAMPPP	T47D breast cancer cells pre-treated with 2 uM Tamoxifen exposed to platelet-poor plasma
Model 2	
Additional Blood Constituent Controls	
Abbreviation	Explanation
WBTAM	Whole blood treated with 2 uM Tamoxifen
PRPTAM	Platelet-rich plasma treated with 2 uM Tamoxifen
PPPTAM	Platelet-poor plasma treated with 2 uM Tamoxifen
Experimental	
Abbreviation	Explanation
WBTAMM	Whole blood treated with 2 uM Tamoxifen co-incubated with MCF7 breast cancer cells
PRPTAMM	Platelet-rich plasma treated with 2 uM Tamoxifen co-incubated with MCF7 breast cancer cells
PPPTAMM	Platelet-poor plasma treated with 2 uM Tamoxifen co-incubated with MCF7 breast cancer cells
WBTAMT	Whole blood treated with 2 uM Tamoxifen co-incubated with T47D breast cancer cells
PRPTAMT	Platelet-rich plasma treated with 2 uM Tamoxifen co-incubated with T47D breast cancer cells
PPPTAMT	Platelet-poor plasma treated with 2 uM Tamoxifen co-incubated with T47D breast cancer cells

3.2.6 Thrombin Activity Assay

3.2.6.1 Sample Preparation

The release of thrombin as a procoagulant factor during co-culture was tested using a Thrombin Activity Assay Kit (Abcam ab 197006). 180 µl of supernatant from samples was aspirated from their respective wells and placed into 1 mL eppendorfs, snap-frozen in liquid nitrogen and kept at -80°C until assayed. On the day of assay, samples were thawed on ice and diluted at 1/10 with assay buffer, determined to be the optimal dilution through optimisation (see Appendix). Two sets of wells in a 96-well plate were prepared: standard wells and sample wells. 6 standards were prepared by adding 2.5 ng/µl and assay buffer to the wells to get thrombin concentrations of; 0 ng/well, 5 ng/well, 10 ng/well, 15 ng/well, 20 ng/well, 25 ng/well. Diluted samples were added to their respective wells at 50 µl. Both standard and sample wells were incubated with thrombin substrate diluted in assay buffer. Fluorescence was measured at Ex/Em=350/450 using a GloMax Promega Plate Reader in a kinetic mode every 2 minutes for 60 minutes at 37°C, Department of Molecular Medicine and Haematology, University of the Witwatersrand.

3.2.6.2 Data Analysis

Data obtained was exported into Microsoft Excel and further analysed using Statistica v23. A Shapiro-Wilk test of normality determined that the data was not normally distributed. Non-parametric tests were also used due to their robusticity for limited sample sizes. The non-parametric Friedman's test was conducted to determine whether thrombin release from breast cancer cells altered in response to hormone-therapy, following which a *post-hoc* Wilcoxon Matched Pairs test was conducted to determine whether these differences were significant ($p < 0.05$). Furthermore, a matched paired Wilcoxon Signed Ranks test was conducted to assess differences between Models. All tests made use of a confidence interval of 95%, and a significance level of 0.05.

3.2.7 Assessment of platelet activation

3.2.7.1 Sample Preparation for flow cytometry

WB samples were prepared as per Chapter 2, section [2.2.8](#). To assess platelets at later stages of activation PE-Cy7 mouse anti-human CD63 antibody at a concentration of 1:20 (5 µl) was added to all samples, as determined by titration. To analyse platelets within PRP, samples were resuspended in 150 µl Tyrode's buffer and labelled with APC-conjugated mouse anti-

human CD41a, FITC-conjugated mouse anti-human CD62P at a concentration of 1:20, for each antibody; and PE-Cy7 mouse anti-human CD63 at a concentration of 1:20. Samples were fixed as per Chapter 2, section 2.2.8.1 prior to assessment on the LSR Fortessa. Technical controls included unlabelled WB and PRP samples and single antibody labelled samples to determine autofluorescence and fluorescence overlap.

3.2.7.2 Gating Strategy and Data Acquisition

Data were acquired as per Chapter 2, section [2.2.8.2](#) with the addition of a late stage marker of platelet activation (CD63).

Events were recorded at 100 000 events per sample using FACSDiva Software (BD Biosciences), with platelets initially gated on size and granularity, using a forward scatter voltage of 300V and a side scatter voltage of 275V. The singlet population was identified and gated making use of forward scatter height and forward scatter area, at a voltage of 300V. Platelets were identified and gated based on the expression of the CD41a and separated using the APC voltage at 565V. Quadrant plots were drawn for identification of active platelet populations. Early stages of platelet activation within this population were assessed using CD62P and separated by making use of the FITC voltage at 469V. Late stage platelet activation was assessed by making use of CD63, a platelet activation marker released by dense granules; discriminated by making use of the PE-Cy7 voltage at 645V. Interval gates were drawn to classify a graded level of platelet activation determined using Geometric Mean Fluorescence Intensity (gMFI) of the platelet population (Figure 3.2, 3.3 and 3.4). Progression of platelet activation was assessed by basing the quadrant plot of either early (CD62P) (Figure 3.2) or late stage (CD63) (Figure 3.3) activation on the positive platelet population (CD41a), with interval gates drawn to assess graded levels of activation progression dependent on CD62P and CD63 gMFI. Interval gates for both, CD62P and CD63 markers, as well as for populations expressing both markers; were drawn identically to ascertain correct intervals when statistically analysing and comparing groups.

The interval gating strategy employed allowed mapping of the spread of CD63, in addition to ascertaining total CD63 expression with a right shift along the X-axis reflecting higher levels of CD62P expression (Figure 3.4). Quadrants (Q): Q1 represents the inactive platelet population (CD41a⁺CD63⁻) population, while the activated population of interest

(CD41a⁺CD63⁺) was within the Q2 quadrant. Q3 represents other cell types within the sample (CD41a⁻CD63⁻), while Q4 is postulated to present CD63⁺ microparticles within the sample. Interval gates (I1-I10) were drawn within the Q2 quadrant, indicative of increasing CD63 expression. Interval gates 1 and 2 (I1 and I2) represents the bulk of CD41a⁺ platelets with low CD63 expression (CD41a⁺CD63⁺), while interval gates 3 and 4 (I3 and I4), represent platelets with moderate CD63 expression (CD41a⁺CD63⁺⁺), with interval gate 5 (I5) presenting the smallest proportion of platelets with the greatest expression of CD62P (CD41a⁺CD63⁺⁺⁺) (Figure 3.3).

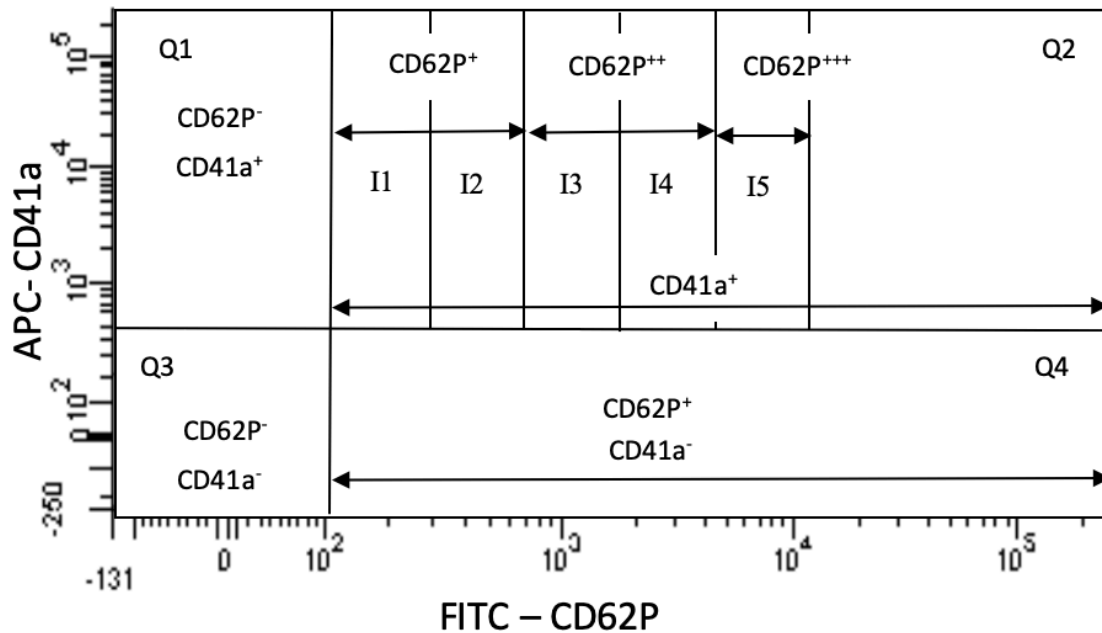


Figure 3.2: Representative scatter plot showing the spread of platelet activation across the interval gates. Quadrant 1 (Q1) is the inactive platelet population (CD41a⁺CD62P⁻) population, while the activated population of interest (CD41a⁺CD62P⁺) was within the 2nd quadrant (Q2). Interval gates (I1-I5) were drawn within the quadrant, indicative of increasing CD62P expression. The 3rd quadrant (Q3) depicts the negative cell population, while the 4th quadrant (Q4) is postulated to show CD62P⁺ microparticles.

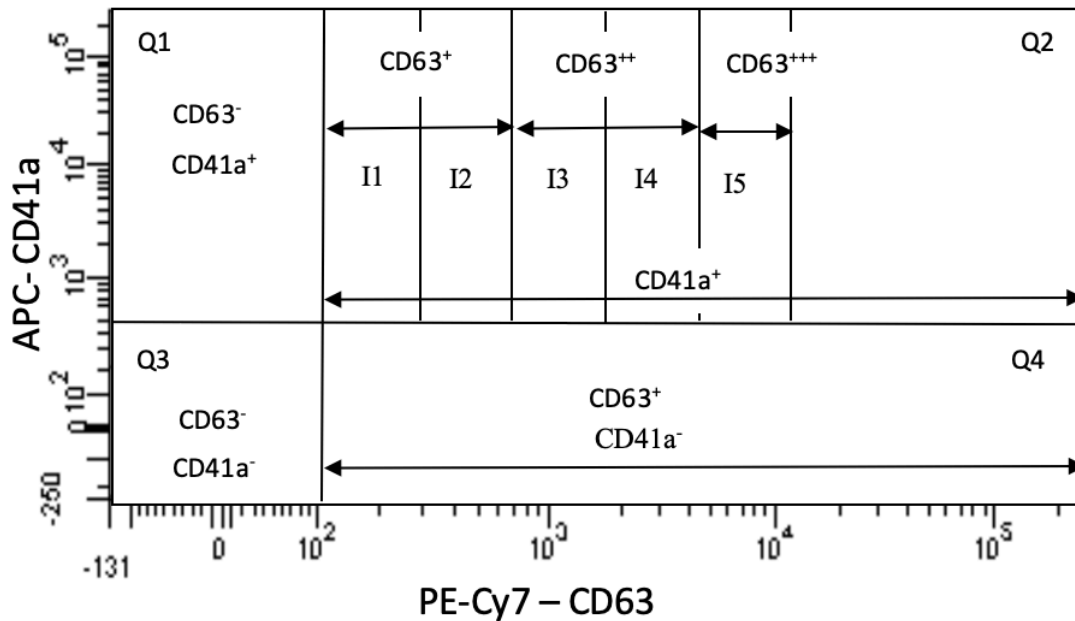


Figure 3.3: Representative scatter plot showing the spread of platelet activation across the interval gates. Quadrant 1 (Q1) is the inactive platelet population ($CD41a^+CD63^-$) population, while the activated population of interest ($CD41a^+CD63^+$) was within the 2nd quadrant (Q2). Interval gates (I1-I5) were drawn within the quadrant, indicative of increasing CD63 expression. The 3rd quadrant (Q3) depicts the negative cell population, while the 4th quadrant (Q4) is postulated to show $CD63^+$ microparticles

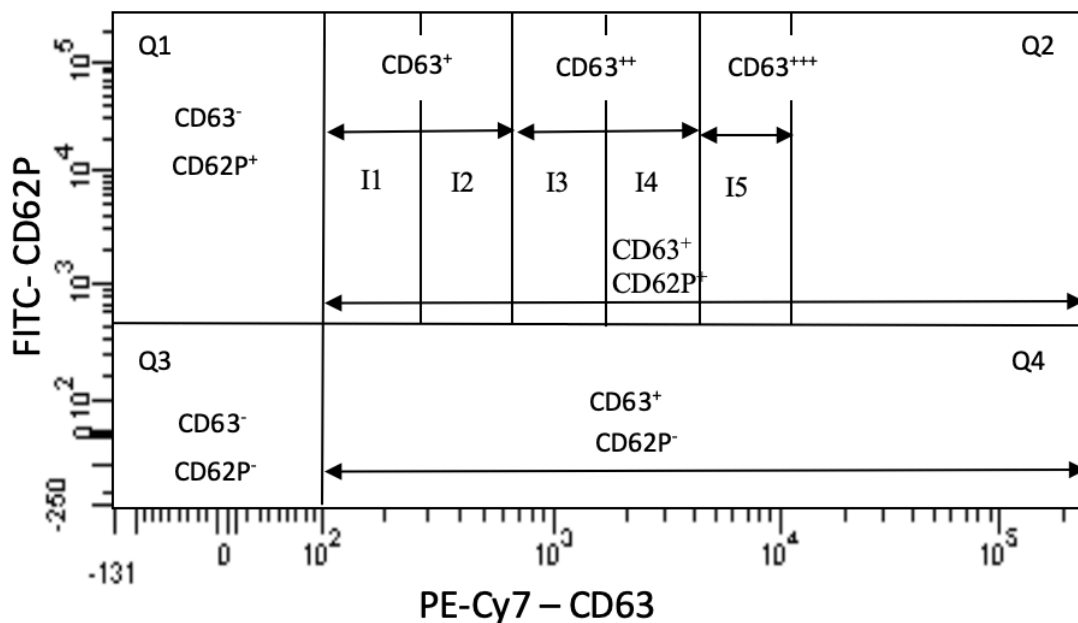


Figure 3.4: Representative scatter plot based on $CD41^+$ platelets, showing the spread of platelet activation across the interval gates. Quadrant 1 (Q1) is the platelet population at early stages ($CD62P^+CD63^-$), while the population of interest ($CD62P^+CD63^+$) was within the 2nd quadrant (Q2). Interval gates (I1-I5) were drawn within the quadrant, indicative of platelets expression both markers. The 3rd quadrant (Q3) depicts the negative cell population, while the 4th quadrant (Q4) shows the $CD62P^-CD63^+$ population.

3.2.7.3 Statistical Analysis

Data acquired using FACSDiva software was exported into Microsoft Excel and analysed using Statistics v23 statistical software.

The index of platelet activation (IPA) was first calculated such that:

Early Activation: $IPA = gMFI (CD62P) \times n(CD41a^+CD62P^+ \text{ events})$

Late Activation: $IPA = gMFI (CD63) \times n(CD41a^+CD63^+ \text{ events})$

With platelet activation identified as a ratio, such that;

Ratio: IPA =

$$\frac{gMFI (CD62P) \times n(CD41a^+CD62P^+ \text{ events})}{gMFI (CD63) \times n(CD41a^+CD63^+ \text{ events})}$$

A Shapiro-Wilk test of normality determined that the data was not normally distributed. The non-parametric Friedman test, based on the dependency of blood constituents, was conducted to determine whether IPA altered in response to hormone-therapy and blood constituent co-incubation, following which a *post-hoc* Wilcoxon Matched Paired test was conducted to determine whether these differences were significant ($p < 0.05$). Furthermore, a matched paired Wilcoxon Signed Ranks test was conducted to assess differences between Models. By making use of a Spearman's Rank Order Correlation, IPA from matched samples was correlated with thrombin detection.

3.2.8 SEM

WB samples were prepared as Chapter 2, section [2.2.8.4](#). PRP and PPP samples were removed from their corresponding wells and 20µl placed onto glass coverslips. All samples then underwent preparation and viewing as per 2.2.8.4.

3.2.9 Immunolocalisation of ER α and ER β protein expression in hormone-dependent breast cancer cells.

Images presented in the results are overlays of ER α (green) and ER β (red) protein expression where yellow indicates domination by ER α and orange indicates domination by ER β ; DAPI staining is not shown as it obscured nuclear localisation of ERs. All images; which included individual sample images of ER α (green) and ER β (red) and DAPI (blue) are presented in the Appendix.

3.2.9.1 Sample Preparation

Breast cancer cells were washed three times with 1M PBS pH 7.4, fixed in 4% paraformaldehyde for 10 minutes at RT and washed with 0.1% Tween20/PBS. Cells were then permeabilised with 0.2% Triton X-100/PBS for 10 minutes at RT, and washed three times with 0.1% Tween20/PBS, for 5 minutes for each wash. Cells were then incubated in 1% BSA (Bovine Serum Albumin) in Tween20/PBS for 30 minutes at RT. Anti-ER β (Abcam, ab288) and anti-ER α (Abcam, ab66102) primary antibodies, at concentrations 1:200 and 1:400 respectively (concentrations were determined by conducting a dilution assay, see Appendix), were diluted in Tween20/PBS and incubated with the cells at 4°C overnight. Cells were then washed three times with Tween20/PBS and incubated with fluorescently labelled secondary antibodies Alexa Fluor 488 anti-rabbit (Life Technologies, Johannesburg, South Africa, A11008) and Alexa Fluor 594 anti-goat (Life Technologies, A11005), at 1:1000 respectively, for 2 hours at RT in the dark. Cells were again washed three times with Tween20/PBS and nuclei were counterstained with DAPI. After a final wash with Tween20/PBS coverslips were mounted onto slides using Fluoromount (Sigma-Aldrich, F4680). Standard immunocytochemical technical controls were included.

3.2.9.2 Data Acquisition

Photomicrographs were obtained using an Olympus iX51 Inverted Fluorescent Microscope with CellSens Software, School of Anatomical Sciences, University of the Witwatersrand. Photomicrographs were taken using filters: U-MNU2 to detect fluorescence from DAPI staining, U-MWIBA3 to detect cells stained with and expressing Alexa Fluor 488 (ER α) and U-MWIY2 to detect cells stained with and expressing Alexa Fluor 594 (ER β). Immunocytochemistry controls indicated that the primary and secondary antibodies were correctly conjugated and there was no autofluorescence within the cells (Appendix).

Photomicrographs were obtained in grayscale at 40X magnification and quantitatively analysed using CellProfiler software (Carpenter *et al.*, 2006) and further statistically analysed using Statistica v23.

3.2.9.3 Cell Profiler

Photomicrographs taken using the U-MWIB2 filter for DAPI fluorescence were processed using the “EnhanceOrSuppressFeatures” module to suppress background noise found between the nuclei. The “IdentifyPrimaryObjects” module was used to identify differences between groups of pixels, and isolate high-intensity pixel regions as nuclei of cells or primary objects. The nuclei that were identified were isolated to a range between 40 and 100 pixels in diameter (Figure 3.5 A). Pixels that did not fall in this range or were on the boundary were discarded. The Background method was used for intensity thresholding and weighted variance was minimized with a non-adjusted “Threshold correction factor” indicated as 1. The lower bound was indicated at 0 and the upper bound was indicated at 1. Objects that were clumped were distinguished and separated using the “Intensity” method. Primary objects that were identified were outlined according to their size and shape in the photomicrograph that was loaded. The “IdentifyAlphaObjects” identified the cells labelled with Alexa Flour 488, expressing ER α , within the photomicrographs (Figure 3.5 B). The Background method was used for intensity thresholding, again and weighted variance was minimized with a “Threshold correction factor” of 0.99. The “IdentifyBetaObjects” identified the cells labelled with Alexa Flour 488, expressing ER β , within the photomicrographs (Figure 3.5 C). The Background method was used for intensity thresholding, again and weighted variance was minimized with a “Threshold correction factor” of 0.99. The “IdentifyColocalization” module was then used to process both images obtained using the “IdentifyAlphaObjects” and “IdentifyBetaObjects” modules to find the cytoplasm between the nuclei and the edge of the cells. The “MeanIntensity” of the markers expressed was measured using the “MeasureObjectIntensity” module and cell size and shape was measured using the “MeasureObjectSizeShape” module. Images obtained from the “Identify” modules were saved using the “SaveImages” modules, and finally, all data obtained using the “Measure” modules were exported to an excel spreadsheet using the “ExportToSpreadsheet” module and was analysed using the Statistica v24.

A Shapiro-Wilk test of normality determined that the data was not normally distributed. Thus, the non-parametric Friedmans test was conducted to determine whether ER alterations in response to hormone-therapy and blood constituent exposure was different, following which a *post-hoc* Wilcoxon Matched Pairs test was conducted to determine whether these differences were significant ($p < 0.05$). Furthermore, a matched paired Wilcoxon Signed Ranks test was conducted to assess differences between Models. By making use of a Spearman Rank Order Correlation, ER expression from matched samples was correlated with thrombin detection and IPA.

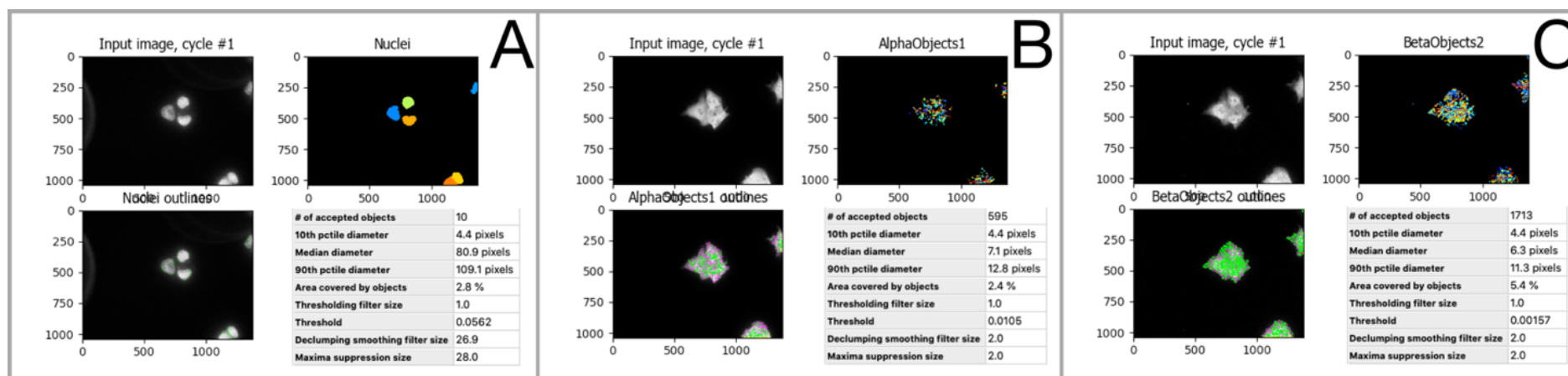


Figure 3.5: Representative CellProfiler generated images to show pipeline. A: Identifying the nucleus within the cells. B: Assessing $ER\alpha$ expression within the cells. C: Assessing $ER\beta$ expression within the identified cells.

3.3 Results

3.3.6 Non-tumorigenic breast epithelial cell line, MCF10A, induction of hypercoagulation and alterations in ER expression

3.3.6.1 *Thrombin Release from MCF10A cells*

MCF10A breast epithelial cells were employed as a control cell line to represent normal breast cell function and transformation. There was little to no detectable levels of thrombin in the culture media conditioned by MCF10A breast epithelial cells. When WB, PRP or PPP was introduced to the MCF10A cells a decrease in thrombin availability was noted in all samples compared to matched blood samples not exposed to breast epithelial cells (Figure 3.6(1)). WB co-incubation resulted in thrombin levels which were comparable to that of the WB alone. Both PRP and PPP co-incubation showed a decrease in thrombin availability compared to untreated PRP and PPP, respectively. This decrease in detectable thrombin levels could be due to binding of thrombin by the activated platelets within PRP. With the reduction in PPP postulated to be due to the binding of thrombin onto the cells themselves, and may relate to tissue factor dependence (Berny-Lang *et al.*, 2011).

3.3.6.2 *Induced Platelet Activation by MCF10A cells*

Overall IPA levels, IPA per interval gate and induction capability of MCF10A breast epithelial cells were assessed in comparison to their matched untreated controls, WB and PRP (Figure 3.6(2)). Co-incubation of WB or PRP with MCF10A cells did not induce significant changes in IPA of either marker overall. Upon ultrastructural assessment platelets from WB exposed to MCF10A breast epithelial cells were mostly in resting phase with some showing a smooth membrane with few surface folds and filipodia extending outward (Figure 3.6(3)A). Samples from both PRP and PPP exposed to MCF10A elicited a heightened response, with the formation of fibrin plaques and in the PRP samples, obscuring platelets within the sample (Figure 3.6(3)B & C). However, both CD62P and CD63 overall marker expression showed no significant changes (Figure 3.6(2)). Furthermore, ultrastructurally, pores in the fibrin plaque can be seen within the PRP co-incubated sample; while in the PPP co-incubated sample, more pores can be seen suggesting a more established plaque formation has taken place (Figure 3.6(3)B & C). This could explain the low marker expression noted within the samples, with the formation of plaques both obscuring the platelets and indicating hypercoagulation. This would be expected in these samples due to the centrifugation steps required to elicit PPP,

with more available fibrinogen to become fibrin in these samples (Ghassab *et al.*, 2015; Kwaan *et al.*, 2019). Upon further assessment of marker expression slight increases were noted between the untreated and WB and PRP co-incubated samples, with greater IPA noted within the low interval gates, tapering off as it reaches the higher interval gates, excluding that of CD63 in the WB co-incubated sample, which indicated a significant increase within the I5, high⁺ interval gate. These results indicate that while MCF10A cells are used as a “normal” control epithelial cell line, they are able to induce low levels of platelet activation and induce fibrin formation.

3.3.6.3 ER Expression in MCF10A Cells

MCF10A cells typically have a rounded cellular appearance. ER expression in untreated MCF10A cells (Figure 3.6(4)A) indicated ER α (green fluorescence) and ER β (red fluorescence) concentrated within the nucleus, as confirmed by assessing nuclear expression of DAPI (Appendix). The quantitative assessment indicated a high ER α expression with low ER β expression (Figure 3.6(5)). Co-incubation with blood constituents altered the morphology of the cells, with cells exposed to WB having a more star-shaped, elongated appearance compared to the untreated control (Figure 3.6(4)B). ER α and ER β expression was concentrated within the nuclear region, with more expression noted extending outward within the cellular processes. The quantitative assessment confirmed a significant increase in ER β expression within the cytoplasm of the cells on exposure with WB (Figure 3.6(5)). MCF10A cells exposed to PRP (Figure 3.6(4)C) showed elongated cellular processes, with both ER expression concentrated within the nuclear region; however, some expression was noted within the cellular processes, more notably so of ER α . MCF10A cells exposed to PPP followed the same trend in which cellular processes were more elongated with ER expression concentrated within the nucleus, and while scarce more ER α expression was noted within the cellular processes. The quantitative assessment indicated an increase ($p>0.05$) in ER β expression when compared to ER β expression in untreated MCF10A cells (Figure 3.6(5)).

Due to the variable results noted; alterations in thrombin availability, induction of low levels of platelet activation and alterations in not only morphology of cells but ERs as well, the MCF10A cell line was not used further as a control cell line in this study.

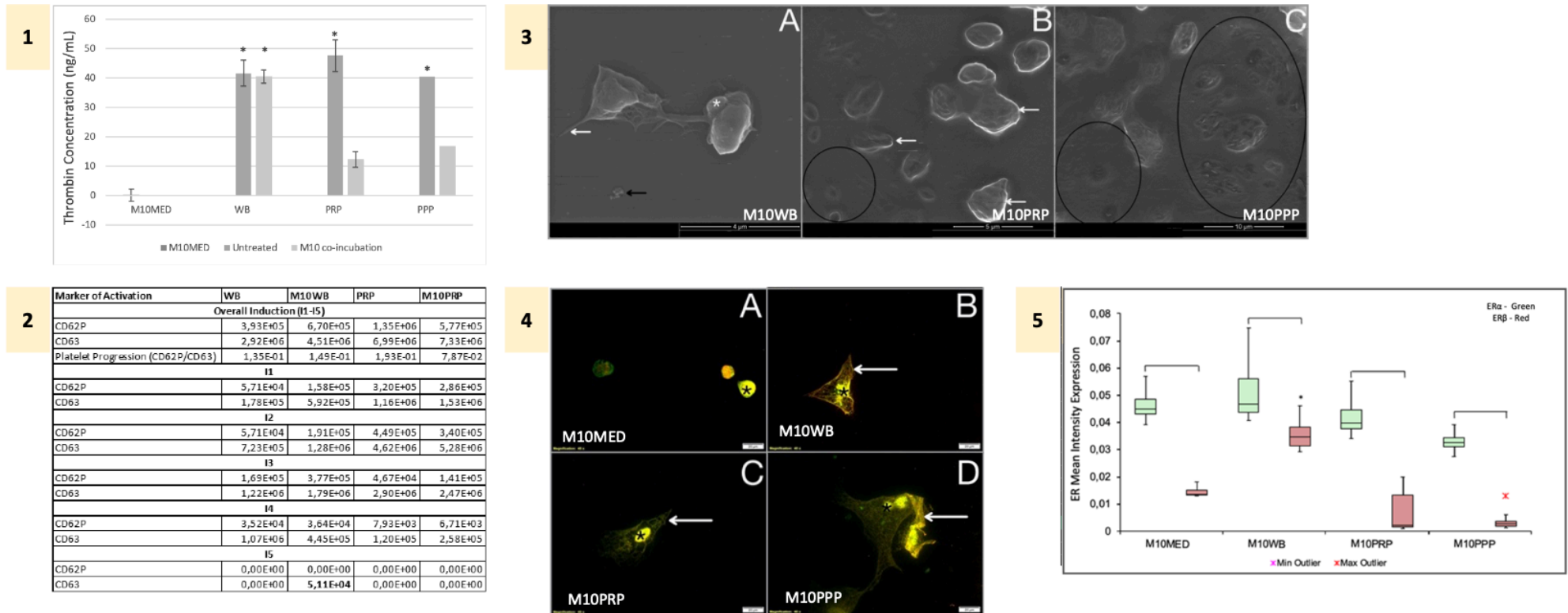


Figure 3.6: MCF10A effects on coagulation and ER expression. Key: WB – untreated whole blood; PRP: platelet-rich plasma; PPP: platelet-poor plasma; M10MED: MCF10 cells in conditioned media; M10WB: WB co-incubated with MCF10A cells; M10PRP – PRP co-incubated with MCF10A cells. (1) Bar-graph showing thrombin concentrations. Low detectable levels of secreted thrombin in conditioned media (M10MED) was noted. Untreated blood constituents (WB, PRP and PPP) showed significantly higher thrombin availability than MCF10A cells alone. On MCF10A co-incubation with WB detectable thrombin levels increased significantly. Co-incubation with both PRP and PPP induced only a slightly higher level of thrombin availability; * $p < 0.05$. (2) Index of platelet activation (IPA) markers CD62P and CD63, and CD62P/CD63 ratio of blood constituents exposed to MCF10A cells. Overall levels and per interval gate levels were assessed. Bold indicates samples significantly ($p < 0.05$) different from untreated matched control groups. (3) Platelet ultrastructural alterations in blood constituents exposed to MCF10A cells. A: M10WB: platelets with a smooth membrane and membrane folds (*), filopodia extension (white arrow) with microparticles (black arrow). B: M10PRP: fibrin plaque formation (white arrows) and pores (black circle). C: M10PPP: plaque formation with many pores (black circles). (4) Immunocytochemistry images (40X magnification) showing co-localisation of ERα (green) and ERβ (red) in MCF10A cells. A: M10MED: nuclear ERα and ERβ. B: M10WB: nuclear ERα and cytoplasmic ERβ. C: M10PRP: ERα in the nucleus (*) and cytoplasm (white arrow) and ERβ minimally in both. D: M10PPP: nuclear ERα (*) and low ERβ in the cytoplasm (white arrow). (5) Box and whisker plots representing quantified ERα (green) and ERβ (red) expression in MCF10A cells. * indicates significant ($p < 0.05$) differences between matched ERs compared to untreated MCF10A cells.

3.3.2 Baseline levels of coagulation in blood constituent controls.

Baseline levels of thrombin availability and platelet activation marker expression (CD62P and CD63) was assessed in untreated WB, PRP and PPP samples. The levels detected indicate standard detectable levels of thrombin and platelet activity, evident without positive induction of activation. Thus, this inherent level of observed availability and activation was treated as standard and an increase above this level would indicate a change in “normal”.

The positive controls (WB and PRP exposed to exogenous thrombin (0.1 U/mL)) allowed for assessment of thrombin activity associated with the induction of hypercoagulation.

3.3.2.1 *Thrombin availability in untreated blood constituent controls*

Thrombin activity assessment of blood constituent control samples indicated baseline levels of thrombin concentration availability in WB at 41.7 ng/mL (Figure 3.7). The separated components indicated that PRP presented with significantly ($p < 0.05$) greater levels of thrombin availability at 47.7 ng/mL reflecting levels of platelet activation; where the IPA of both CD62P and CD63 compared to WB (Table 3.2) was heightened within PRP (Table 3.3). PPP had a slightly decreased level of thrombin availability, at 40.4 ng/mL, as expected (Figure 3.7). For the positive control, the addition of thrombin to WB increased thrombin availability slightly to 41.9 ng/mL; however, this was not significant ($p > 0.05$) and could be attributed to the binding of the added, exogenous thrombin to the platelets within the WB. However, the addition of exogenous thrombin to PRP induced significantly heightened levels of thrombin availability, much higher than the detectable levels of the assay itself, resulting in a negative value of -45.8 ng/mL (Weibrich *et al.*, 2004; Everts *et al.*, 2006). Since PRP contains high numbers of platelets this allows for interaction rates with thrombin to be heightened as platelet activation proceeds (Figure 3.7). Moreover, the generation of PRP may have further induced activation within the platelets making them more receptive to the added thrombin. Exogenous thrombin, however, was not added to PPP, as this component would not contain many platelets; the centrifugation would have also activated the platelets fully allowing for the release of granules.

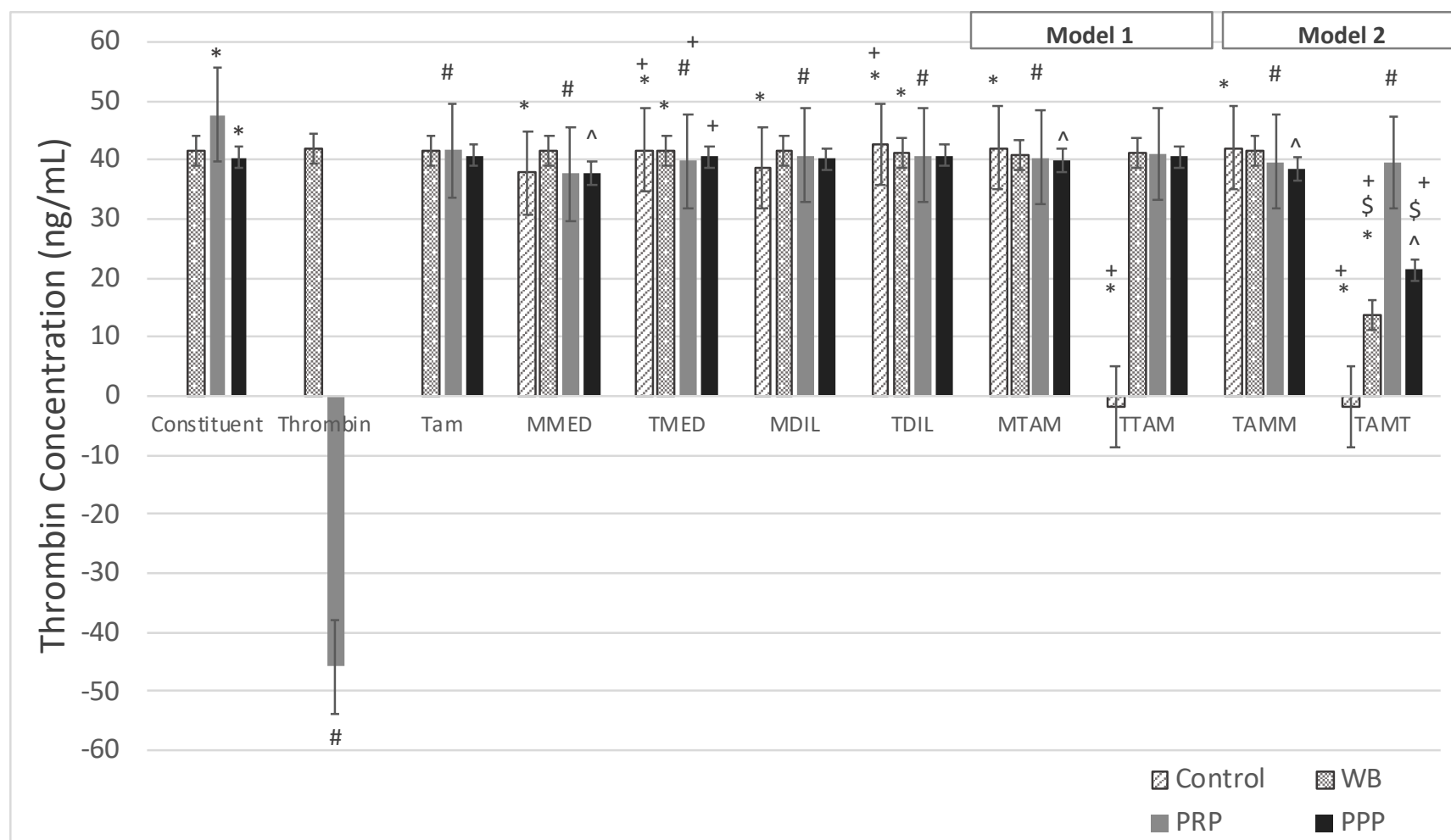


Figure 3.7: Bar-graph showing thrombin generation in MCF7 and T47D cells co-incubated with blood constituent samples, treated with cumulative (Model 1) or circulatory Tamoxifen-therapies (Model 2). Constituent: blood constituent controls; WB, PRP and PPP. Thrombin: positive controls, with WB and PRP, incubated with 0.1 U/mL of thrombin. M/T MED: Conditioned media control of MCF7/T47D cells. M/T DIL: MCF7/T47D cells treated with 0.1% DMSO, denoting the diluent control. Tam: Tamoxifen-treatment of blood constituents. M/T TAM: Tamoxifen-treated MCF7/T47D cells. TAM M/T: Tamoxifen-treated blood constituents exposed to MCF7/T47D cells. $p < 0.05$ indicated * compared to untreated WB; # compared to untreated PRP; ^ compared to untreated PPP; + compared to matched cell line; \$ between Models.

Table 3.2: Index of platelet activation markers CD62P and CD63, as well activation marker ratio (CD62P/CD63) of Tamoxifen-treatment of MCF7 and T47D cells exposed to WB (Model 1) and Tamoxifen treatment of WB exposed to MCF7 and T47D cells (Model 2). Overall levels (Q2) and per interval gate levels; CD62^{low} and CD63^{low} (I1 and I2), CD62^{medium} and CD63^{medium} (I3), CD62^{high} and CD63^{high} (I4 and I5) were assessed. Key: WB – untreated whole blood; WBThr: positive control, whole blood incubated with 0.1 U/ml of thrombin. WBTam: Tamoxifen-treated WB. MMEDWB: WB exposed to MCF7 cells. MDILWB: WB exposed to MCF7 cells treated with 0.1% DMSO, denoting the diluent control. MTAMWB: WB exposed to Tamoxifen-treated MCF7. WBTAMM: Tamoxifen-treated WB exposed to MCF7. TMEDWB: WB exposed to T47D cells. TDILWB: WB exposed to T47D cells treated with 0.1% DMSO, denoting the diluent control. TTAMWB: WB exposed to Tamoxifen-treated T47D. WBTAMT: Tamoxifen-treated WB exposed to T47D. Bold indicates samples which were significantly ($p < 0.05$) different from untreated WB and (*) indicates samples significantly different to matched media cell lines (MMEDWB or TMEDWB). Italics refers to samples significantly different between Models.

Marker of Activation	Controls							Model 1		Model 2	
	WB	WBThr	WBTam	MMEDWB	TMEDWB	MDILWB	TDILWB	MTAMWB	TTAMWB	WBTAMM	WBTAMT
Q2											
CD62P	3,93E+05	4,99E+05	4,93E+05	6,33E+05	4,31E+05	5,48E+05	3,63E+05	6,24E+05	7,78E+05*	6,26E+05	6,01E+05*
CD63	2,92E+06	3,35E+06	3,01E+06	3,65E+06	4,08E+06	3,15E+06	2,80E+06	3,07E+06	4,87E+06	3,64E+06	3,69E+06
Ratio (CD62P/CD63)	1,35E-01	1,49E-01	1,64E-01	1,74E-01	1,06E-01	1,74E-01	1,30E-01	2,03E-01	1,60E-01	1,72E-01	1,63E-01
I1											
CD62P	5,71E+04	4,78E+04	9,85E+04	1,67E+05	1,02E+05	1,19E+05	1,16E+05	1,55E+05	1,76E+05	1,30E+05*	2,17E+05*
CD63	1,78E+05	2,80E+05	5,63E+05	6,59E+05	4,53E+05	4,58E+05	4,38E+05	5,07E+05	6,66E+05*	5,70E+05	6,62E+05*
I2											
CD62P	5,71E+04	2,28E+05	2,64E+05	3,41E+05	1,74E+05	2,93E+05	1,73E+05	3,58E+05	3,71E+05*	3,26E+05	3,71E+05*
CD63	7,23E+05	1,07E+06	1,57E+06	1,70E+06	1,17E+06	1,09E+06	1,01E+06	1,64E+06	1,57E+06	1,64E+06	1,48E+06
I3											
CD62P	1,69E+05	1,87E+05	1,27E+05	2,40E+05	2,23E+05	2,15E+05	1,44E+05	1,80E+05	2,57E+05	2,14E+05	1,95E+05
CD63	1,22E+06	2,40E+06	1,28E+06	2,32E+06	2,02E+06	1,80E+06	1,24E+06	1,40E+06*	2,76E+06	1,84E+06	1,99E+06
I4											
CD62P	3,52E+04	2,30E+05	9,43E+03	3,59E+04	5,79E+04	4,59E+04	1,97E+04	3,02E+04	6,49E+03*	1,48E+04*	7,09E+03*
CD63	1,07E+06	1,22E+06	3,92E+05	7,76E+05	1,25E+06	6,61E+05	5,58E+05	7,46E+05	2,82E+05*	3,43E+05*	3,98E+05*
I5											
CD62P	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00
CD63	0,00E+00	0,00E+00	0,00E+00	4,27E+04	4,26E+04	0,00E+00	5,97E+04	0,00E+00	0,00E+00	0,00E+00	0,00E+00

Table 3.3: *Index of platelet activation markers CD62P and CD63, as well activation marker ratio (CD62P/CD63) of Tamoxifen treatment of MCF7 and T47D cells exposed to PRP (Model 1) and Tamoxifen treatment of PRP exposed to MCF7 and T47D cells (Model 2). Overall levels (Q2) and per interval gate levels; CD62P^{low} and CD63^{low} (I1 and I2), CD62P^{medium} and CD63^{medium} (I3), CD62P^{high} and CD63^{high} (I4 and I5) were assessed. Key: PRP – untreated Platelet-rich plasma; PRPThr: positive control, platelet-rich plasma incubated with 0.1 U/ml of thrombin. PRPTam – Tamoxifen-treated PRP. MMEDPRP: PRP exposed to MCF7 cells. MDILPRP: PRP exposed to MCF7 cells treated with 0.1% DMSO, denoting the diluent control. MTAMPRP: PRP exposed to Tamoxifen-treated MCF7. PRPTAMM: Tamoxifen-treated PRP exposed to MCF7. TMEDPRP: PRP exposed to T47D cells. TDILPRP: PRP exposed to T47D cells treated with 0.1% DMSO, denoting the diluent control. TTAMPRP: PRP exposed to Tamoxifen-treated T47D. PRPTAMT: Tamoxifen-treated PRP exposed to T47D. Bold indicates samples which were significantly (p<0.05) different from untreated PRP and (*) indicates samples significantly different to matched media cell lines (MMEDPRP or TMEDPRP). Italics refers to samples significantly different between Models.*

Marker of Activation	Controls								Model 1		Model 2	
	PRP	PRPThr	PRPTam	PPP	MMEDPRP	TMEDPRP	MDILPRP	TDILPRP	MTAMPRP	TTAMPRP	PRPTAMM	PRPTAMT
Q2												
CD62P	1,35E+06	7,60E+05*	7,73E+05*	1,44E+05	1,20E+06	1,20E+06	7,07E+05*	1,76E+06	8,62E+05*	2,08E+06*	7,16E+05*	1,52E+06
CD63	6,99E+06	8,41E+06*	9,04E+06*	5,35E+06	1,25E+07	8,39E+06	7,93E+06*	1,14E+07	6,11E+06*	1,23E+07*	7,92E+06*	1,01E+07*
Ratio (CD62P/CD63)	1,93E-01	9,04E-02*	8,55E-02*	2,69E-02	9,62E-02	1,43E-01	8,92E-02	<i>1,54E-01</i>	1,41E-01*	1,69E-01	9,03E-02	1,50E-01
I1												
CD62P	3,20E+05	2,27E+05	5,60E+05	2,37E+05	4,94E+05	4,14E+05	3,06E+05	4,46E+05	3,39E+05	4,75E+05	4,80E+05	8,39E+05*
CD63	1,16E+06	1,10E+06	4,37E+06*	3,19E+06	1,47E+06	1,49E+06	9,75E+05*	1,70E+06	7,80E+05*	1,02E+06	2,59E+06	3,61E+06*
I2												
CD62P	4,49E+05	3,79E+05	2,60E+05*	1,07E+05	8,43E+05	8,82E+05	4,94E+05*	1,24E+06	6,08E+05	1,49E+06*	3,11E+05*	7,52E+05
CD63	4,62E+06	3,38E+06	4,53E+06	2,19E+06	8,34E+06	5,30E+06	5,32E+06	5,57E+06	4,02E+06*	7,07E+06*	4,59E+06	5,70E+06
I3												
CD62P	4,67E+04	1,66E+05	2,87E+04*	1,30E+04	1,77E+05	1,16E+05	4,68E+04	1,44E+05	6,05E+04	2,84E+05	1,93E+04*	8,34E+04*
CD63	2,90E+06	4,65E+06	1,33E+06	8,28E+05	5,59E+06	3,85E+06	4,26E+06	6,37E+06	2,39E+06	5,58E+06	1,63E+06*	2,25E+06
I4												
CD62P	7,93E+03	9,29E+03	4,84E+03*	0,00E+00	4,91E+03	5,06E+03	0,00E+00	0,00E+00	3,07E+03	3,71E+03	0,00E+00	4,16E+03
CD63	1,20E+05	1,02E+06*	1,76E+05	1,11E+05	2,39E+05	1,06E+05	1,47E+05	2,88E+05	1,22E+05	4,56E+05*	1,04E+05	2,44E+05
I5												
CD62P	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00
CD63	0,00E+00	3,57E+04*	0,00E+00	0,00E+00	4,02E+04	2,09E+04	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00

3.3.2.2 Platelet activation in blood constituent controls

Overall IPA

Overall IPA represents levels of platelet activation from interval gates 1 to 5 (I1 – I5) in the positive quadrant ((Q2) Figure 3.2 – 3.4). Q4 is postulated to represent microparticles positive for CD62P and CD63 and can be seen in the quadrant plots.

Baseline levels of platelet activation were determined by assessing IPA in the untreated control samples (Table 3.2 & 3.3). Both early stage (CD62P IPA) and late stage (CD63 IPA) assessment showed low IPA in the untreated WB control (Table 3.2), while concentrated PRP showed a significant increase in IPA compared to WB (Table 3.3). To further assess baseline levels, despite the low number of platelets expected in PPP, IPA was assessed (Table 3.3). PPP showed the lowest levels of IPA in early stages, while late stage assessment indicated a significant increase in IPA compared to the WB control which could be attributed to the presence of CD63⁺ microparticles derived from samples that had been centrifuged two-fold. As such the centrifugal force would have contributed to greater levels of platelet activation and the subsequent release of microparticles.

A ratio between CD62P IPA and CD63 IPA was used to explain the capacity of platelets to progress to later stages of activation; where a lower ratio indicated a later stage. The ratio indicated lower levels of progression along the activation pathway in WB, comparable to that of PRP (Table 3.2 and Table 3.3.), while PPP indicates a significantly decreased response and progression to a later stage of activation, as expected due to the presence of microparticles expressing platelet activation markers (Table 3.3).

The addition of thrombin in the positive controls specifically to WB only induced slightly higher CD62P overall IPA, while a significant induction was noted in CD63 IPA, including a slight decrease in the ratio of activation markers indicating that platelets are progressing towards a later stage (Table 3.2). The addition of thrombin to PRP, however, significantly reduced CD62P IPA, which may be attributed to greater platelet aggregation, and significantly increased CD63 IPA, when compared to untreated PRP (Table 3.3). The ratio of markers was also seen to be significantly lower than that induced by PRP alone, indicating that platelets within this sample were not only at later stages of activation but remained responsive to the effects of agonists.

IPA per interval gate

IPA per interval gate was assessed to determine the spread of marker expression throughout the interval gates (Table 3.2 & 3.3). The untreated control samples were used to assess baseline marker expression spread. Untreated WB indicated high IPA in the CD62P^{low} interval gate (I1 and I2); and CD62P^{medium} (I3) gates, with decreasing IPA in the CD62P^{high} interval gates (I4 and I5). CD63 displayed similar spread; however, the IPA was much higher than that of CD62P. Untreated PRP showed higher IPA levels of both CD62P and CD63 throughout all interval gates, compared to the untreated WB control. PPP, however, indicated very low marker expression throughout the interval gates, with greater CD63 expression, noted at higher levels than that of WB in I1 and I2.

The addition of thrombin in the positive control induced a greater spread of expression of markers through the interval gates (Table 3.2 & 3.3). This spread, however, was dependent on the blood constituent being assessed. WB induced a greater spread, while PRP induction was tighter in appearance with the addition of thrombin, increasing the expression throughout the gates.

Corresponding platelet ultrastructure

Analysis of platelet ultrastructure confirmed flow cytometry results. Ultrastructural assessment of lysed WB (Figure 3.8A) showed inactive platelets, with a smooth membrane appearance and few surface folds. As expected, in the positive control the addition of thrombin at 0.1U/ml induced greater membrane folds and extending filipodia from the platelet body, with the presence of few microparticles (Figure 3.8B). Untreated PRP (Figure 3.8C) showed platelets displaying early stages of activation, with extending filipodia and lamellipodia and a smoother membrane appearance; with some platelets displaying the early stages of hyalomere spread with the presence of pores. Some aggregation is evident. The addition of thrombin to PRP (Figure 3.8D), induced greater levels of platelet aggregation showing greater microparticles and fibrin filament formation. Platelets also displayed a smoother membrane appearance and hyalomere spread. Platelets at this heightened level of expression correspondingly lose CD62P, however, CD63 persists (Table 3.3). PPP (Figure 3.8E), had many crenated red blood cells noted, with remnants from fully spread platelets and fibrin clots seen; these remnants are postulated to be expressing platelet activation markers with

the lower levels of CD62P noted, indicating that loss of expression occurred through full activation (Table 3.3).

3.3.2.3 Thrombin Availability Altered by Tamoxifen-treatment of blood constituents (Model 2 controls)

We further assessed baseline thrombin levels specifically in Model 2 where circulating Tamoxifen effects in blood constituents were mimicked (Figure 3.7). To assess samples from Model 2; WB was treated with Tamoxifen and separated into blood constituents and analysed. Tamoxifen-treatment of WB induced levels of thrombin availability, 41.67 ng/mL, comparable to that of untreated WB (41.65 ng/mL). Tamoxifen-treatment significantly reduced thrombin availability in PRP samples to 41.53 ng/mL, compared to PRP at 47.66 ng/mL. The opposite is noted within PPP samples, in which thrombin availability was slightly increased ($p>0.05$) from 40.43 ng/mL in untreated PPP to 40.69 ng/mL in Tamoxifen-treated PPP samples.

3.3.2.4 Platelet activation following Tamoxifen-treatment of blood constituents (Model 2 controls)

Overall IPA

On assessment of the baseline effects of circulating Tamoxifen (Model 2) on early and late stage platelet activation (Table 3.2 and 3.3), it was noted that Tamoxifen-treatment of WB induced an increase in IPA of both CD62P and CD63, compared to that of untreated WB (Table 3.2). The ratio of activation markers (CD62P/CD63) in Tamoxifen-treated WB, remained within early stages of activation, similar to the untreated control (Table 3.2). However, the activation marker ratio in Tamoxifen-treated PRP was significantly lower than untreated PRP, indicating a shift to a later stage of platelet activation (Table 3.3); and was confirmed ultrastructurally (Figure 3.8).

IPA per interval gate

Tamoxifen treatment of WB elicited higher IPA in the CD62P^{low} and CD63^{low} interval gates (I1 and I2), with significantly higher CD63 IPA compared to that of untreated WB (Table 3.2). In both CD62P^{medium} and CD63^{medium} interval gate (I3) slight changes are noted within the IPA of the markers, with CD62P decreasing and CD63 increasing, however, this was not significant. In the CD62P^{high} CD63^{high} interval gates (I4 and I5), a reduction in IPA is noted, with CD62P significantly lower when compared to untreated WB.

Tamoxifen-treated PRP (Table 3.3), showed significantly heightened CD62P and CD63 IPA in the CD62P^{low} and CD63^{low} interval gates (I1 and I2), respectively, compared to untreated PRP. The CD62P^{medium} interval gate showed a significant reduction in CD62P IPA, with the CD63^{medium} interval gate indicating slight reduction noted in CD63 IPA. CD62P^{high} interval gates indicated a reduction in CD62P IPA, significantly in I4, with the CD63^{high} interval gate showing an increase in CD63 IPA; however, this was not significant. I5, for both markers, indicated no expression and shows no extremely high levels of activation as seen in the positive control.

Corresponding platelet ultrastructure

Ultrastructurally, platelets from both Tamoxifen-treated WB and PRP (Figure 3.8 F & G), were at lower levels of activation when compared to that of samples from the matched positive, thrombin control. Nevertheless, platelets from Tamoxifen-treated WB were noted to have a folded membrane surface with many filipodia extensions (Figure 3.8 F). In Tamoxifen-treated PRP, significant increases in both CD62P IPA and CD63 IPA is noted, compared to untreated PRP (Table 3.3). Ultrastructurally, Tamoxifen-treated PRP samples (Figure 3.8 G) showed platelet membrane budding, with lamellipodia and filipodia extension with some platelets showing a hyalomere spread and evidence of aggregation.

The ratio of activation markers (CD62P/CD63) indicated that platelets in Tamoxifen-treated WB remained within early stages of activation (Table 3.2); while the ratio in Tamoxifen-treated PRP indicated a shift to a later stage of platelet activation (Table 3.3). This difference between Tamoxifen-treated WB and PRP was noted ultrastructurally, with platelets at later stages of activation in treated PRP samples (Figure 3.8 F). Furthermore, Tamoxifen-treated PPP (Figure 3.8 H) showed fewer but active platelets with filipodia extension and the presence of microparticles.

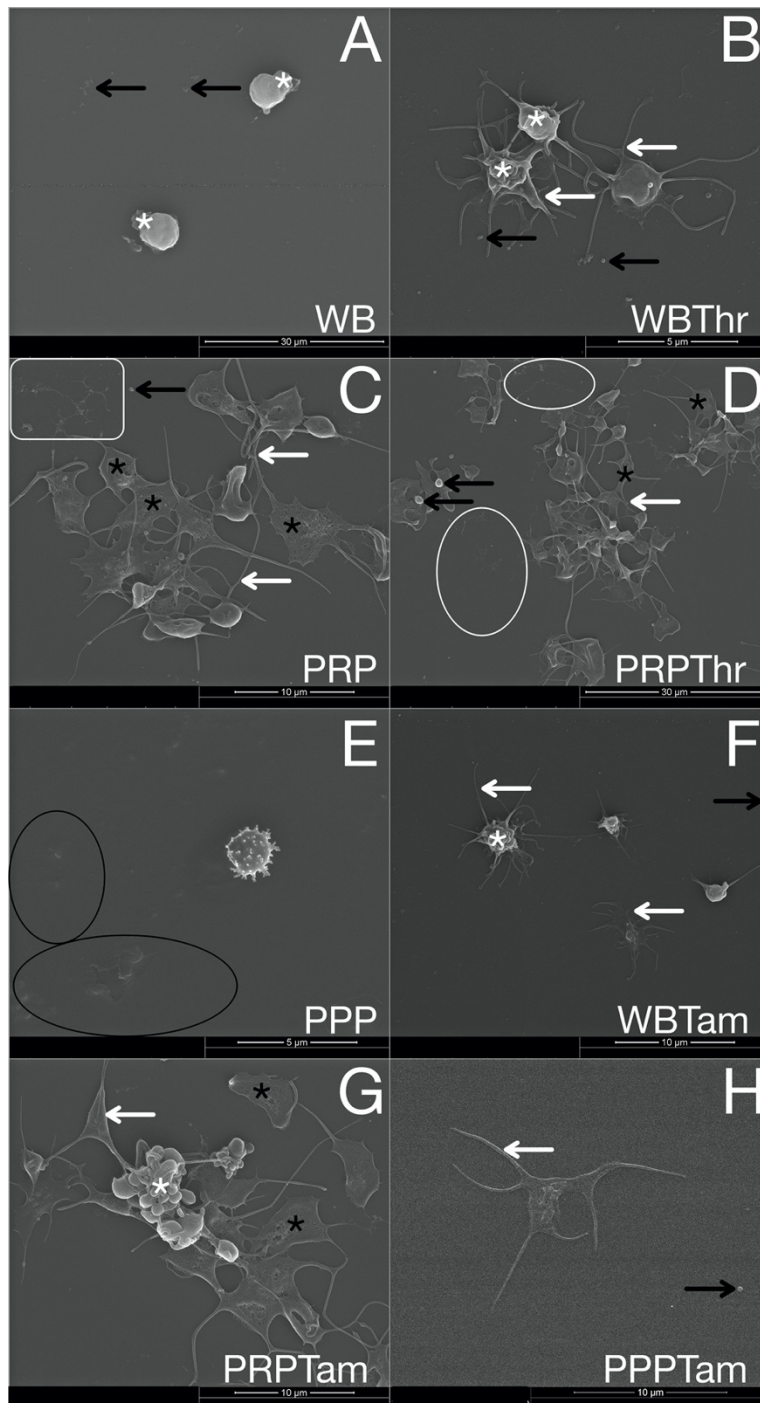


Figure 3.8: Control Blood Constituent Samples. Platelet ultrastructural alterations. A: lysed WB showing inactive platelets, with a smooth membrane (*) and few surface folds. Note microparticles (black arrow). B: Positive control, the addition of thrombin at 0.1U/ml, showed platelets with greater membrane folds (*) and extending filipodia (white arrows) from the platelet body. Note microparticles (black arrows). C: PRP showed platelets with a hyalomere spread with the presence of pores (*), extending filipodia and lamellipodia (white arrows) and the presence of fibrin (white circle). D: Positive control; addition of thrombin to PRP induced platelet aggregation with platelets displaying a hyalomere spread (*), filipodia extension (white arrow) and microparticles (black arrow) and fibrin filament formation (white circle). E: untreated PPP showed with crenated red blood cells, remnants from fully spread platelets and fibrin clots (black circle). F: Tamoxifen-treated WB showing platelets with membrane surface folds (*) with many filipodia extensions (white arrow) and few microparticles (black arrow). G: Tamoxifen-treated PRP shows platelet membrane budding (white *), with lamellipodia and filipodia extension (white arrow) with platelets showing hyalomere spread (black *). H: Tamoxifen-treated PPP showed platelets with filipodia extension (white arrow). Note microparticles (black arrow).

3.3.3 Breast cancer cell-mediated effects on hypercoagulation

Baseline levels of platelet activation were determined by thrombin activity (Figure 3.7), IPA (Table 3.2 & 3.3) and platelet ultrastructure (Section 3.3.2; Figure 3.8). This section presents the effects of breast cancer cells on these parameters.

3.3.3.1 Breast cancer cell line thrombin generation

Thrombin availability, as previously discussed, indicates the levels of endogenous detectable thrombin within the blood constituent samples. Thrombin generation refers to the generation of thrombin by components not within the blood itself – but in the model systems of this study with MCF7 and T47D breast cancer cells.

On assessment of thrombin generation by control, untreated cells (Figure 3.7), MCF7 breast cancer cell-secreted thrombin in conditioned media (37.77 ng/mL), was significantly lower than that present in the WB control (41.66 ng/mL) and the positive thrombin control (41.91 ng/mL). Furthermore, MCF7 secreted-thrombin was also significantly lower than that detectable in both PRP at 47.66 ng/mL and PPP at 40.43 ng/mL control samples. Conversely, T47D breast cancer cells secreted levels of thrombin slightly lower and comparable to that of the WB control at 41.61 ng/mL. Thrombin secreted by T47D cells; however, was only significantly lower than the PRP control but higher than that of PPP. Both MCF7 and T47D cells secreted what appeared to be higher levels of thrombin compared to the positive PRP control (-45.8 ng/mL). However, as noted in blood constituents, the negative value of the positive PRP control noted is attributable to higher than detectable levels of thrombin availability as such this result indicated a *lower level* of generated thrombin by MCF7 and T47D cells.

Control, diluent samples (0.1% DMSO) induced thrombin generation only slightly higher than that of their matched untreated cell line (Figure 3.7); MCF7 at 38.59 ng/mL and T47D at 42.57 ng/mL. This slight increase was not significant compared to their matched untreated cellular controls, and as such hormone-therapy treated samples were compared to their matched untreated cell lines. Diluent samples are not traditionally presented; however, we have noted variability in some techniques and as such diluent results have been presented. Furthermore, it was noted that the T47D cell line generated levels of thrombin significantly greater than that of the MCF7 cell line; indicating its ability to potentially induce heightened levels of

platelet activity. Establishing the level of thrombin secreted by breast cancer cells allowed for assessment of thrombin when blood constituents were then exposed to breast cancer cells (Figure 3.7).

3.3.3.2 Thrombin generation affected by exposure of breast cancer cells to blood constituents

MCF7 breast cancer cells generated thrombin at 37.77 ng/mL, significantly lower than thrombin available in the WB control; however, MCF7 cells exposed to WB increased the level of detectable thrombin significantly to 41.48 ng/mL, when compared to untreated MCF7 cells (Figure 3.7). This greater level of available thrombin could be due to low levels of platelet activation evident in untreated WB. However, when PRP and PPP were exposed to MCF7 breast cancer cells, slight decreases in detectable thrombin levels is noted ($p>0.05$), with PRP exposure at 37.59 ng/mL and PPP exposure at 37.72 ng/mL when compared to the MCF7 control; and significantly lower than that of untreated PRP and PPP, respectively. While only slight, these decreases could be due to binding of available thrombin within the PRP, concentrated with platelets, resulting in lower detectable levels, and further the generation of fibrin as we identified ultrastructurally (Figure 3.9(1)C).

On assessment of blood constituents exposed to T47D breast cancer cells, detectable thrombin levels were lower than secreted levels by the cells themselves (41.61 ng/mL), as well as to their matched blood constituent controls, WB exposure inducing 41.48 ng/mL; PRP exposure inducing significantly lower thrombin at 37.59 ng/mL and PPP exposure at 37.72 ng/mL, compared to their matched blood constituent controls (Figure 3.7). This significant decrease could be attributed to the binding of exogenous thrombin to the platelets, as well as binding to and converting serum fibrinogen to fibrin within the plasma of the blood constituents itself. Furthermore, the exposure of blood constituent to T47D cells; specifically, PRP and PPP, significantly heightened the induced levels of thrombin generation compared to PRP and PPP exposed to MCF7 cells; further correlating with the pro-thrombotic nature of the T47D cell line itself (Figure 3.7).

3.3.3.3 Breast Cancer Cell Line Induced Platelet Activation

Overall IPA and platelet ultrastructure

Platelet activation was noted in WB samples exposed to untreated MCF7 cells higher than that of the untreated control, as well as the positive control (Table 3.2). CD62P IPA was significantly greater than that of the control, while CD63 IPA was only slightly higher. Furthermore, the ratio of activation markers was only slightly lower than that of the WB control, indicating while platelet activation was plentiful, the process remained at an earlier stage. The diluent-treated MCF7 cells induced a similar, yet lower response, with platelet activation remaining at comparable levels to that of untreated MCF7 cells. On ultrastructural assessment, platelets from WB exposed to MCF7 cells (Figure 3.9(1)A) displayed a smooth membrane with many filipodia extensions from the platelet body, with the presence of microparticles; while platelets from WB exposed to diluent-treated MCF7 cells (Figure 3.9(1)B), displayed aggregated platelets with fibrin formation.

On assessment of the PRP component, a consistently higher level of platelet activation was noted. PRP exposed to MCF7 cells (Table 3.3) showed an increase in CD62P IPA and a significant increase in CD63 IPA, with a significant decrease in the ratio of the activation markers compared to untreated PRP, indicating a shift to later stages of activation. Lower levels of platelet activation and progression are noted in PRP samples exposed to diluent treated MCF7 cells, compared to MCF7 cells alone. Ultrastructurally, PRP exposed to MCF7 cells (Figure 3.9(1)C) induced heightened platelet activation with platelets exhibiting hyalomere spreading and extending filipodia, with evidence of aggregation. Platelets from PRP exposed to diluent-treated MCF7 cells (Figure 3.9(1)D) expressed platelets with a folded membrane and microparticles evident in the extracellular space. e PPP exposed to both untreated MCF7 cells and diluent-treated MCF7 cells (Figure 3.9(1)E & F) showed remnants of platelets, with plaque and fibrin pores as well as fibrinogen evident and indicative of hypercoagulation.

Platelet activation was noted in WB samples exposed to untreated T47D cells, the levels induced were higher than that of untreated WB, as well as the positive control (Table 3.2). CD62P IPA, was slightly greater ($p>0.05$), while CD63 IPA was significantly higher than that of untreated WB. However, the ratio of activation markers was noted to be only slightly lower

than that of the WB control, indicating activation remaining at earlier stages. Ultrastructurally, platelets from WB exposed to T47D cells (Figure 3.10(1)A) displayed a smooth membrane with many filipodia extensions from the platelet body, with the presence of microparticles; as opposed to untreated WB (Figure 3.8A) which showed platelets with only a few membrane folds and no extensions. The diluent-treated T47D cells induced CD62P and CD63 IPA levels comparable to that of the untreated WB control (Table 3.2). Ultrastructurally, platelets from WB exposed to diluent-treated T47D cells (Figure 3.10(1)B), displayed a relatively smooth but folded platelet membrane, as well as the presence of filipodia extending outward from the platelet body.

PRP exposed to T47D cells (Table 3.3) showed a decrease in CD62P IPA and a significant increase in CD63 IPA, with a slight decrease in the ratio of activation markers compared to untreated PRP, reflecting a later stage of platelet activation. This was apparent in ultrastructural assessment, with platelets from PRP exposed to T47D cells (Figure 3.10(1)C) exhibiting a folded membrane surface with hyalomere spread and extending filipodia. However higher levels of IPA ($p>0.05$) is noted in PRP samples exposed to diluent-treated T47D cells, compared to that T47D cells alone. Ultrastructurally, platelets from PRP exposed to diluent-treated T47D cells (Figure 3.10(1)D) showed a folded membrane and many microparticles, confirming this increase. Furthermore, ultrastructural assessment of PPP exposed to T47D cells (Figure 3.10(1)E) showed platelets displaying higher levels of activation with many filipodia extensions and the presence of a hyalomere spread. PPP exposed to diluent-treated T47D cells (Figure 3.10(1)F) showed the presence of plaque formation with extending projections. It was further noted that the T47D cell line exposed to WB induced ($p>0.05$) a slight reduction in CD62P IPA and an increase in CD63 IPA compared to that induced by MCF7 cells; and carried through to PRP samples as well, thus affirming the pro-thrombotic nature of this cell line.

IPA per interval gate

Induced platelet activation levels in the CD62P^{low} interval gates (I1 and I2) showed CD62P IPA significantly higher in WB samples exposed to MCF7 cells when compared to that of the untreated control. CD63 IPA while not significant was noted to be lower in the CD63^{low} interval gates when compared to the untreated control (Table 3.2). However, upon assessment of

both CD62P^{medium} and CD63^{medium} (I3); and CD62P^{high} and CD63^{high} (I4 and I5) interval gates, WB exposed to MCF7 cells showed an increase in both CD62P and CD63 IPA. CD63^{high} interval gate I5 indicated a significant increase in CD63 IPA when compared to the untreated control (Table 3.2). CD62P^{low} and CD63^{low} interval showed higher CD62P and CD63 IPA when compared to untreated PRP. However, a significant increase is noted in CD62P and CD63 IPA, in CD62P^{medium} and CD63^{medium} interval gates (I3), respectively. PRP exposed to MCF7 cells in the CD62P^{high} interval gate indicated a reduction in CD62P IPA, with a significant increase in CD63 IPA in the CD63^{high} interval gates (Table 3.3).

Induced platelet activation levels in CD62P^{low} and CD63^{low} interval gates (I1 and I2) by T47D cells was higher than that of untreated control WB; with CD63 IPA indicating a significant increase (Table 3.2). On assessment of both CD62P^{medium} and CD63^{medium} interval gates, an increase ($p>0.05$) is noted in WB samples exposed to T47D cells, compared to untreated WB; while WB exposed diluent-treated T47D cells exhibited expression similar to that of the untreated WB. In CD62P^{high} and CD63^{high} interval gates, WB exposed to T47D cells induced an increase in both CD62P and CD63 IPA with I5, indicating a significant increase in CD63 IPA when compared to the untreated WB. These results are further heightened in PRP samples exposed to T47D cells (Table 3.3). CD62P^{low} and CD63^{low} interval gates (I1 and I2) showed consistently higher IPA when compared to untreated PRP. This was also evident in the CD62P^{medium} and CD63^{medium} interval gates (I3), with significant increases in both IPA's, compared to untreated PRP. Furthermore, CD63^{high} persisted, with significantly greater expression in PRP exposed to T47D cells indicating platelets within the sample were at later stages of activation.

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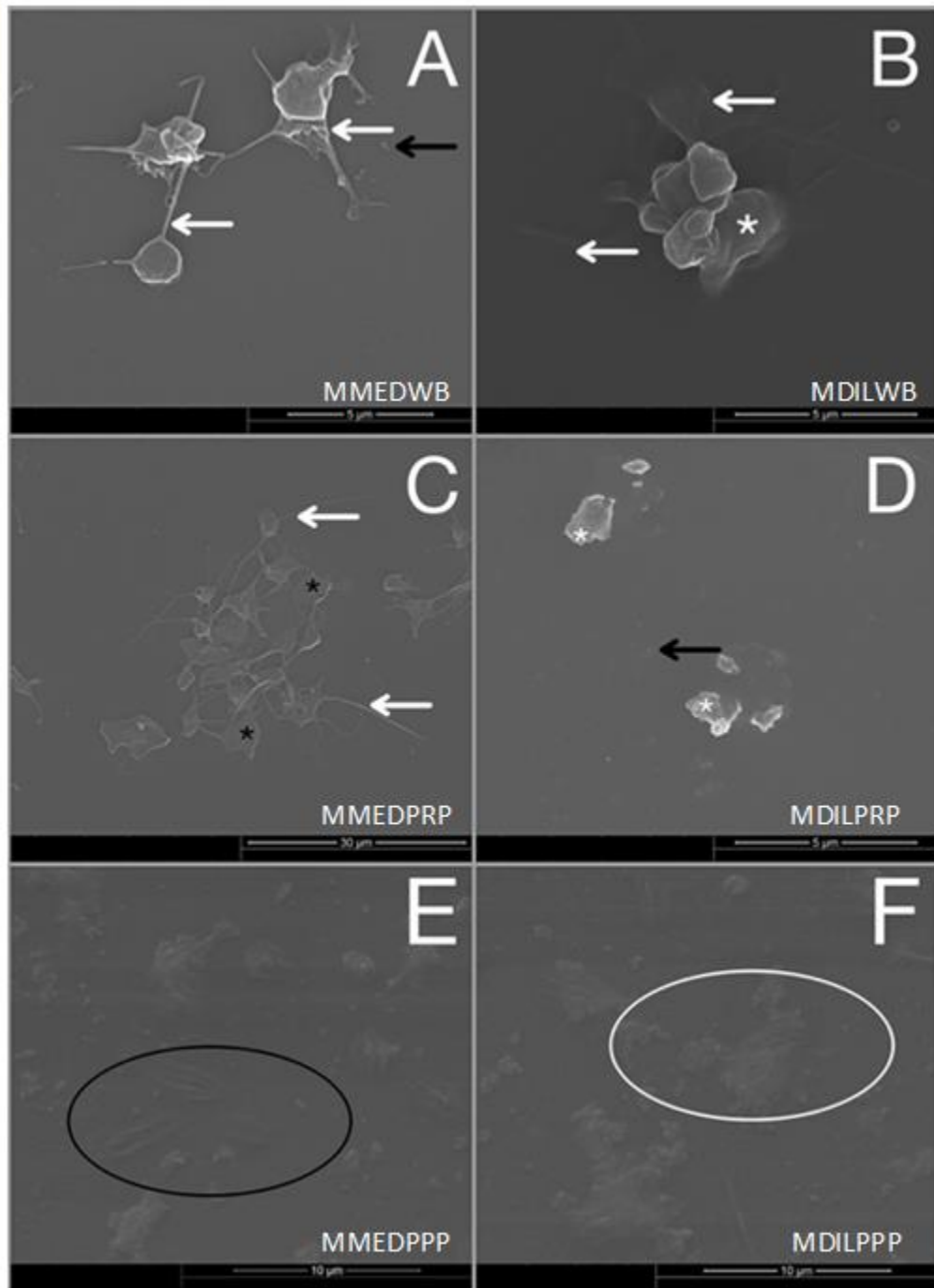


Figure 3.9: **Effects of blood constituent incubation with MCF7 cells - controls.** Key: MMED- media-treated MCF7 cells; MMEDWB: WB exposed to media-treated MCF-7 cells; MMEDPRP – PRP exposed to media-treated MCF-7 cells; MMEDPPP – PPP exposed to media-treated MCF-7 cells; MDIL- diluent-treated MCF7 cells; MDILWB: WB exposed to diluent-treated MCF-7 cells; MDILPRP – PRP exposed to diluent-treated MCF-7 cells; MDILPPP – PPP exposed to diluent-treated MCF-7 cells. **(1)** Platelet ultrastructural alterations. Key: white * - membrane folds, black * - hyalomere spread, white arrow - extending filopodia, black arrow – microparticles, white circle -fibrin, black circle - fibrin pores A: MMEDWB - active platelets, smooth membrane, extending filopodia and microparticles. B: MDILWB – aggregated platelets with fibrin. C: MMEDPRP – spread platelets and filopodia. D: MDILPRP – platelets with membrane folds and microparticles. E: MMEDPPP and F: MDILPPP - remnants of platelets and fibrinogen deposits.

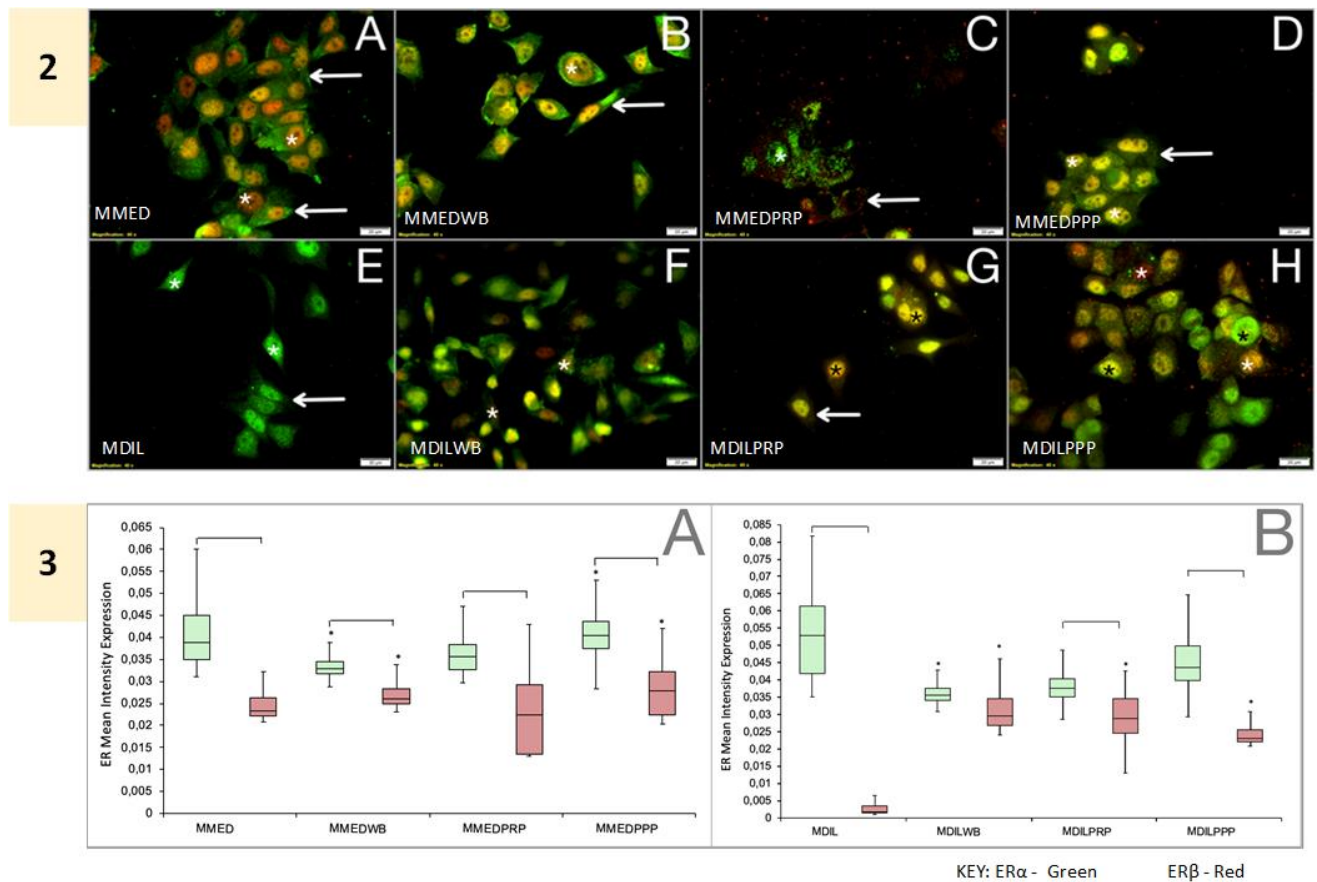


Figure 3.9: Effects of blood constituent incubation with MCF7 cells - controls. *Key:* MMED- media-treated MCF7 cells; MMEDWB: WB exposed to media-treated MCF-7 cells; MMEDPRP – PRP exposed to media-treated MCF-7 cells; MMEDPPP – PPP exposed to media-treated MCF-7 cells; MDIL- diluent-treated MCF7 cells; MDILWB: WB exposed to diluent-treated MCF-7 cells; MDILPRP – PRP exposed to diluent-treated MCF-7 cells; MDILPPP – PPP exposed to diluent-treated MCF-7 cells. **(2)** Co-localisation of ERα (green) and ERβ (red) in MCF7 cells, following exposure to blood constituents. Yellow - ERα dominating; Orange - ERβ dominating A: MMED – cytoplasmic (white arrow) ERα and nuclear (*) ERβ. B: MMEDWB - cellular processes extend, cytoplasmic ERα and nuclear ERβ. C: MMEDPRP – diffuse ERα and ERβ. D: MMEDPPP- cytoplasmic ERα and nuclear ERβ. E: MDIL – nuclear and cytoplasmic ERα, nuclear ERβ. F: MDILWB -nuclear and cytoplasmic ERα, nuclear ERβ. G: MDILPRP and H: MDILPPP – ERα and ERβ nuclear expression. **(3)** Box and whisker plots showing quantitative analysis of ERα (green) and ERβ (red) expression in MCF7 cells, following exposure to blood constituents. A: Media-treated. B: Diluent-treated (0.1% DMSO). [$p < 0.05$ between ERα and ERβ within the treatment group. * $p < 0.05$ between matched ERs compared to untreated MCF7.

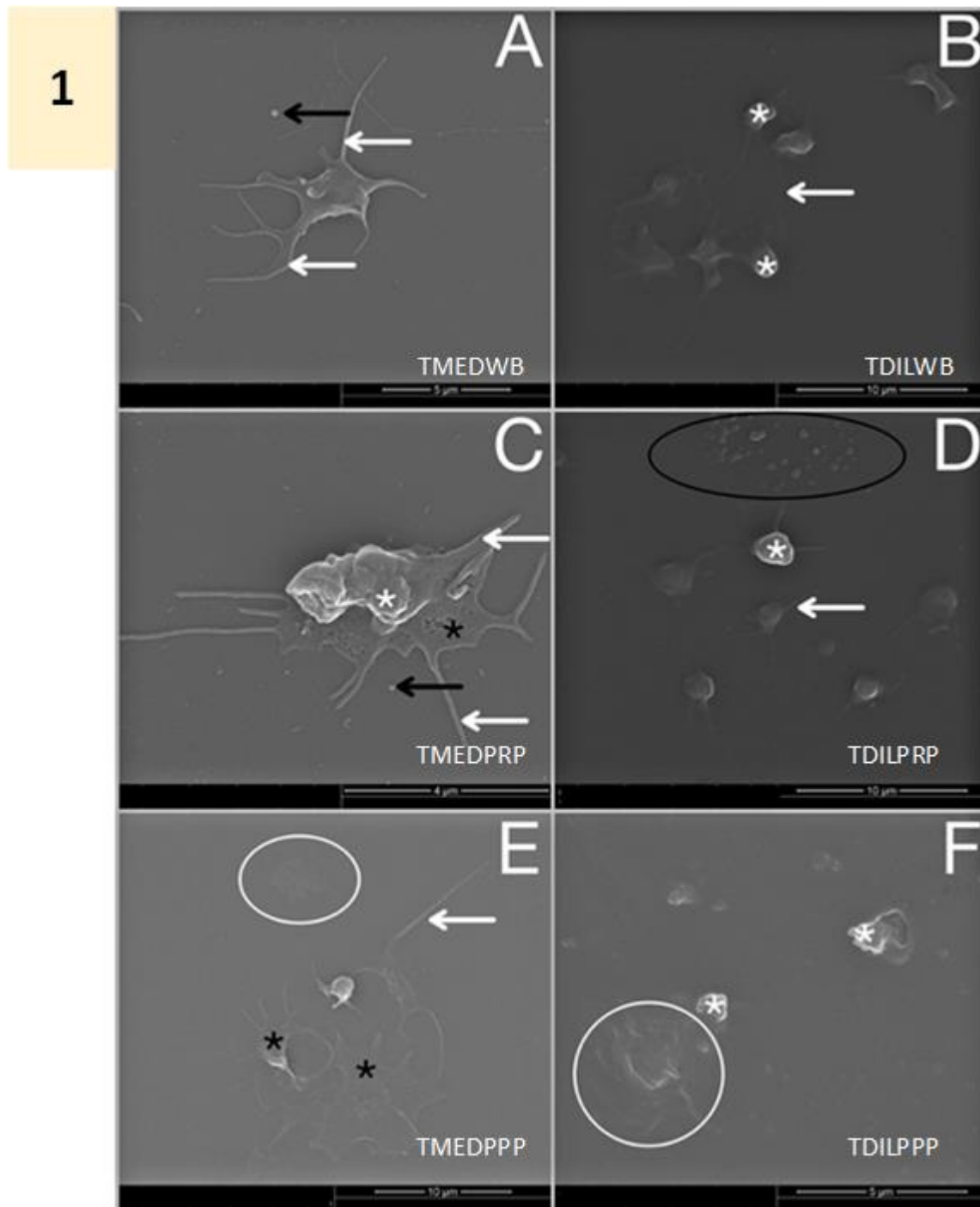


Figure 3.10: **Effects of blood constituent incubation with T47D cells - controls.** Key: TMED- media-treated T47D cells; TMEDWB: WB co-incubated with media-treated T47D cells; TMEDPRP – PRP co-incubated with media-treated T47D cells; TMEDPPP – PPP co-incubated with media-treated T47D cells; TDIL- diluent-treated T47D cells; TDILWB: WB co-incubated with diluent-treated T47D cells; TDILPRP – PRP co-incubated with diluent-treated T47D cells; TDILPPP – PPP co-incubated with diluent-treated T47D. **(1).** Platelet ultrastructural alterations. Key: white * - membrane folds, black * - platelet hyalomere spread, white arrow - extending filipodia, black arrow – microparticles, white circle -fibrin, black circle - fibrin deposits. A: TMEDWB – slightly active platelet with filipodia and microparticles; B: TDILWB – mostly resting platelets; C: TMEDPRP – spread platelet, filipodia and microparticles. D: TDILPRP – platelets with smooth membrane and extending filipodia, with microparticles. E: TMEDPPP: fully spread platelets with fibrin deposits. F: TDILPPP: fibrin plaque formation.

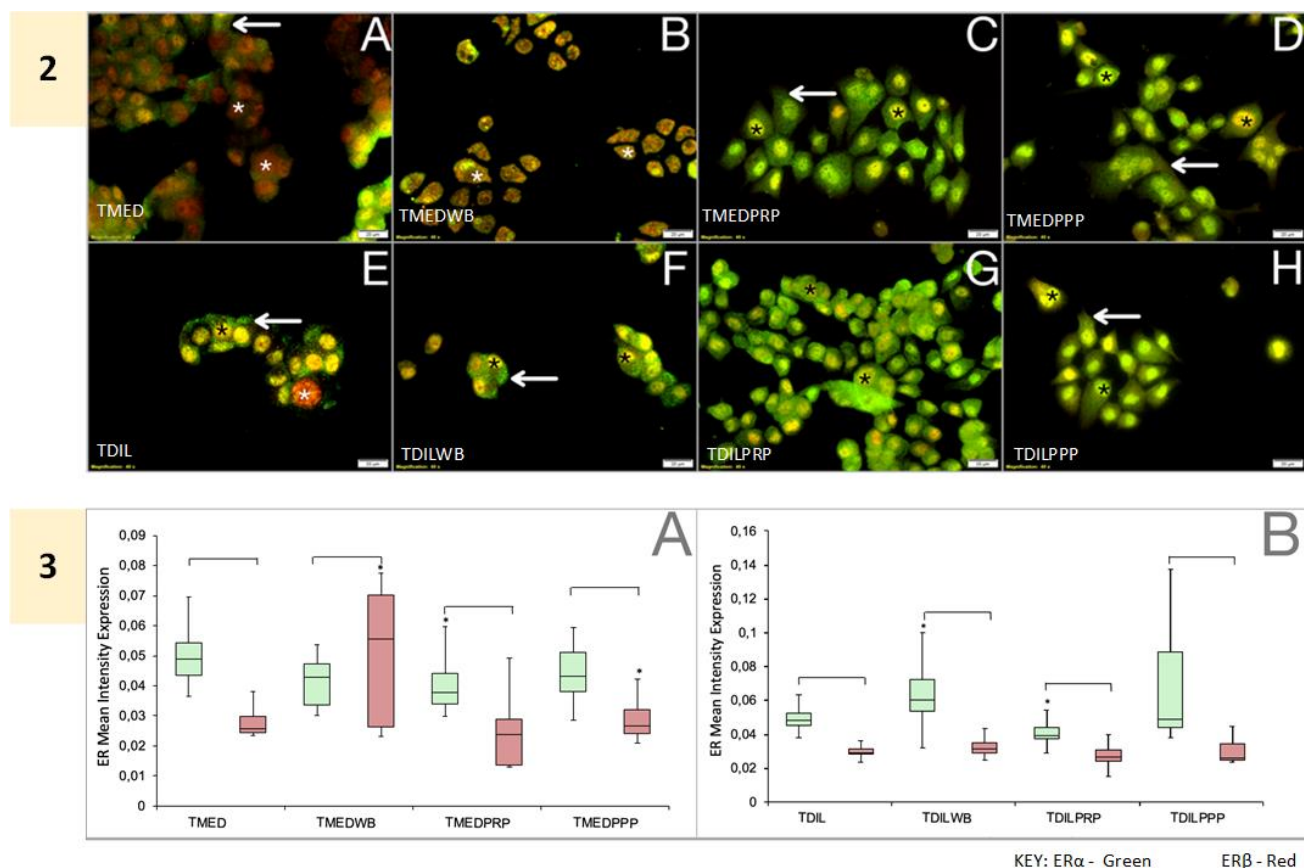


Figure 3.10: Effects of blood constituent incubation with T47D cells - controls. Key: TMED- media-treated T47D cells; TMEDWB: WB co-incubated with media-treated T47D cells; TMEDPRP – PRP co-incubated with media-treated T47D cells; TMEDPPP – PPP co-incubated with media-treated T47D cells; TDIL- diluent-treated T47D cells; TDILWB: WB co-incubated with diluent-treated T47D cells; TDILPRP – PRP co-incubated with diluent-treated T47D cells; TDILPPP – PPP co-incubated with diluent-treated T47D. **(2)** Co-localisation of ERα (green) and ERβ (red) in T47D cells, following exposure to blood constituents. Yellow - ERα dominating; Orange - ERβ dominating. A: TMED: primarily cytoplasmic ERα (white arrow) and nuclear ERβ expression (*). B: TMEDWB: Nuclear ERα and ERβ expression (*). C: TMEDPRP: cytoplasmic and nuclear ERα expression (arrow) and nuclear ERβ (*). D: TMEDPPP: high cytoplasmic ERα (white arrow) and nuclear ERβ (*). E: TDIL – diffuse cytoplasmic and nuclear ERα (white arrow), nuclear ERβ (*). F: TDILWB – cytoplasmic and nuclear ERα (white arrow), higher nuclear ERβ (*). G: TDILPRP – cytoplasmic and nuclear ERα and nuclear ERβ (*). H: TDILPPP – high nuclear and cytoplasmic ERα (black *) and higher nuclear ERβ (white *). **(3)** Box and whisker plots showing quantitative analysis of ERα (green) and ERβ (red) expression in T47D cells, following exposure to blood constituents. A: Conditioned media-treated T47D cells. B: Diluent-treated T47D cells (0.1% DMSO). [indicates significant differences between ERα and ERβ within the treatment group. * indicates significant ($p < 0.05$) differences between matched ERs compared to untreated T47D.

3.3.4 Tamoxifen-Treatment Compounds Breast Cancer Cell-Induced hypercoagulation

3.3.4.1 *Thrombin Generation by Breast Cancer Cells Altered by Tamoxifen-treatment*

Model 1

Tamoxifen-treatment of MCF7 breast cancer cells (Model 1) induced significantly greater secretion of thrombin, at 41.96 ng/mL when compared to conditioned media from the untreated cell line control, 37.77 ng/mL; however, this level was similar to that of untreated WB (41.66 ng/mL) (Figure 3.7). Tamoxifen-treated MCF7 breast cancer cells exposed to blood constituents, induced a reduction in thrombin generation: WB exposure significantly reduced thrombin generation to 40.96 ng/mL, with PRP reducing it to 40.43 ng/mL and PPP further reducing this to 39.91 ng/mL; all of which were significantly lower than that detected in the conditioned media of Tamoxifen-treated MCF7 cells. Furthermore, PRP and PPP exposed to Tamoxifen-treated MCF7 cells induced significantly lower levels of thrombin generation compared to matched untreated blood constituent controls (PRP (47.66 ng/mL) and PPP (40.43 ng/mL)) as well as matched Tamoxifen-treated blood constituent controls (PRP (41.53 ng/mL) and PPP (40.69 ng/mL)).

T47D breast cancer cells treated with Tamoxifen (Model 1) significantly heightened levels of thrombin generation greater than the detectable levels of the assay itself, resulting in a negative value of -1.83 ng/mL, similar to that of the positive PRP control but not as high (Figure 3.7). When exposed to blood constituents this level of thrombin generation was noted to be detectable. WB exposure to pre-treated T47D cells significantly reduced thrombin generation to 41.07 ng/mL; PRP to 41.00 ng/mL and PPP to 40.51 ng/mL. When compared to matched, untreated T47D cells exposed to blood constituents; Tamoxifen-treatment of T47D cells exposed to WB reduced thrombin generation, while PRP exposure significantly increased thrombin generation and PPP exposure to only slightly increased detectable thrombin.

Model 2

Pre-treatment of blood constituents with Tamoxifen (Model 2), induced varying levels of thrombin generation. When compared to Tamoxifen-treated MCF7 cells, pre-treated WB exposed to MCF7 cells induced a slightly higher level of thrombin generation (41.63 ng/mL), while pre-treated PRP (39.65 ng/mL) and PPP (38.39 ng/mL) induced significantly lower thrombin generation. When compared to matched Tamoxifen-treated blood constituents, pre-treated WB exposed to MCF7 cells induced only a slight decrease in thrombin availability,

while both PRP and PPP induced a significant reduction (Figure 3.7). Tamoxifen-treatment of blood constituents, again, resulted in a reduction of thrombin generation by MCF7 breast cancer cells; however, this could be due to endogenous binding capacity in PRP and the generation of fibrin formation in PPP.

Tamoxifen-treated blood constituents exposed to T47D cells altered thrombin generation to detectable levels which were at significantly lower levels than all control samples; Tamoxifen-treated T47D cells matched untreated T47D cells exposed to blood constituents, matched treated blood constituents and untreated blood constituents. Pre-treated WB exposed to T47D cells reduced thrombin generation to 13.58 ng/mL, pre-treatment of PRP to 39.51 ng/mL while PRP pre-treatment and exposure reduced this to 21.39 ng/mL (Figure 3.7). Tamoxifen-treatment not only induced negative detectable levels of thrombin on treatment of T47D cells but also altered this to detectable levels on exposure to blood constituents; suggesting that Tamoxifen heightens thrombin generation.

Thrombin Generation Alters between Models

Assessment of induction of thrombin generation between Models revealed that Tamoxifen-treatment of MCF7 cells (Model 1) and Tamoxifen-treatment of blood constituents (Model 2) with subsequent exposure to MCF7 cells, did not elicit any significant differences in thrombin generation (Figure 3.7).

However, upon assessment of the T47D cell line; it was noted that Tamoxifen-treatment of blood constituents (Model 2) resulted in variable levels of thrombin generation with both Tamoxifen-treated WB and PPP inducing significantly lower levels of thrombin generation compared to matched samples in Model 1 (Figure 3.7). This reduction was reflected in CD62P and CD63 IPA assessment, with both markers lower than that expressed from WB exposed to Tamoxifen-treated T47D cells ($p>0.05$) (Table 3.2). Tamoxifen-treatment was also noted to induce reduced levels of thrombin generation in T47D cells compared to that of MCF7 cells, as mentioned previously, this reduction may not be solely indicative of reduced generation but of generation greater than that of detectable levels (Figure 3.7).

3.3.4.2 Platelet activation

Model 1: Overall IPA and Platelet Ultrastructure

Cumulative effects of Tamoxifen were assessed in WB samples exposed to Tamoxifen treated MCF7 cells (Table 3.2). These samples showed a significant increase in CD62P IPA, with a slight increase in CD63 IPA, when compared to untreated WB and comparable to that of WB exposed to MCF7 cells. The ratio of activation markers was also seen to have only decreased slightly, therefore indicating that platelets within this sample remained at an earlier stage of activation. Ultrastructural assessment indicated that platelets from WB exposed to Tamoxifen-treated MCF7 cells had hyalomere spread with a further extension of pseudopodia, microparticle presence and fibrin pores (Figure 3.11(1)A).

PRP exposed to Tamoxifen-treated MCF7 cells induced a significant decrease in CD62P IPA as well as a decrease in CD63 IPA ($p>0.05$); compared to untreated PRP, with platelets within the sample remaining at earlier stages of activation (Table 3.3). On comparison to PRP exposed to untreated MCF7 cells, PRP exposed Tamoxifen-treated MCF7 cells induced a significant decrease in both CD62P and CD63 IPA, as well as a reduction in the ratio of these markers. This loss was reflected ultrastructurally with platelets from PRP exposed to Tamoxifen-treated MCF7 cells showing active platelets with a smooth membrane and extending filipodia (Figure 3.11(1)C).

WB exposed to Tamoxifen-treated T47D cells (Table 3.2) indicated a significant increase in CD62P IPA and CD63 IPA, when compared to untreated WB. The ratio of activation markers was also seen to have only increased slightly, therefore indicating that platelets within this sample remained at an earlier stage of activation. Ultrastructurally, platelets from WB exposed to Tamoxifen-treated T47D cells exhibited a smooth membrane surface with some folds and extension of pseudopodia outward from the platelet body (Figure 3.12(1)A).

PRP exposed to Tamoxifen-treated T47D cells induced a significant increase in CD62P IPA and CD63 IPA (Table 3.3); with ultrastructural assessment showing platelets with a smooth membrane and extending lamellipodia and filipodia, outward toward other platelets representing early aggregation (Figure 3.12(1)C).

Model 1: IPA per interval gate

On assessment of IPA per interval gate, WB exposed to Tamoxifen-treated of MCF7 cells indicated an increase in CD62P^{low} ($p>0.05$) and a significant increase in CD63^{low} interval gates (I1) compared to untreated WB; as well as an increase ($p>0.05$) when compared to WB exposed to MCF7 cells (Table 3.2). An increase is noted in the CD62P^{medium} interval gate while a decrease is noted within the CD63^{medium} interval gate (I2 -I3), compared to untreated WB. In the CD62P^{high} and CD63^{high} (I4 – I5) interval gates, however, a decrease in both IPA in response to Tamoxifen-treatment of MCF7 cells when compared to the untreated WB was found. PRP exposed to Tamoxifen-treated MCF7 cells showed an increase in CD62P^{low} ($p>0.05$) and a significant decrease in CD63^{low} interval gates, compared to untreated PRP; however, on comparison to PRP exposed to MCF7 cells a significant decrease is noted within the CD63^{low} interval gate (Table 3.3). Both CD62P^{medium} and CD63^{medium} interval gates indicated an increase ($p>0.05$) in IPA, compared to the untreated PRP control. A decrease, however, is noted within the CD62P^{high} and CD63^{high} interval gates, compared to both untreated PRP and PRP exposed to MCF7 cells.

WB from cumulative Tamoxifen treatment of T47D cells indicated a significant increase in CD62P^{low} and CD63^{low} interval gates compared to untreated WB (Table 3.2). An increase in CD62P^{medium} and CD63^{medium} interval gates was noted compared to the untreated control. In the CD62P^{high} and CD63^{high} interval gates, decreases in both CD62P IPA and CD63 IPA in response to Tamoxifen-treatment of T47D cells when compared to untreated WB were found. PRP exposed to Tamoxifen-treated T47D cells showed significant increases in CD62P^{low} and CD63^{low} interval gates, with the CD62P^{medium} and CD63^{medium} interval gates showed significant increases in CD62P and CD63 IPA (Table 3.3). The CD62P^{high} and CD63^{high} interval gates; however, showed a decrease in CD62P IPA and significant increase CD63 IPA, compared to untreated PRP.

Model 2: Overall IPA and platelet ultrastructure

When assessing circulating effects of Tamoxifen-treated WB samples exposed to MCF7 cells (Table 3.2), both CD62P and CD63 IPA were heightened ($p>0.05$) compared to untreated WB, similar to that of the cumulative effects (Model 1). The low ratio of activation markers indicated that platelets remained at an earlier stage of activation. Ultrastructurally, this was

confirmed with platelets from Tamoxifen-treated WB showing active platelets with extending filipodia (Figure 3.11(1)B). Tamoxifen-treated PRP exposed to MCF7 cells induced a significant decrease in CD62P IPA overall, and only a slight increase in CD63 IPA ($p>0.5$); inducing the ratio of activation markers to be lower than that of untreated PRP, indicating a shift to later stages of activation (Table 3.3). This was reflected ultrastructurally with platelets from Tamoxifen-treated PRP exposed to MCF7 cells exhibiting much higher levels of platelet activation with platelets displaying a hyalomere spread with lamellipodia and filipodia extension, as well as levels of platelet aggregation, (Figure 3.11(1)D). Furthermore, PPP exposed to Tamoxifen-treated MCF7 cells showed heightened levels of fibrin plaque formation with many pores being visible (Figure 3.11(1)E). Tamoxifen-treated PPP exposed to MCF7 cells, however, showed lower but still visible fibrin plaque formation with fewer pores visible (Figure 3.11(1)F).

When assessing circulating effects of Tamoxifen; in Tamoxifen-treated WB samples exposed to T47D cells (Table 3.2), both CD62P and CD63 IPA were significantly heightened compared to untreated WB, similar to that of the cumulative effects. The ratio of platelet activation markers was slightly lower indicating a shift in the stage of activation, visualised ultrastructurally as platelets with a folded membrane surface and filipodia extension. Early formation of fibrin meshwork was evident (Figure 3.12(1)B).

Tamoxifen-treated PRP exposed to T47D cells induced a significant increase in CD62P and CD63 IPA; however, the ratio indicated that platelets remained at early stages similar to untreated PRP, (Table 3.3) this was not noted ultrastructurally, where platelets were aggregated with filipodia extension (Figure 3.12(1)D). Furthermore, PPP exposed to Tamoxifen-treated T47D cells showed early levels of fibrin plaque formation with few pores being visible (Figure 3.12(1)E). Tamoxifen-treated PPP exposed to T47D cells, however, induced fibrinogen deposits (Figure 3.12(1)F).

Model 2: IPA per interval gate

Circulating effects of Tamoxifen-treated WB exposed to MCF7 cells showed an increase in CD62P^{low} as well as a significant increase in CD63^{low} interval gates. In the CD62P^{medium} and CD63^{medium} interval gates, Tamoxifen-treatment of WB exposed to MCF7 cells induced only a

slight increase ($p>0.05$) when compared to untreated WB. The CD62P^{high} and CD63^{high} interval gates; however, showed a reduction, with CD63 IPA significantly lower than that of untreated WB. These observations persisted in Tamoxifen-treated PRP, with the most notable difference being the loss of expression in the CD62P^{high} interval gate compared to that of untreated PRP (Table 3.3).

Circulating effects of Tamoxifen-treated WB exposed to T47D cells showed a significant increase in CD62P^{low} and CD63^{low} interval gates, compared to untreated WB. In the CD62P^{medium} and CD63^{medium} interval gates, Tamoxifen-treated WB exposed to T47D cells induced a significant increase in both CD62P and CD63 IPA; however, the CD62P^{high} and CD63^{high} interval gates showed significantly lower IPA compared to untreated WB. These observations persisted in Tamoxifen-treated PRP, with CD63 IPA only persisting in CD63^{high} interval gate I4 at lower levels than that of untreated PRP (Table 3.3).

Comparison between Cumulative (Model 1) and Circulating (Model 2) effects of Tamoxifen on Platelet Activation.

Assessment between Models indicated no significant difference between Cumulative (Model 1) and Circulating (Model 2) effects of Tamoxifen in both MCF7 and T47D cell lines on assessment of CD62P and CD63 IPA in the WB component (Table 3.2). Similarly, when assessing the PRP component, no significant differences were noted overall between Models of both cell lines, although a reduction of overall CD62P and CD63 IPA was noted ($p>0.05$). This reduction was identified on assessment per interval gate; however, only significant differences were noted in the T47D cell line. In the CD62P^{low} and CD63^{low} interval gates, significant reductions are noted in CD62P and CD63 IPA, respectively in Tamoxifen-treated PRP (Model 2) exposed to T47D cells, compared to Model 1 (Table 3.3). This reduction was further highlighted in both CD62P^{medium} and CD62P^{high} interval gates, where significantly lower CD62P IPA was noted in Tamoxifen-treated PRP samples exposed to T47D compared to Tamoxifen-treated T47D cells exposed to PRP (Table 3.3). Platelet ultrastructural analysis confirmed platelet activity to a point of aggregation indicating a loss of CD62P, correlating with these results (Figure 3.12(1)D). Furthermore, in Model 1 it was identified that Tamoxifen-treatment heightened the pro-thrombotic induction capability of the T47D cell

line compared to that of the MCF7 cell line; however, this was not as evident in Model 2 samples.

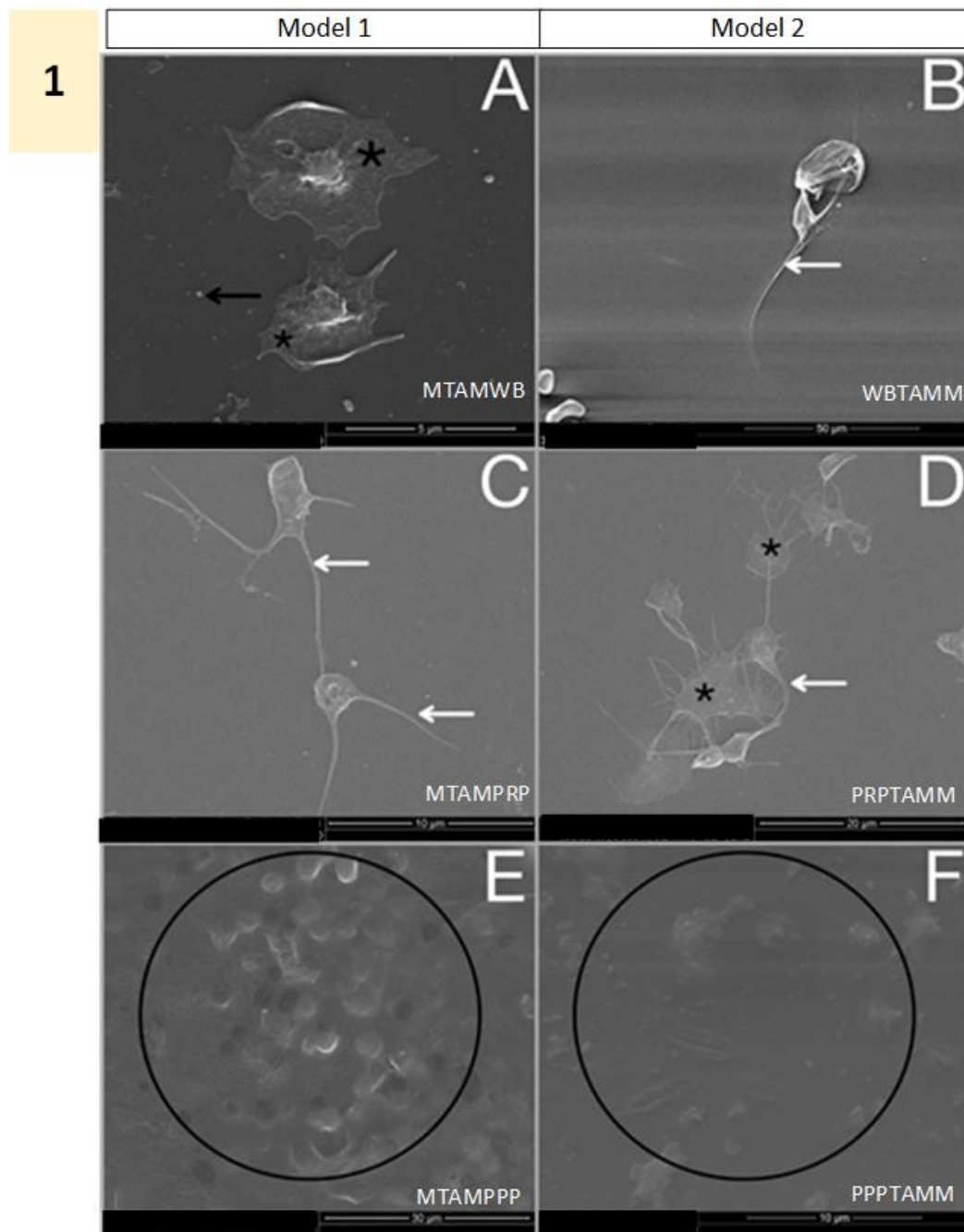
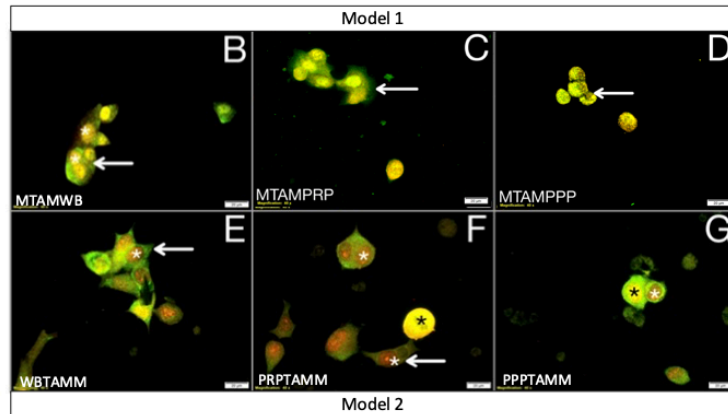
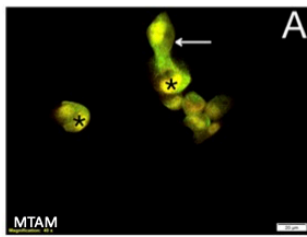


Figure 3.11: Effects of Tamoxifen- treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with MCF7 cells. Key: MTAM: Tamoxifen-treated MCF7 cells. MTAMWB: Tamoxifen-treated MCF7 cells with WB. MTAMPRP: Tamoxifen-treated MCF7 cells with PRP. MTAMPPP: Tamoxifen-treated MCF7 cells with PPP. WBMTAM: Tamoxifen-treated WB exposed to MCF7 cells. PRPTAMM: Tamoxifen-treated PRP exposed to MCF7 cells. PPPTAMM: Tamoxifen-treated PPP exposed to MCF7 cells. (1) Platelet ultrastructural alterations. Key: black * - platelet hyalomere spread, white arrow - extending filipodia, black arrow - microparticles, black circle - fibrin clots and plaques with pores. A: MTAMWB - active platelets with a hyalomere spread and microparticles. B: WBMTAM - active platelets with a folded membrane and extending filipodia. C: MTAMPRP - platelets with a hyalomere spread and extending filipodia. D: PRPTAMM - platelets in early stages of aggregation, with extending filipodia. E: MTAMPPP - fibrin plaque formation with some pores. F: PPPTAMM- fibrin pores and remnants of fibrin.

2



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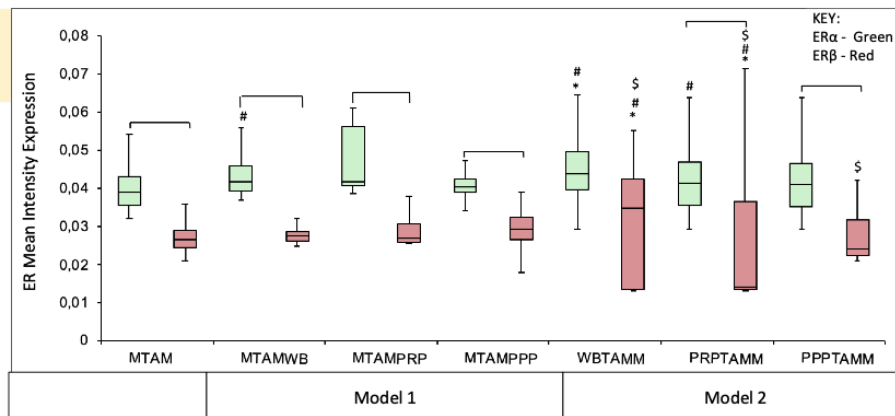


Figure 3.11: Effects of Tamoxifen- treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with MCF7 cells. **Key:** MTAM: Tamoxifen-treated MCF7 cells. MTAMWB: Tamoxifen-treated MCF7 cells with WB. MTAMPRP: Tamoxifen-treated MCF7 cells with PRP. MTAMPPP: Tamoxifen-treated MCF7 cells with PPP. WB TAMM: Tamoxifen-treated WB exposed to MCF7 cells. PRPTAMM: Tamoxifen-treated PRP exposed to MCF7 cells. PPPTAMM: Tamoxifen-treated PPP exposed to MCF7 cells. **(2)** Co-localisation of ERα (green) and ERβ (red) in MCF7 cells. Yellow - ERα dominating; Orange - ERβ dominating. **A:** MTAM – cytoplasmic and nuclear ERα (arrow) and nuclear ERβ (*). **B:** MTAMWB – nuclear and cytoplasmic ERα (arrow) and nuclear ERβ (*). **C:** MTAMPRP – nuclear and low cytoplasmic ERα (arrow) and nuclear ERβ (*). **D:** MTAMPPP – low cytoplasmic and high nuclear ERα (arrow) and nuclear ERβ (*). **E:** WB TAMM – cytoplasmic ERα (arrow) and nuclear ERβ (*). **F:** PRPTAMM – cytoplasmic ERα (arrow) and nuclear ERβ (white *), combined expression (black *). **G:** PPPTAMM – cytoplasmic ERα with nuclear of ERβ (*). **(3)** Box and whisker plots representing quantitative analysis of ERα (green) and ERβ (red) expression in MCF7 cells. [$p < 0.05$ between ERα and ERβ within the treatment group. # $p < 0.05$ between matched ERs compared to untreated MCF7. * $p < 0.05$ between matched ERs compared to Tamoxifen- treated MCF7. \$ $p < 0.05$ between matched ERs and blood constituent groups compared between Models.

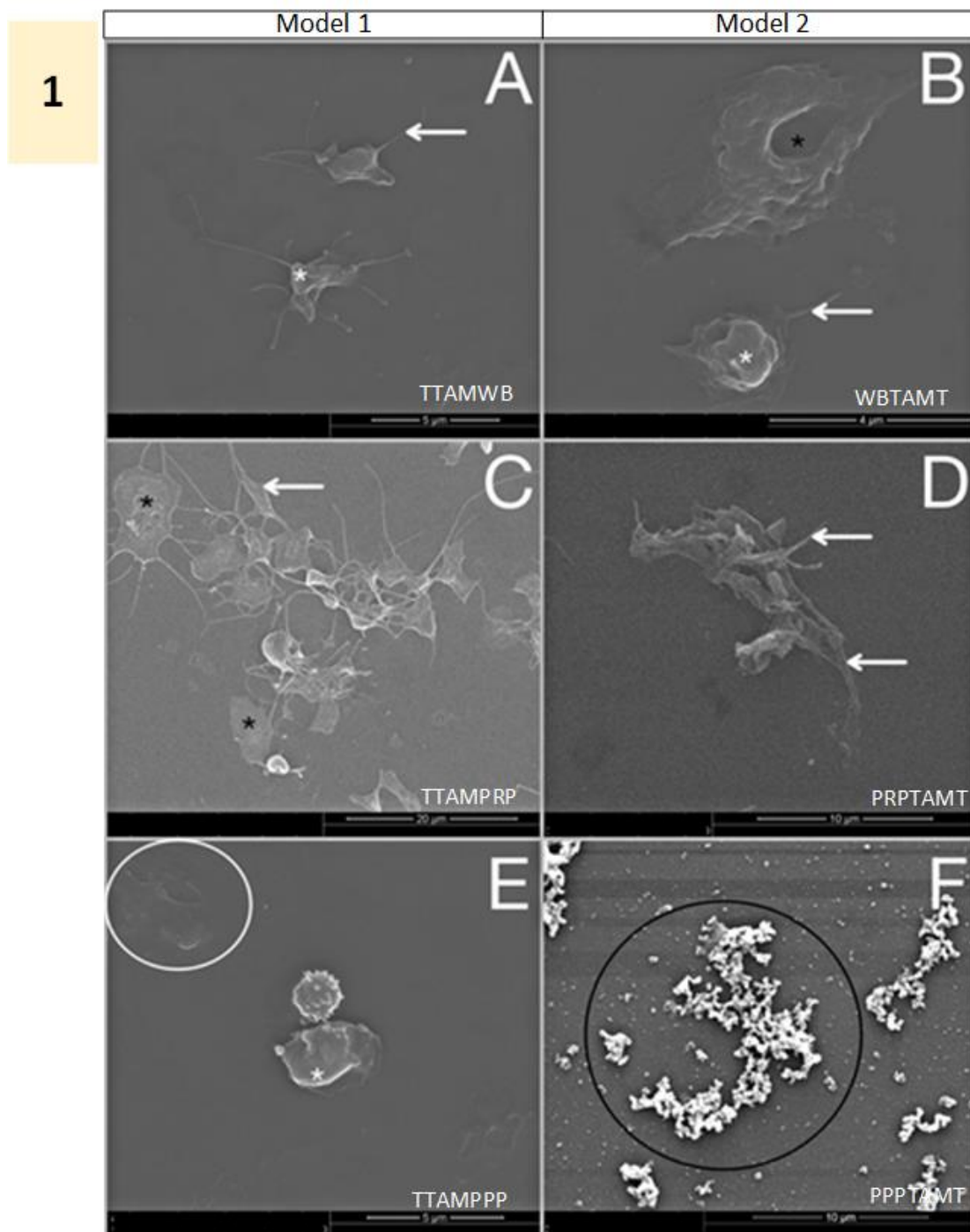
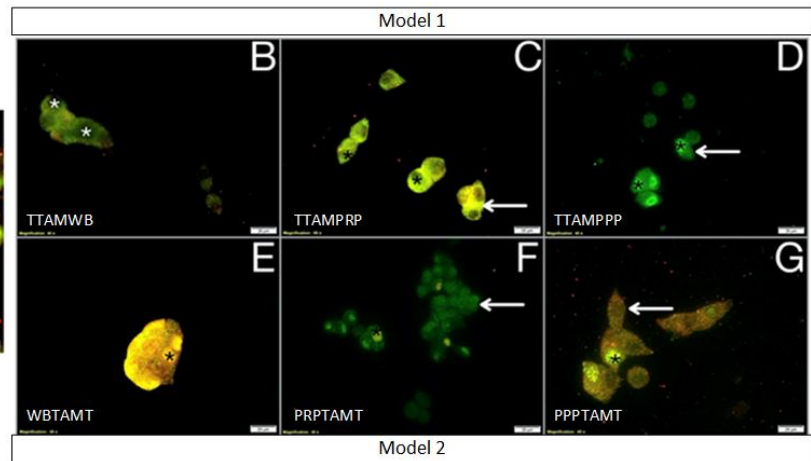
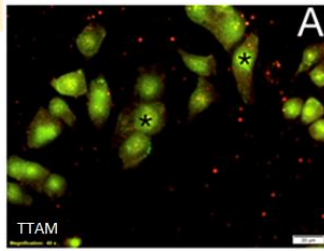


Figure 3.12: Effects of Tamoxifen treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with T47D cells. Key: TTAM: Tamoxifen-treated T47D cells. TTAMWB: Tamoxifen-treated T47D cells with WB. TTAMPRP: Tamoxifen-treated T47D cells with PRP. TTAMPPP: Tamoxifen-treated T47D cells with PPP. WBTAMT: Tamoxifen-treated WB with T47D cells. PRPTAMT: Tamoxifen-treated PRP exposed to T47D cells. PPPTAMT: Tamoxifen-treated PPP exposed to T47D cells. **(1)** Platelet ultrastructural alterations. Key: white * - membrane folds, black * - platelet hyalomere spread, white arrow - extending filipodia, black arrow - microparticles, white circle - fibrin, black circle - fibrin deposits. A: TTAMWB - active platelets with a folded membrane and extending filipodia. B: WBTAMT - active platelets with a folded membrane and extending filipodia with fibrin deposits and pores C: TTAMPRP - platelets with a hyalomere spread and extending filipodia. D: PRPTAMT - aggregated platelets with extending filipodia. E: TTAMPPP - fibrin deposits and plaque formation with some pores. F: PPPTAMT- fibrinogen deposits.

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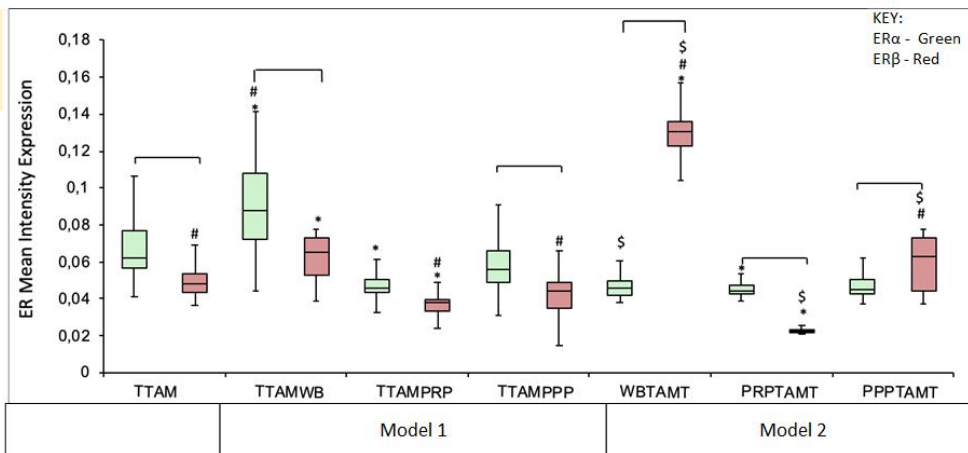


Figure 3.12: **Effects of Tamoxifen treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with T47D cells.** **Key:** TTAM: Tamoxifen-treated T47D cells. TTAMWB: Tamoxifen-treated T47D cells with WB. TTAMPRP: Tamoxifen-treated T47D cells with PRP. TTAMPPP: Tamoxifen-treated T47D cells with PPP. WBTAMT: Tamoxifen-treated WB with T47D cells. PRPTAMT: Tamoxifen-treated PRP exposed to T47D cells. PPPTAMT: Tamoxifen-treated PPP exposed to T47D cells. **(2)** Co-localisation of ER α (green) and ER β (red) in T47D cells. Yellow - ER α dominating; Orange - ER β dominating. A: TTAM – cytoplasmic and nuclear ER α (*) and ER β dispersed. B: TTAMWB – cytoplasmic ER α and nuclear ER β , some nuclei devoid of expression (*). C: TTAMPRP – cytoplasmic ER α (arrow) and ER β , with the nucleus devoid of expression (*). D: TTAMPPP – cytoplasmic and nuclear ER α (arrow) with nuclear ER β (*). E: WBTAMT – cytoplasmic ER α and nuclear ER β (*). F: PRPTAMT – cytoplasmic ER α (arrow) with nuclear ER α and ER β (*). G: PPPTAMT – cytoplasmic and nuclear ER α (*), and cytoplasmic ER β (arrow). **(3)** Box and whisker plots representing quantitative analysis of ER α (green) and ER β (red) expression in T47D cells. [$p < 0.05$ between ER α and ER β within the treatment group. # $p < 0.05$ between matched ERs compared to untreated T47D. * $p < 0.05$ between matched ERs compared to Tamoxifen- treated T47D. \$ $p < 0.05$ between matched ERs and blood constituent groups compared between Models.

3.3.5 Tumour invasion profiles marked by ER Responses in Breast cancer cells

3.3.5.1 *Breast Cancer Cell ER Expression alters in response to blood constituents*

MCF7 breast cancer cells typically have an epithelial-like morphology with thicker cellular processes protruding from their cell body. In untreated MCF7 breast cancer cells ER α is concentrated within the cytoplasm and along the edges of the cell, with some nuclear expression, while ER β can be seen persisting within the nucleus (Figure 3.9(2)A). Quantitative assessment showed overall, greater ER α expression than that of ER β (Figure 3.9(3)A). On exposure to WB, greater ER α expression in the perinuclear cytoplasmic region and cytoplasm was noted; while ER β persisted within the nucleus (Figure 3.9(2)B), this increase in ER β was significantly higher than that of the untreated control (Figure 3.9(3)A). However, when MCF7 cells were exposed to PRP (Figure 3.9(2)C), ER expression altered considerably: ER α remained in the cytoplasm of the cells, very dispersed with high expression and ER β although more dispersed (Figure 3.9(2)C) presented with significantly high intensity expression when compared to that of the control (Figure 3.9(3)A). PPP exposure (Figure 3.9(2)D), induced greater nuclear expression of ER β which was significantly higher than the control (Figure 3.9(3)A), with a more diffuse cytoplasmic expression of ER α , greater than the control but not significantly so (Figure 3.9(3)A).

T47D breast cancer cells are more rounded in shape and demonstrate a highly cohesive cobblestone appearance. This cell line also expressed ER β consistently higher compared to that of MCF7 cells, corroborating the analysis of ER gene expression highlighted in Chapter 2. In untreated T47D breast cancer cells ER α is noted to be diffusely expressed within the cytoplasm while ER β can be seen persisting within the nucleus (Figure 3.10(2)A). Quantitative assessment indicates ER α to be highly expressed, which can be seen toward the cytoplasmic edges of some cells. On exposure to WB expression sites are noted to have altered, with both ER α and ER β present within the nucleus, with ER β being expressed significantly higher than untreated cells (Figure 3.10(3)). However, when T47D cells were exposed to PRP (Figure 3.10(2)C), ER α expression was noted in both the nucleus and cytoplasm, with ER β expression remaining in the nucleus. Mean intensity expression of markers, however, was noted to be lower than the untreated control (Figure 3.10(3)A). PPP exposure (Figure 3.10(2)D), induced similar expression, with higher ER α and ER β mean intensity expression observed (Figure 3.10(3)A).

The diluent (Figure 3.10(2)E), 0.1% DMSO, induced greater ER α expression concentrated primarily in the nucleus with diffuse cytoplasmic expression. ER β expression; however, was only minimally expressed within the nucleus, correlating with quantitative assessment. On exposure to blood constituents; WB induced greater nuclear expression of ER β and diffuse cytoplasmic expression of ER α , however, this was significantly lower than the diluent control (Figure 3.10(3)B). PRP heightened ER β nuclear expression significantly with some diffuse cytoplasmic staining, and lower ER α expression than the diluent control; and PPP (Figure 3.10(2)H) induced greater ER β nuclear and some diffuse cytoplasmic expression with equivalent ER α expression. On assessment of matched media and diluent samples; a significant difference is noted only between ER α and ER β expression of untreated MCF7 cells and diluent-treated MCF7 cells. 0.1% DMSO induced significantly greater ER α expression, while significantly reducing ER β expression when compared to the untreated control.

Diluent-treated T47D cells (Figure 3.10(2)E), showed greater ER α expression within both the nucleus and cytoplasm, with highly concentrated expression points within the cytoplasm itself; while ER β was seen persisting in the nucleus. Quantitatively, the mean intensity of both ERs was noted to be lower than that of untreated T47D cells. On exposure to blood constituents; WB (Figure 3.10(2)F) induced greater cytoplasmic expression of ER α , with high expression points within the cytoplasm while ER β was noted to be only expressed within the nucleus itself qualitatively and quantitatively (Figure 3.10(3)B). Both PRP (Figure 3.10(2)G) and PPP (Figure 3.10(2)H); induced similar expression levels with greater ER α expression diffusely in the cytoplasm with some nuclear expression, while ER β was expressed primarily in the nucleus. Quantitative assessment shows an increase in ER α mean intensity expression on exposure to PPP, with greater variability of the data (Figure 3.10(B)).

3.3.2.1 Tamoxifen induced ER alterations on exposure to blood constituents

Model 1

Tamoxifen-treatment induced consistently higher ER α expression throughout all treatment groups, this was also seen in quantitative assessment (Figure 3.11(3)). Tamoxifen-treated MCF7 cells (Figure 3.11(2)A) expressed ER α both in the nucleus and the cytoplasm, while ER β

was noted to be highly expressed in the nucleus. Following exposure to WB (Figure 3.11(2)B), Tamoxifen-treated MCF7 cells expressed ER α in the cytoplasmic and nuclear regions, while ER β expression remained in the nucleus, albeit heightened; quantitatively ER α mean intensity was slightly higher than that of Tamoxifen-treated MCF7 cells while ER β remained the same (Figure 3.11(3)). PRP exposure (Figure 3.11(2)C), induced higher ER β expression in the nucleus with more diffuse cytoplasmic ER α expression, substantiated by quantitative results; with greater variability noted within ER α . PPP exposure (Figure 3.11(2)5D) induced nuclear expression of ER α and ER β .

Tamoxifen treatment induced consistently higher ER α expression in T47D cells (Figure 3.12(3)), s similarly seen in MCF7 cells (Figure 3.11(3)). Tamoxifen-treatment of T47D cells (Model 1) (Figure 3.12(2)A) induced ER α expression both in the nucleus and the cytoplasm, with heightened nuclear expression, while low ER β expression was noted to be in the nucleus, with some extracellular binding postulated to be from lysed cells due to our methodology. Mean intensity expression indicated ER α to be higher than that of ER β expression, as expected (Figure 3.12(3)). Following exposure to WB (Figure 3.12(2)B), Tamoxifen-treated T47D cells expressed ER α primarily in the cytoplasm, significantly higher expression than that of Tamoxifen-treated T47D cells alone (Figure 3.12(3)). ER β expression was primarily in the nucleus (Figure 3.12(2)B), and the mean intensity was also significantly higher than that of Tamoxifen-treated cells (Figure 3.12(3)).

PRP exposure (Figure 3.12(2)C), induced greater ER α expression in the nucleus with more diffuse cytoplasmic expression, however, the overall mean intensity was significantly lower when compared to that of Tamoxifen-treated cells; ER β was present only in the cytoplasm of cells seen to be clumped together and was also noted to have a mean intensity significantly lower than that of Tamoxifen-treated cells (Figure 3.12(3)). PPP exposure (Figure 3.12(2)D) induced diffuse cytoplasmic expression of ER α with heightened nuclear expression, while ER β expression was noted only slightly within the nucleus.

Model 2

Tamoxifen-treated WB (Figure 3.11(2)E) induced heightened perinuclear ER α and greater ER β nuclear expression of MCF7 cells. Quantitative assessment showed a significant increase in both ER α and ER β mean intensity (Figure 3.11(3)), with greater variability noted within ER β . Tamoxifen-treated PRP (Figure 3.11(2)F), induced reduced ER β nuclear expression with lower diffuse ER α in the cytoplasm. Tamoxifen-treated PPP (Figure 3.11(2)G) induced expression similar to that induced by treated WB, slightly lower with persistent, yet lower, nuclear ER β expression (Figure 3.11(3)).

T47D cells exposed to Tamoxifen-treated WB (Figure 3.12(2)E) revealed heightened nuclear and cytoplasm ER α and ER β expression, with much greater levels of ER β expression and mean intensity significantly higher than that of Tamoxifen- treated cells (Figure 3.12(3)) and the untreated control (Figure 3.9(3)). Tamoxifen-treated PRP (Figure 3.12(2)F), induced greater ER α cytoplasmic and nuclear expression, with focused expression within the nucleus. ER β expression was only present in the nucleus of some cells; however, the mean intensity of both markers was significantly lower than the control (Figure 3.12(3)). Tamoxifen-treated PPP (Figure 3.12(2)G) induced ER α expression primarily in the nucleus, with some diffuse cytoplasmic staining with heightened diffuse ER β expression present within the cytoplasm of the cells.

Alterations in ER expression between Cumulative (Model 1) and Circulating (Model 2) Tamoxifen-treatment.

On assessment of ER expression between models, it was noted that ER β was significantly heightened in Tamoxifen-treated WB and PRP exposed to MCF7 cells (Model 2), respectively when compared to matched samples from Model 1 (Tamoxifen-treated MCF7 cells exposed to blood constituents) (Figure 3.11(3)). This increase was further noted in photomicrographs, where ER β is noted to be more highly expressed in the nucleus in samples from Model 2 (Figure 3.11(2) E & F) compared to less intense expression noted within samples from Model 1 (Figure 3.11 (2) B & C). However, a significant reduction in ER β is noted in Tamoxifen-treated PPP samples compared to matched Tamoxifen-treated MCF7 cells exposed to PPP (Figure 3.11(3)); this was reflected in photomicrographs (Figure 3.11(2)G).

Tamoxifen-treatment of blood constituents exposed to T47D cells (Model 2) elicited differential effects; with Tamoxifen-treatment of WB inducing significantly reduced ER α expression and significantly heightened ER β expression compared to Tamoxifen-treated T47D cells exposed to WB (Model 1) (Figure 3.12(3)), this difference was further noted in photomicrographs (Figure 3.12(2) B & E). A significant reduction in ER β expression was noted in Tamoxifen-treated PRP samples exposed to T47D cells; while a significant increase was noted in Tamoxifen-treated PPP samples exposed to T47D cells compared to matched Model 1 samples (Figure 3.12(3)); and was confirmed through protein analysis (Figure 3.12(2)).

3.3.6. The Correlation between hypercoagulation and ER expression

Overall, the non-parametric Spearman's correlation test identified significant ($p < 0.05$), positive ($r > 0.5$) correlation between CD62P and CD63 IPA (Appendix), further not correlated to thrombin detection (Table 3.7). While a significant negative correlation was also noted between ER β and thrombin detection, this was notably weak (Table 3.4). On analysis per treatment group a significant ($p < 0.05$) positive ($r > 0.5$) correlation between thrombin generation and CD63 IPA is noted in Model 2; Tamoxifen-treated WB exposed to MCF7 cells. When assessing ER correlation to IPA, a significant ($p < 0.05$) positive ($r > 0.5$) correlation is noted between ER α and CD63 IPA and CD62P IPA in Tamoxifen-treated PRP exposed to MCF7 cells. Notably, significant ($p < 0.05$) negative ($r < 0.5$) correlation between ER β and CD63 IPA and CD62P IPA in Tamoxifen-treated PRP exposed to MCF7 cells, was noted. While other correlations are shown, these appear to be weak. Significant correlations are consistently noted within MCF7 samples from Model 2 indicating a role for circulatory Tamoxifen-treatment in the induction of platelet activation associated with ER expression, dependent on subphenotype. Furthermore, the negative correlations noted between thrombin generation and ER β ; illustrates the opposing actions of ER β to that of ER α .

Table 3.4: *Tabulation showing r values obtained from the nonparametric Spearman's correlation between CD62P, CD63 IPA and ER α and ER β , with thrombin detection for MCF7 and T47D cells.* Key: MMEDWB: MCF7 cells exposed to WB. MMEDPRP: MCF7 cells exposed to PRP. MTAMWB: Tamoxifen-treated MCF7 cells exposed to WB. MTAMPRP: Tamoxifen-treated MCF7 cells exposed to PRP. WBTAMM: MCF7 cells exposed to Tamoxifen-treated WB. PRPTAMM: MCF7 cells exposed to Tamoxifen-treated PRP, TMEDWB: T47D cells exposed to WB. TMEDPRP: T47D cells exposed to PRP. TTAMWB: Tamoxifen-treated T47D cells exposed to WB. TTAMPRP: Tamoxifen-treated T47D cells exposed to PRP. WBTAMT: T47D cells exposed to Tamoxifen-treated WB. PRPTAMT: T47D cells exposed to Tamoxifen-treated PRP. Red $p < 0.05$ compared to thrombin detection, italics $p < 0.05$ compared to CD62P IPA, bold $p < 0.05$ compared to CD63 IPA.

Spearman Rank Order Correlations																	
Highlighted correlations are significant at $p < 0.05$. $r = 0.6$																	
	Overall	Control				Model 1						Model 2					
		MME DWB	MME DPRP	TMED WB	TMED PRP	MCF7 Overall	T47D Overall	MTAM WB	MTAM PRP	TTAM WB	TTAM PRP	MCF7 Overall	T47D Overall	WBTA MM	PRPTA MM	WBTA MT	PRPTA MT
Thrombin Generation																	
CD62P IPA	-0,075	-0,314	0,085 7	-0,028	0,257	-0,242	0,1319	-0,2	0,7714	0,3	0,3142	-0,24795	-0,06761	- 0,6572	-0,486	-0,579	0,4
CD63 IPA	<i>-0,047</i>	-0,314	<i>0,028</i> 5	<i>0,314</i>	<i>0,428</i>	<i>-0,195</i>	<i>0,1018</i>	<i>-0,429</i>	0,771	-0,7	-0,086	<i>-0,17414</i>	<i>-0,03075</i>	<i>0,8986</i>	-0,4857	0,6	0,1
ER- Alpha	0,016	-0,714	<i>0,029</i> 4	0,257	0,25	<i>0,16584</i>	<i>0,0425</i>	0,4857	-0,4857	0,3	0,1426	<i>0,173056</i>	0,01357	0,3142	<i>0,7714</i>	-0,657	-0,1
ER- Beta	<i>-0,189</i>	-0,2	-0,264	-0,257	0,142	<i>-0,00069</i>	<i>-0,037</i>	0,485	-0,1428	0,4	-0,314	<i>-0,2009</i>	-0,10588	0,4286	<i>-0,6</i>	-0,486	-0,6

3.3 Discussion

Tamoxifen, is known to increase the risk of a thromboembolic event in breast cancer patients, compounding the risk associated with breast cancer itself (Decensi *et al.*, 2005; Howell *et al.*, 2005; Dobryднева *et al.*, 2007; Walker *et al.*, 2017; Lin *et al.*, 2018). However, recent *in vitro* studies indicate contradictory results pointing to a role for Tamoxifen rather in the inhibition of platelet activation (Chang *et al.*, 2011; Johnson *et al.*, 2017). Given that Tamoxifen is also used as a chemopreventive agent (Chang, 2012; Waters *et al.*, 2012), its associated risk with thrombosis may not readily be taken into account in future clinical scenarios, despite published clinical studies indicating the converse (Meier & Jick 1998; Onitilo *et al.* 2012; Walker *et al.* 2016; MacLellan *et al.*, 2012). These contradictory results may be due to varying methodologies including platelet washing and centrifugation. Tamoxifen used in the pre-treatment of washed platelets indicated inhibition of cell line induced platelet activation *in vitro* (Chang *et al.*, 2011; Johnson *et al.*, 2017). Whereas in Chapter 2 we indicate a role for Tamoxifen in the induction of platelet activation, higher than that of breast cancer cells alone; dependent on phenotype reflecting that of clinical studies (Walker *et al.*, 2017). Furthermore, we indicate that the loss of plasma, which occurs during the washing process, inhibits tumour-cell induced platelet activation and subsequent aggregation (Zarà *et al.*, 2018). As such, the methodology in this Chapter allowed for mimicking of both accumulation of Tamoxifen at the tumour site (Model 1) and recreated intravenously administered Tamoxifen (Model 2) (Ishitobi *et al.*, 2013; Kisanga, Gjerde, Guerrieri-gonzaga, *et al.*, 2004; Ishitobi *et al.*, 2013). We further tested blood constituents WB, PRP and PPP to ascertain potential mechanisms associated with retaining plasma, greater and lower numbers of platelets including higher levels of fibrinogen in tumour-induced platelet activation and hypercoagulation. Fibrinogen is a glycoprotein complex circulating within the bloodstream; once activated through their PAR-1 receptors by thrombin, it cleaves fibrin which forms networks and ultimately stabilizes the platelet plug and forms plaque, noted highly in diseased states (Pretorius *et al.*, 2007, 2011; Weisel and Litvinov, 2017). Studies have indicated that the removal of plasma, limiting the effects of thrombin and the induction of calcium influx, during platelet washing inhibits tumour cell-induced platelet activation and subsequent aggregation (Zarà *et al.*, 2018), favouring a methodological approach which complements the physiological environment so as not to negate such effects. Thus, this chapter aimed to determine the effect of Tamoxifen

treatment modelled on the physiological environment and the effects on ER expression and their relationship to hypercoagulation *in vitro*, as well as to determine varying effects of blood constituents.

Modelling the physiological environment in this way allows for an investigation of cellular responses with reduced interference and potentially more controlled mechanisms; however, certain techniques and methodological approaches may be limited and would affect the outcome. Our results indicated baseline levels of activation of platelets in both models, with platelets in the PRP component being more active identified both morphologically and by expression of CD62P and CD63 markers. We further noted that platelets within PRP were still responsive to agonists, specifically thrombin, since PRP was generated by soft centrifugation with a limited number of subsequent centrifugations prior to fixation (Augustine *et al.*, 2016), compared to the contradictory studies (Chang *et al.*, 2011; Johnson *et al.*, 2017). PRP also indicated higher detectable levels of thrombin, via granular release from platelets (Haemmerle *et al.*, 2018), compared to that of WB. This potentially reflects the greater number of platelets contained within this fraction as opposed to WB, with detected thrombin further compounding breast cancer cell-induced hypercoagulation.

Platelets are known to play a key role in priming tumour cells for metastasis in the tumour microenvironment, both before clinical diagnosis (Massagué and Obenauf, 2016) and during the metastatic process itself (Li *et al.*, 2014; Qi *et al.*, 2015; Mezouar *et al.*, 2016; Michael *et al.*, 2017; Schlesinger, 2018). During this process, secretion of proinflammatory cytokines and release of granules causes changes in coagulatory mechanisms and may lead to thromboembolic complications (Rugo, 2016); further compounded by Tamoxifen treatment (Miller *et al.*, 2005; Fabian., 2007; Masakazu *et al.*, 2009; Walker *et al.*, 2016). Breast cancer cells are able to activate the coagulation system through induction of platelet activation by releasing platelet-activating mediators including ADP and thrombin, as well as tissue factor which leads to thrombin generation and ultimately causes thrombi (Nash *et al.*, 2002); thus, creating a hypercoagulable environment (Caine *et al.*, 2002; Bauer, 2019). Our results indicate the ability of breast cancer cells to secrete thrombin, dependent on phenotype and exposure of blood constituent; with T47D cells secreting higher levels of thrombin compared to that of MCF7 cells.

Thrombin was not readily generated by the MCF10A mammary epithelial cell line, which is one of the most commonly used “normal” breast cancer cell models (Qu *et al.*, 2015), correlating with other studies which also indicate that MCF10A cells express low to no PAR1 receptors (Booden *et al.*, 2004; Qu *et al.*, 2015; Reddel, Tan and Chen, 2019). This suggests that the low levels of platelet activation and fibrin formation we identified on exposure to blood constituents were dependent on a different agonist. One study has identified coagulation induction by the MCF10A cell line in a Tissue Factor dependent manner (Berny-Lang *et al.*, 2011); however, the exact mechanism by which it exerts this effect is not well understood. Our results also indicate alterations in ER α and ER β expression on exposure to blood constituents in MCF10A cells. Studies have suggested that MCF10A cells exhibit a basal-like phenotype, with similar features to that of mesenchymal cancer cell lines (Neve *et al.*, 2006). These cells are capable of expressing luminal and stem-like markers in culture (Qu *et al.*, 2015) or are undergoing epithelial-mesenchymal transition (Sarrio *et al.*, 2008); raising the question of whether the MCF10A cell line should be used as a standard for modelling normal breast epithelial tissue. Our results indicate that the MCF10A cell line requires further investigation in this regard.

Cell line models of breast cancer subtypes are required for *in vitro* studies due to their heterogeneity that stems from their origin (Keller *et al.*, 2010). , proper. Expression of ER defines luminal phenotype cancers and cell lines (Begam, Jubie and Nanjan, 2017); these cancers are typically treated with hormone-therapy, with the “gold standard” in premenopausal patients undergoing first-line treatment being Tamoxifen (Zhai *et al.*, 2013; Begam, Jubie and Nanjan, 2017). Our results highlight switched responses in the MCF7 and T47D cell line: with MCF7 cells having greater ER α expression, while T47D cells had a higher ER β expression, comparatively. Additionally, our results indicate an increase in thrombin generation in breast cancer cells treated with Tamoxifen, corresponding with studies which indicate that hormone-therapies can induce heightened sensitivity to thrombin by upregulating PAR1 receptors, therefore increasing associated thromboembolic risk (Jick *et al.*, 2000; Herkert *et al.*, 2001; Nag and Bar-Shavit, 2018). Both cells lines are able to secrete Platelet Activating Factor (PAF) under thrombin stimulation, which in addition to mediating platelet aggregation in an autocrine manner, also enhances tumour progression (Bussolati *et*

al., 2000; da Silva Junior *et al.*, 2018). T47D cells, however, induced a more hypercoagulatory environment than MCF7 cells. This capability was heightened under Tamoxifen treatment in both Models, compared to that of MCF7 cells. Furthermore, Tamoxifen-treated T47D cells induced later stages of platelet activation, associated with a loss of CD62P IPA and persistence of CD63 IPA reflecting hypercoagulation in both PRP and WB, compared to that of MCF7 cells. This is related to the activation process in which platelets undergo morphological alterations and release of granular contents (Haemmerle *et al.*, 2018; Smith *et al.*, 2018); CD62P is released from platelet α -granules early in the activation process and is exposed on the plasma membrane and is lost as activation progresses (Augustine *et al.*, 2016; Smith *et al.*, 2018), as indicated in Chapter 2. CD63, however, is released from dense and lysosomal granules later during the process of activation (Baaten *et al.*, 2017; Haemmerle *et al.*, 2018). We identified that its expression is sustained throughout the process of activation with ultrastructural alterations coupled with marker expression analysis indicating CD63 expression at earlier stages of activation as well, complementing studies which indicate the stability of CD63 through its activation (Marquardt *et al.*, 2002; Saboor, Moinuddin and Ilyas, 2013).

Models mimicked the physiological environment, on assessment per Model; Tamoxifen-treatment of blood constituents (Model 2) resulted in comparable levels of induced hypercoagulation by MCF7 cells as that of Model 1 (Tamoxifen-treatment of breast cancer cells); however, T47D cells consistently induced a much higher level of hypercoagulation comparatively. This level was highlighted by the loss of both platelet activation markers, potentially due to their role in mediating platelet aggregation or shed as microparticles, causing full activation of platelets (van der Zee *et al.*, 2006). To potentially address why hypercoagulation was variably induced, we further assessed ERs as a marker for subphenotype alterations in MCF7 and T47D cells. ER α expression was noted to be higher than ER β expression in both cell lines, as expected. ER α has been extensively studied in relation to hormone-dependent breast cancers, with the focus being primarily on genomic and positive prognostic outcomes indicating an ability to respond to hormone-therapy (Altundag and Ibrahim, 2006; Giacinti *et al.*, 2006; Bahreini *et al.*, 2017; Begam, Jubie and Nanjan, 2017); while the role of ER β has not been studied extensively and presents with conflicting results. Recent studies have implicated high ER β expression in breast tumour

development as well as Tamoxifen-resistance (Begam, Jubie and Nanjan, 2017); and has been associated with a poorer outcome in patients treated with hormone-therapies (Strom *et al.*, 2004) while older studies implicate low ER β in Tamoxifen-resistance (Fox, Davis and Shupnik, 2008). Additionally, some studies have indicated that ER β expression may be associated with a more favourable response to treatment (Omoto *et al.*, 2003; Elebro *et al.*, 2017), and that its reduction is associated with the transition to a more invasive phenotype (Iwao *et al.*, 2000; Begam, Jubie and Nanjan, 2017). Our results, however, indicated the opposite with greater ER β expression noted in T47D cells, which we regarded as more invasive due to their expression of proteins associated with tumorigenesis, growth and prevention of apoptosis (Adjo Aka and Lin, 2012), supporting studies indicating a role for heightened ER β in tumour progression (Tee *et al.*, 2004; Chai *et al.*, 2019). However, our study is limited, with markers of invasion and migration not presently looked at; this could be a future aspect of studies to come.

Clinically, hormone-dependent breast cancers are identified by “conventional” ER or ER α overexpression, and the “gold standard” treatment, Tamoxifen, is able to mediate ER α activity (Begam, Jubie and Nanjan, 2017). In response to Tamoxifen treatment breast cancer cells employ a strategy by which they downregulate their receptor in an attempt to reduce binding, this is a common strategy employed and is associated with the transition to a more aggressive phenotype that does not respond to therapy (Begam, Jubie and Nanjan, 2017). Our results indicate a significant increase in ER β expression on exposure to WB in both cell lines, however, in T47D cells a translocation of ER α to the nucleus is noted when exposed to WB. This translocation may reflect engagement of genomic activities (Begam, Jubie and Nanjan, 2017) and corresponds with an increase in *ESR1* in T47D cells exposed to WB noted in Chapter 2. While WB reflects a more circulatory environment; many laboratory studies make use of PRP to isolate effects of platelets alone and thus would not reflect a physiological environment (Yin *et al.*, 2016; Hechler *et al.*, 2019). Alterations in both ERs are noted on exposure to blood constituents, postulated to be due to the generation of platelet-rich plasma, which even with soft centrifugation, provides a more concentrated source of growth factors (El-Sharkawy *et al.*, 2007). Furthermore, platelets predominantly express ER β

(Khetawat *et al.*, 2000; M L Nealen *et al.*, 2001), thus indicating a role for its' expression in heightened levels of platelet activation as treated directly with Tamoxifen (Model 2).

ER-positive breast tumours are associated with tailored therapeutic approaches status (Carey *et al.*, 2006; El Sayed *et al.*, 2019), which increase the risk for thromboembolic complications; while we had intended to correlate ER expression to hypercoagulation as a means to potentially predict future outcomes, our results were not as favourable and indicated further investigation be done. Our results indicate a correlation between ERs and IPA in Model 2 for MCF7 cells, positive for ER α , involved in breast cancer proliferation, and conversely negative for ER β , reiterating its' potential opposing effects to that of ER α (Strom *et al.*, 2004), heightened by Tamoxifen treatment. This substantiates clinical studies indicating that Tamoxifen induces a hypercoagulatory environment mediated by platelet interactions with breast cancer cells (Meier & Jick 1998; Onitilo *et al.* 2012; Walker *et al.* 2016; MacLellan *et al.*, 2012). We further highlight the important role platelets play in relation to breast cancer cells and ultimately within the tumour microenvironment mediated by factors including changes in nuclear and cytoplasmic ER α relating to the metastatic potential of breast cancer cells. We further present that breast cancer cells (regardless of cell line) facilitate coagulatory mechanisms, including thrombin release to induce a hypercoagulable state compounded by Tamoxifen effects and cytotoxicity (Chen and Geng, 2006; Falanga, Russo and Verzeroli, 2013; Mezouar *et al.*, 2016; Zhang and Newman, 2019). These results further highlight the procoagulant and heightened invasive nature of T47D cells compared to that of MCF7 cells, extending and correlating with results presented in Chapter 2, and indicates a role for subphenotyping clinically. Our results ultimately indicate an association between tumour invasion profiles, represented by ER expression and defining hypercoagulation, further extending and substantiating results from Chapter 2 and correlating with clinical studies indicating that patients undergoing Tamoxifen treatment are more likely to suffer a thromboembolic event (Fennerty, 2006; Dobryднева *et al.*, 2007; Lin *et al.*, 2018).

4 CHAPTER 4: Cumulative and circulating Anastrozole effects on ER expression and the relationship to hypercoagulation.

4.1 Introduction

Targeted breast cancer treatment strategies involve drugs which block the growth of cancer by altering the function of molecules and proteins involved in tumour cell proliferation and tumour survival (Mittendorf *et al.*, 2016; Rugo, 2016; Masoud and Pagès, 2017). Breast cancer cells overexpress receptors, such as ERs which are expressed in 75% of breast cancers, which when activated initiate downstream signalling thereby promoting expression of genes involved in cell proliferation and growth (Pal, Childs and Pegram, 2011; Williams and Lin, 2013). Targeted therapies for hormone-dependent breast cancers overexpressing ERs include aromatase inhibitors (AI)'s, such as Anastrozole, which can be taken orally or intravenously, depending on the severity, staging and patient preferences (Ishitobi *et al.*, 2013); and are known to marginally increase a patient's risk for thrombosis, therefore increasing associated levels of platelet activation and aggregation (Fennerty, 2006; Fabian, 2007; Gonnelli and Petrioli, 2008; Karimi and Cohan, 2010).

4.1.1. Hormone-dependent breast cancer and Anastrozole treatment

Hormone-dependent breast cancers, modelled by the MCF7 and T47D cell lines *in vitro*, are commonly treated using hormone-therapies such as non-steroidal AI's, which prevent breast cancer proliferation by reducing oestradiol levels (Mokbel, 2002; Carey *et al.*, 2006; Holliday and Speirs, 2011; Masoud and Pagès, 2017). Anastrozole is commonly used as second-line therapy in adjuvant treatment strategies in postmenopausal women (Mokbel, 2002; Campos, 2004; Kümler *et al.*, 2016). However, when used as first-line therapy for advanced breast cancers, AI's have proven to be as effective as Tamoxifen; and have further shown benefit in patients initially treated with Tamoxifen (Bonneterre *et al.*, 2000; Nabholz *et al.*, 2000; Mouridsen *et al.*, 2001; Smith and Dowsett, 2003). Furthermore, studies have proposed switching women currently on Tamoxifen treatment to Anastrozole or other AI's, to potentially pre-empt the development of Tamoxifen resistance, increase tolerability of the drug and possibly reduce thrombotic effects associated with Tamoxifen (Mokbel, 2002; Hillner, 2004; Francesco *et al.*, 2005; Howell *et al.*, 2005).

Clinical studies have shown that patients being treated with Anastrozole have a reduced risk of developing venous thromboembolisms, compared to the risk associated with cancer itself (Fennerty, 2006; Fabian, 2007; Gonnelli and Petrioli, 2008). *In vitro* studies have supported clinical studies and indicate the antiproliferative effects of Anastrozole on the MCF7 cell line (Chen *et al.*, 2015); however, upon further investigation, cytotoxic effects were noted at increasing concentrations of the drug over a 24-hour period (Hassan *et al.*, 2017). As Anastrozole is metabolized within 3 days of treatment, these cytotoxic effects pose a significant increase in thromboembolic risk (Hassan *et al.*, 2017), contradictory to earlier studies. Limited information exists on the effects of Anastrozole on the T47D cell line, with more information on Letrozole, a non-steroidal type II AI being available (Gupta *et al.*, 2013; Hole *et al.*, 2015). Letrozole, as opposed to Anastrozole, has been associated with a greater lipid profile leading to cardiovascular events; therefore, to remove potential effects on the haemostatic system, this study focused on Anastrozole (Bundred, 2005). Additionally, while studies have shown that AI treatment results in suppression of oestrogen synthesis, breast cancer cells themselves are able to increase local oestrogen production and in response may compensate overcome this suppression (Hole *et al.*, 2015). While the effects of Anastrozole as an adjuvant treatment strategy has been extensively studied compared to Tamoxifen; detailed assessment surrounding its' precise effects on ERs and hypercoagulation are not well defined.

4.1.2. Tumour progression and the role of thrombin and platelet activation

The progression of breast cancer from an early stage to that of metastasis involves a cascade of events during which breast cancer cells invade and travel within the bloodstream to distant sites, leading to an accumulation of breast cancer cells in non-breast tissues (Lal, Dittus and Holmes, 2013). This process not only involves the tumour microenvironment but the haemostatic system as well (Lal, Dittus and Holmes, 2013). Recent studies have indicated a role for platelets, the processes of coagulation and fibrinolysis as important mediators of breast cancer progression (Hanahan and Robert A. Weinberg, 2011; Lal, Dittus and Holmes, 2013; Gomes *et al.*, 2017).

It is well known that patients with breast cancer are at an increased risk for thrombosis, (Jurasz, Alonso-Escolano and Radomski, 2004; Kedzierska *et al.*, 2013; Gomes *et al.*, 2017).

Studies have noted an increase in circulating platelet count in patients diagnosed with breast cancer (Sierko and Wojtukiewicz, 2004; Trappenburg *et al.*, 2009). Breast cancer cells themselves are able to facilitate platelet activation and aggregation and the formation of a fibrin-rich network to form thrombi by either direct contact with platelets or by the release of mediators including ADP, thrombin, thromboxane A₂ (TXA₂) or tumour associated proteinases (Nash *et al.*, 2002; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2014); this capacity may potentially be increased by the anti-oestrogenic effects of hormone-therapy (Hassan *et al.*, 2017).

Thrombin induction of platelet activation and aggregation is facilitated through PAR1 and PAR4 expressed on the platelet membrane (Kreutz *et al.*, 2012; Nag and Bar-Shavit, 2018). Thrombin binding induces the coagulation cascade and is said to be the main effector in its induction (Lisman, Weeterings and de Groot, 2005; Chapin and Hajjar, 2015). In response to thrombin binding, platelets undergo the process of activation leading to shape changes, small-molecule and protein release and concomitant membrane alterations (Nash *et al.*, 2002; Zheng *et al.*, 2017). Clinically, β -thromboglobulin and P-selectin (CD62P) are increased in breast cancer patients indicating ongoing platelet activation in response to breast cancer itself (Nash *et al.*, 2002; Zheng *et al.*, 2017). Early in the activation process, CD62P translocates from the α -granule to the platelet membrane allowing it to be used as an identifying marker for activated platelets (McNicol and Israels, 2008; Augustine *et al.*, 2016); it functions by facilitating platelet adhesion and initial recruitment of leukocytes (McNicol and Israels, 2008). Lysosomal-associated membrane protein (CD63/LAMP3) translocates from the dense and lysosomal granules within the platelet to the membrane surface, later during the process of activation (Saboor, Moinuddin and Ilyas, 2013); and modulates platelet spreading and aggregation. Furthermore, during this process, platelets synthesize and release thromboxane (TXA₂), a potent pro-activation and pro-aggregatory, and vasoconstrictor mediator, which plays a role in the formation and stabilisation of thrombi, notwithstanding the release of other pro-coagulants including thrombin (Lisman, Weeterings and de Groot, 2005; Weisel and Litvinov, 2017; Zarà *et al.*, 2018). Platelets *in situ* are also able to influence tumour growth and metastasis, through mechanisms including the synthesis and release of TXA₂ and growth factors, including PDGF, to facilitate tumour cell intravasation into the bloodstream; the formation of platelet aggregates to surround tumour cells; enhancement of tumour cell

adhesion to the endothelium leading to tumour cell arrest and extravasation at secondary sites (McNicol and Israels, 2008; Schlesinger, 2018).

Breast cancer cells can induce platelet activation by releasing platelet-activating mediators, including ADP and thrombin, as well as tissue factor which in turn, leads to thrombin generation (Nash *et al.*, 2002; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2017; Reddel, Tan and Chen, 2019). Furthermore, this ability of breast cancer cells to induce platelet activation and further aggregation, known as tumour cell-induced platelet aggregation (TCIPA), correlates with metastatic potential (Jurasz, Alonso-Escalano and Radomski, 2004). Laboratory studies found that ADP released by MCF7 breast cancer cells induced platelet aggregates (Alonso-Escalano *et al.*, 2004); this induction could potentially be heightened in response to Anastrozoles cytotoxicity (Hassan *et al.*, 2017). Breast cancer cells are also able to produce coagulation factors including Factor V, VIII, IX and XI; leading to thrombin generation, platelet activation, aggregation and hypercoagulation (Nash *et al.*, 2002; Schlesinger, 2018). Clinically, this is noted in patients who present with heightened levels of markers of platelet activation and indicate that at least 1% of breast cancer patients develop VTE's within 2 years post-diagnosis (Caine *et al.*, 2002; Chaari *et al.*, 2012; Kyriazi, 2012; Bauer, 2019).

4.1.3. Breast Cancer Hormone-Therapy effects on platelets

Breast cancer itself is associated with hypercoagulability, with Anastrozole-treatment of both pre-metastatic and metastatic tumours is known to increase a patient's risk for thrombosis, reflecting increasing associated levels of platelet activation and aggregation (Karimi and Cohan, 2010). Anastrozole reduces the synthesis of oestrogen (Campos, 2004; Burstein *et al.*, 2019), which diminish hormone-induced proliferation (Mokbel, 2002; Onitilo *et al.*, 2012). However, while Anastrozole targets oestradiol induced proliferation through the conversion of the aromatase enzyme, the cytotoxic nature of the drug itself contributes to platelet activation; which is further induced through ERs and oestradiol associated with breast cancer (M Jayachandran and Miller, 2003; Kovac *et al.*, 2015).

The formation of breast cancer-associated thrombi is dependent on the interplay between platelets and tumour cells itself, thrombin-mediated responses, hormone-therapy and the

receptor profile of the cancer cells. As previously described in Chapters 2 and 3; this study has identified that hormone-therapies are able to differentially affect breast cancer subphenotypes and ER profiles, inducing levels of platelet activation comparable to that of the positive control. As shown in Chapter 3, the cumulative and circulating effects of Tamoxifen induced alternating ER profiles dependent on the cell line, as well as heightened induced levels of platelet activation by breast cancer cells. Thus, this Chapter expanded on the effects of cumulative and circulating hormone-therapy, specifically, Anastrozole, on ER protein expression and the relationship to hypercoagulation. This study aims to provide evidence for the pro-thrombotic effects of Anastrozole and co-effects on ERs, the data on which is lacking.

To reiterate, two models for assessment were employed; the first in which breast cancer cells were pre-treated with Anastrozole and co-incubated with blood constituents; whole blood (WB), platelet-rich plasma (PRP) and platelet-poor plasma (PPP) (Model 1); and a second in which blood constituents (WB, PRP, PPP) were treated with Anastrozole and co-incubated with untreated breast cancer cells (Model 2). Pre-treatment of breast cancer cells allows for mimicking of the cumulative effect of the drugs, while pre-treatment of blood constituents allows for mimicking of the circulatory effects of the drugs, as would occur *in vivo*.

This chapter presents in both models, the resulting effects on the protein expression of ER α and ER β . We further assess associated hypercoagulation represented by thrombin release and generation by breast cancer cells and ensuing effects on platelet activation progression identified by CD62P and CD63 expression, with corresponding platelet morphological alterations. As previously described by our laboratory, by generating an index of platelet activation (IPA), we describe a parameter that describes CD62P and D63 expression in direct relation to the number of platelets expressing the marker, as well as platelet activation progression by making use of a ratio of the two (Augustine *et al.*, 2016).

4.1.4 Aims and Objectives

The aim of this study was to determine the effect of cumulative (Model 1) and circulating (Model 2) Anastrozole effects on ER expression and their relationship to hypercoagulation *in vitro*. Thus, the objectives were:

- 4.1.4.1 To assess the interactions between platelets and MCF7 and T47D breast cancer cells pre-treated with Anastrozole, indicating the cumulative effects of the drug (Model 1).
- 4.1.4.2 To assess the interactions between platelets and MCF7 and T47D breast cancer cells exposed to Anastrozole-treated blood constituents, indicative of freely available drugs within the bloodstream (Model 2).

This was done by analysing:

- 4.1.4.3 Spontaneous thrombin release from breast cancer cells
- 4.1.4.4 Induced levels of platelet activation defined by CD62P and CD63 expression interpreted by the Index of Platelet Activation (IPA) and confirmed ultrastructurally.
- 4.1.4.5 ER α and ER β protein expression profiles in breast cancer cells
- 4.1.4.6 The correlation between ER protein alterations and spontaneous thrombin release from breast cancer cells, to determine whether ER expression profiles may predict endogenous thrombin availability.
- 4.1.4.7 The correlation between spontaneous thrombin release from breast cancer cells and the Index of Platelet Activation (IPA), to determine whether changes in endogenous thrombin associated with ER expressional alterations may hold predictive value in determining associated platelet activation.

4.2 Materials and Methods

Human Research Ethics Clearance was obtained from the Human Ethics Research Committee (Medical), University of the Witwatersrand, Clearance Certificate Number M160826 (Refer to Appendix for Clearance Certificate). All methods conducted for this Chapter were the same as Chapter 3; however, due to the variable nature of the results obtained from non-tumourigenic MCF10A cell line, it was not used as a control cell line in this study (Chapter 3, Section [3.3.1](#)). Furthermore, this Chapter presents results for Anastrozole.

4.2.1 Methodology Framework – in brief

To conduct the methodology for this aim MCF7 and T47D breast cancer cells were seeded at 1×10^5 cells/well onto glass coverslips. T47D cells were seeded onto glass coverslips coated

with poly-d-lysine to facilitate adhesion. For Model 1: MCF7 and T47D cells were subsequently pre-treated with 1 μ M Anastrozole for 24 hours. For Model 2: 18 ml of peripheral WB was obtained from volunteers and 4.5 mL treated with 1 μ M Anastrozole for 1 hour at RT. WB was then centrifuged and separated into its constituents: WB, PRP and PPP. For Model 1: untreated blood constituents were exposed to Anastrozole-treated cells, and for Model 2: Anastrozole-treated blood constituents were concurrently exposed to untreated cells; for 2.5 minutes. Following this co-incubation breast cancer cells and blood constituents were isolated and prepared for their ensuing techniques (Figure 4.1).

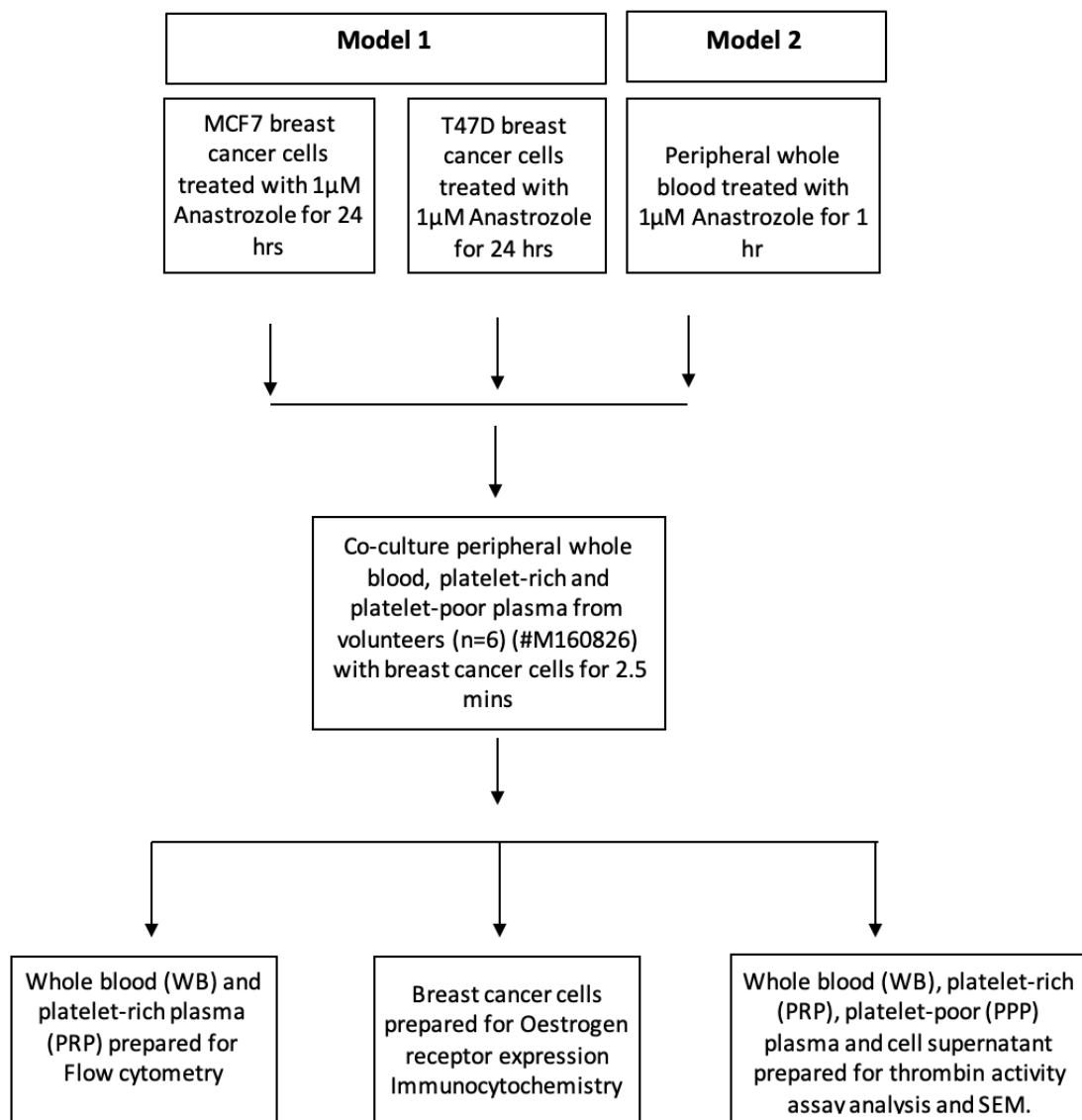


Figure 4.1: Flow diagram indicating the stepwise methodology followed for experimentation. MCF7 and T47D breast cancer cells were seeded at 1×10^5 cells/well onto glass coverslips. MCF7 and T47D cells were subsequently pre-treated with either $1 \mu\text{M}$ Anastrozole. Peripheral WB was obtained from volunteers 4.5 ml was treated with $1 \mu\text{M}$ Anastrozole for 1 hour at RT. WB was separated into its constituents: WB, PRP and PPP. Untreated blood constituents were exposed to Anastrozole- treated cells (Model 1), Anastrozole-treated blood constituents were concurrently exposed to untreated cells (Model 2), for 2.5 minutes. Following co-incubation breast cancer cells and blood constituents were isolated and prepared for their ensuing techniques. Blood constituents were prepared for flow cytometry and scanning electron microscopy to assess levels of platelet activation. Breast cancer cells were prepared for ICC for assessment of ERs, and supernatant including WB, PRP, PPP and cell supernatant were prepared for assessment of thrombin generation.

4.2.2 Cell Culture and Establishment of Stock Populations

MCF7 and T47D breast cancer cells were cultured with stock populations established as per Chapter 2, section [2.2.2](#).

4.2.3 Hormone-Therapy Treatment of Breast Cancer Cells

Cryopreserved cells were thawed, washed once in 1ml 1 M PBS (pH 7.4) followed by resuspension in 1ml of their respective cell culture media, with cell seeding following as per Chapter 2, section [2.2.3](#) paragraph 2. MCF7 cells were seeded directly onto glass coverslips in 24 well plates, while T47D cells were seeded onto glass coverslips coated with 5 mg/ml poly-d-lysine to facilitate attachment.

The maximum effective concentration of Anastrozole was determined as per Chapter 2, section [2.2.4](#). For Model 1, plated cells were rinsed with 1 M PBS and pre-treated with 200 μ l 1 μ M Anastrozole for 24 hours at 37°C and 5% CO₂.

4.2.4 Whole Blood Acquisition, Preparation and Co-culture

Peripheral WB (n=6) was extracted under the same conditions as Chapter 2, section [2.2.5](#). For establishment of Model 2, 4 mL of WB was removed from the sodium citrate vacuette coagulation tubes and treated with 1 μ M Anastrozole in a separate centrifuge tube for 1 hour at RT. The remaining WB was retained untreated for use in Model 1. Subsequently, WB was separated into its component parts; PRP and PPP.

4.2.5 Establishment of a co-culture system

Breast cancer cells were rinsed twice with 1 M PBS and incubated with 200 μ l WB, PRP or PPP for 2.5 minutes. For Model 1: Anastrozole-treated breast cancer cells were incubated with 200 μ l of WB, PRP and PPP for 2.5 minutes. For Model 2: Untreated breast cancer cells were incubated with 200 μ l Anastrozole-treated WB, PRP or PPP for 2.5 minutes. All samples, experimental and control, were prepared in singlet for each subsequent assay (SEM, flow cytometry, ICC and thrombin assessment) with 6 biological replicates (n = 6) (Table 4.1).

Table 4.1: Control and experimental samples, with the abbreviation indicating the sample key used in ensuing analysis. Samples are further grouped into Model 1: assessment of cumulative effects of Anastrozole. Prefix: M: MCF7 breast cancer cells. T: T47D breast cancer cells. Suffix: treatment options employed; and Model 2: assessment of circulating effects of Anastrozole. Prefix: pre-treated blood constituent Suffix: M - MCF7 breast cancer cells. T- T47D breast cancer cells.

Blood Constituent Controls	
Abbreviation	Explanation
WB	Whole Blood
WBThr	Positive control: Whole blood incubated with 0.1 U/mL human thrombin
PRP	Platelet-rich Plasma
PRPThr	Positive control: platelet-rich plasma incubated with 0.1 U/mL human thrombin
PPP	Platelet-poor Plasma
Cell Line Controls	
Abbreviation	Explanation
MMED	MCF7 breast cancer cell media control
MMEDWB	MCF7 breast cancer cells exposed to whole blood
MMEDPRP	MCF7 breast cancer cells exposed to platelet-rich plasma
MMEDPPP	MCF7 breast cancer cells exposed to platelet-poor plasma
MDIL	MCF7 breast cancer cells treated with 0.1% DMSO
MDILWB	MCF7 breast cancer cells treated with 0.1% DMSO exposed to whole blood
MDILPRP	MCF7 breast cancer cells treated with 0.1% DMSO exposed to platelet-rich plasma
MDILPPP	MCF7 breast cancer cells treated with 0.1% DMSO exposed to platelet-poor plasma
MANAS	MCF7 breast cancer cells treated with 1uM Anastrozole
TMED	T47D breast cancer cell media control
TMEDWB	T47D breast cancer cells exposed to whole blood
TMEDPRP	T47D breast cancer cells exposed to platelet-rich plasma
TMEDPPP	T47D breast cancer cells exposed to platelet-poor plasma
TDIL	T47D breast cancer cells treated with 0.1% DMSO
TDILWB	T47D breast cancer cells treated with 0.1% DMSO exposed to whole blood
TDILPRP	T47D breast cancer cells treated with 0.1% DMSO exposed to platelet-rich plasma
TDILPPP	T47D breast cancer cells treated with 0.1% DMSO exposed to platelet-poor plasma
TANAS	T47D breast cancer cells treated with 1uM Anastrozole
Model 1	
Abbreviation	Explanation
MANASWB	MCF7 breast cancer cells pre-treated with 1 uM Anastrozole exposed to whole blood
MANASPRP	MCF7 breast cancer cells pre-treated with 1 uM Anastrozole exposed to platelet-rich plasma
MANASPPP	MCF7 breast cancer cells pre-treated with 1 uM Anastrozole exposed to platelet-poor plasma
TANASWB	T47D breast cancer cells pre-treated with 1 uM Anastrozole exposed to whole blood
TANASPRP	T47D breast cancer cells pre-treated with 1 uM Anastrozole exposed to platelet-rich plasma
TANASPPP	T47D breast cancer cells pre-treated with 1 uM Anastrozole exposed to platelet-poor plasma
Model 2	
Additional Blood Constituent Controls	
Abbreviation	Explanation
WBANAS	Whole blood treated with 1 uM Anastrozole
PRPANAS	Platelet-rich plasma treated with 1 uM Anastrozole
PPPANAS	Platelet-poor plasma treated with 1 uM Anastrozole
Experimental	
Abbreviation	Explanation
WBANASM	Whole blood treated with 1 uM Anastrozole co-incubated with MCF7 breast cancer cells
PRPANASM	Platelet-rich plasma treated with 1 uM Anastrozole co-incubated with MCF7 breast cancer cells
PPPANASM	Platelet-poor plasma treated with 1 uM Anastrozole co-incubated with MCF7 breast cancer cells
WBANAST	Whole blood treated with 1 uM Anastrozole co-incubated with T47D breast cancer cells
PRPANAST	Platelet-rich plasma treated with 1 uM Anastrozole co-incubated with T47D breast cancer cells
PPPANAST	Platelet-poor plasma treated with 1 uM Anastrozole co-incubated with T47D breast cancer cells

4.2.6 Thrombin Activity Assay

The release of thrombin as a procoagulant factor during co-culture was tested using a Thrombin Activity Assay Kit as per Chapter 3; section [3.2.6](#).

4.2.7 Assessment of platelet activation

WB samples were prepared as per Chapter 2, section [2.2.8](#). PRP samples were prepared as per Chapter 3, section [3.2.7.1](#). With gating, acquisition and analysis conducted as per Chapter 3, section [3.2.7.2](#) and section [3.2.7.3](#), respectively.

4.2.7.1 Statistical Analysis

Data acquired using FACSDiva software was exported into Microsoft Excel and analysed using Statistica v23 statistical software.

The index of platelet activation (IPA) was calculated such that:

Early Activation: $IPA = gMFI (CD62P) \times n(CD41a^+CD62P^+ \text{ events})$

Late Activation: $IPA = gMFI (CD63) \times n(CD41a^+CD63^+ \text{ events})$

With progression of platelet activation identified as a ratio, such that;

Activation Progression: $IPA =$

$$\frac{gMFI (CD62P) \times n(CD41a^+CD62P^+ \text{ events})}{gMFI (CD63) \times n(CD41a^+CD63^+ \text{ events})}$$

A Shapiro-Wilk test of normality determined that the data was not normally distributed. Additionally, considering the small sample size, the non-parametric Friedman test, based on the dependency of blood constituents, was conducted to determine whether IPA altered in response to hormone-therapy and blood constituent co-incubation, following which a *post-hoc* Wilcoxon Matched Paired test was conducted to determine whether these differences were significant ($p < 0.05$). By making use of a Spearman's Rank Order Correlation, IPA from

matched samples was correlated with thrombin detection.

4.2.7.2 Scanning Electron Microscopy (SEM)

WB samples were prepared as Chapter 2, section [2.2.8.4](#). PRP and PPP samples were removed from their corresponding wells and 20µl placed onto glass coverslips. All samples then underwent preparation and viewing as per 2.2.8.4.

4.2.8 Immunolocalisation of ER α and ER β protein expression in hormone-dependent breast cancer cells.

Images presented in the results are overlays of ER α (green) and ER β (red) protein expression where yellow indicates domination by ER α and orange indicates domination by ER β ; DAPI staining is not shown as it obscured nuclear localisation of ERs. All images; which included individual sample images of ER α (green) and ER β (red) and DAPI (blue) are presented in the Appendix. All samples were prepared, acquired and analysed as per Chapter 3, section [3.2.8](#).

4.3 Results

For this section, Anastrozole results are presented. Please note that for the methodological procedure all samples including Tamoxifen treatment, Anastrozole treatment and controls were conducted simultaneously. For conceptual presentation, Tamoxifen was presented in Chapter 3 and Anastrozole in Chapter 4; however, the controls as presented in Chapter 3, also apply to Chapter 4. As such, the reader will be referred to control Figures from Chapter 3, and only a summary is provided below.

4.3.1 Baseline levels of platelet activation in blood constituent controls

Previously presented results (Chapter 3), indicated baseline levels of thrombin availability and platelet activation in blood constituent controls; untreated WB, PRP and PPP. These levels indicate the standard detectable levels of thrombin and platelet activity, evident without positive induction of activation; and allow for comparison through deviation from these standard levels.

4.3.1.1 *Thrombin Availability in untreated blood constituent controls*

Thrombin activity assessment of blood constituent control samples indicated baseline levels of thrombin concentration availability in WB at 41.7ng/mL (Figure 4.2), with PRP indicating significantly ($p < 0.05$) greater levels of availability at 47.7ng/mL; corresponding with levels of platelet activation (Table 4.3; PRP Overall Induction (I1-I5)). PPP had a slightly decreased level of thrombin availability, at 40.4 ng/mL, as expected. For the positive controls, the addition of exogenous thrombin to WB increased thrombin availability to 41.9ng/mL ($p > 0.05$); however the addition of exogenous thrombin to PRP induced significantly heightened levels of thrombin availability, much higher than the detectable levels of the assay itself, resulting in a negative value of -45.8ng/mL (Weibrich *et al.*, 2004; Everts *et al.*, 2006). Exogenous thrombin, however, was not added to PPP, as this component does not contain many platelets. Additionally, the centrifugation would have activated the platelets fully allowing for the release of granular contents (Ghassab *et al.*, 2015).

4.3.1.2 *Platelet activation in blood constituent controls*

Overall IPA and Platelet Ultrastructure

Overall IPA represents levels of platelet activation from interval gates 1 to 5 (I1 – I5) in the positive quadrant ((Q2) Chapter 3; Figure 3.2 – Figure 3.4). Baseline levels of platelet

activation were determined by assessing IPA in the untreated control samples (Table 4.2). Both early stage (CD62P IPA) and late stage (CD63 IPA) were low in the untreated WB control, while concentrated PRP (Table 4.3) showed a significant increase in IPA compared to WB. As expected with reference to detection of microparticles, PPP showed the lowest levels of IPA in early stages compared to the WB control. The ratio of activation markers revealed more dominant early stage activation in untreated WB. Corresponding platelet ultrastructural assessment of lysed WB (Figure 4.3A) showed inactive platelets, with a smooth membrane appearance and few surface folds. Untreated PRP (Figure 4.3C) showed platelets displaying early stages of activation, with extending filipodia and a smoother membrane appearance; with some platelets displaying the early stages of hyalomere spread and evidence of platelet aggregation. PPP (Figure 4.3E), presented with crenated red blood cells noted with platelet remnants and microparticles evident. These microparticles are postulated to be expressing platelet activation markers and as such is associated with the loss of marker expression noted (Table 4.3).

The addition of thrombin to the positive controls heightened IPA of both CD62P and CD63. Thrombin added to WB only induced slightly higher CD62P overall IPA, while a significant induction was noted in CD63 IPA (Table 4.2). Comparatively, the addition of thrombin to PRP significantly reduced CD62P IPA, which may be attributed to greater platelet aggregation, and significantly increased CD63 IPA, when compared to untreated PRP (Table 4.3). Ultrastructurally, thrombin-induced greater levels of activation with platelets in WB showing greater membrane folds and extending filipodia from the platelet body, with the presence of few microparticles (Figure 4.3B). The addition of thrombin to PRP (Figure 4.3D), induced greater levels of platelet aggregation showing greater microparticle release and fibrin formation. Platelets also displayed a smoother membrane appearance and hyalomere spread. Platelets at this heightened level of expression lost CD62P; however, CD63 persisted (Table 4.3).

IPA per interval

IPA per interval gate was assessed to determine the spread of marker expression throughout the interval gates. Untreated WB indicated high IPA in the CD62P^{low} interval gate (I1 and I2); decreasing further throughout the interval gates. CD63 IPA displayed similar expression spread; however, this was much higher than that of CD62P (Table 4.2). Untreated PRP showed

higher IPA levels of both CD62P and CD63 throughout all interval gates (Table 4.3), compared to the untreated WB control. PPP, however, indicated very low marker expression throughout the interval gates, as expected (Table 4.3). The addition of thrombin in the positive controls, induced a greater spread of expression of markers through the interval gates (Table 4.2 & 4.3), dependent on blood constituent.

4.3.1.3 Thrombin availability following treatment of blood constituents with Anastrozole (Model 2 controls)

On assessment of Model 2, which describes the effects of circulating Anastrozole, it was noted that Anastrozole-treatment of WB induced levels of thrombin availability comparable to that of untreated WB (41.66 ng/mL), at 41.61 ng/mL (Figure 4.2). Significantly reduced levels of available thrombin are noted within the Anastrozole-treated PRP and PPP samples; 41.06 ng/mL and 41.30 ng/mL respectively, compared to untreated WB. This was also noted to be lower than that of the positive, WB thrombin control. In comparison to matched untreated samples, Anastrozole pre-treatment of PRP significantly reduced levels of available thrombin, from 47.66 ng/mL to 41.06 ng/mL; while pre-treatment of PPP significantly increased available thrombin levels from 40.43 ng/mL to 41.30 ng/mL. Anastrozole-treated PRP significantly heightened levels of thrombin availability compared to the positive, PRP thrombin control; however, given that this was much higher than the detectable levels of the assay itself, Anastrozole-treatment of PRP would thus have reduced levels of detectable thrombin comparatively.

4.3.1.4 Platelet activation following Anastrozole-treatment of blood constituents (Model 2)

Overall IPA

Assessment of circulating Anastrozole effects on early and late stage platelet activation (Table 4.2 & 4.3), indicated pre-treatment of WB induced heightened levels of IPA of both CD62P and CD63, compared to that of untreated WB; although this was not significant. On assessment of ultrastructural alterations (Figure 4.3), platelets from WB treated with Anastrozole showed fibrin plaque formation, contrasting with untreated WB (Figure 4.3F), in which platelets were inactive and had a smooth membrane surface. The Anastrozole-treated PRP sample (Table 4.3), indicated significantly higher IPAs of both markers, compared to the WB control (Table 4.2). However, when compared to the PRP control; a significant decrease was noted in CD62P IPA while a significant increase was noted in the IPA of CD63; similar to

that of thrombin control. This indicates progression to a later stage of activation indicated by the significant decrease noted by the ratio of activation markers (Table 4.3). This progression was not as evident in the Anastrozole-treated WB sample, with only a slight decrease indicative of most platelets remaining at an earlier stage of activation. Ultrastructural analysis of Anastrozole-treated PRP samples (Figure 4.3G) showed platelets with extending filipodia with the presence of microparticles. The formation of fibrin plaques was heightened in Anastrozole-treated PPP samples (Figure 4.3H), and many pores can be seen.

IPA per interval gate

Anastrozole treatment of WB elicited significantly higher IPA in the CD62P^{low} and CD63^{low} interval gates (I1 and I2), compared to untreated WB (Table 4.2). However, only a slight increase in the CD62P^{medium} and CD63^{medium} interval gate (I3) is noted. IPA within the CD62P^{high} and CD63^{high} interval gates (I4 and I5) was higher than untreated WB; however, this was not significant.

Anastrozole-treated PRP showed significantly heightened IPA in CD62P^{low} and CD63^{low} intervals compared to untreated PRP (Table 4.3). CD62P^{medium} and CD63^{medium} interval gate showed a reduction in IPA, significantly so regarding CD62P. CD62P^{high} and CD63^{high} interval gates showed a further reduction in IPA of CD62P with only a slight increase in CD63. This reduction of CD62P in both CD62P^{medium} and CD62P^{high} intervals could be due to the loss of CD62P following full activation of platelets and fibrin formation obscuring platelets, as seen ultrastructurally (Figure 4.3G); however, the presence of microparticles could account for detection of the marker.

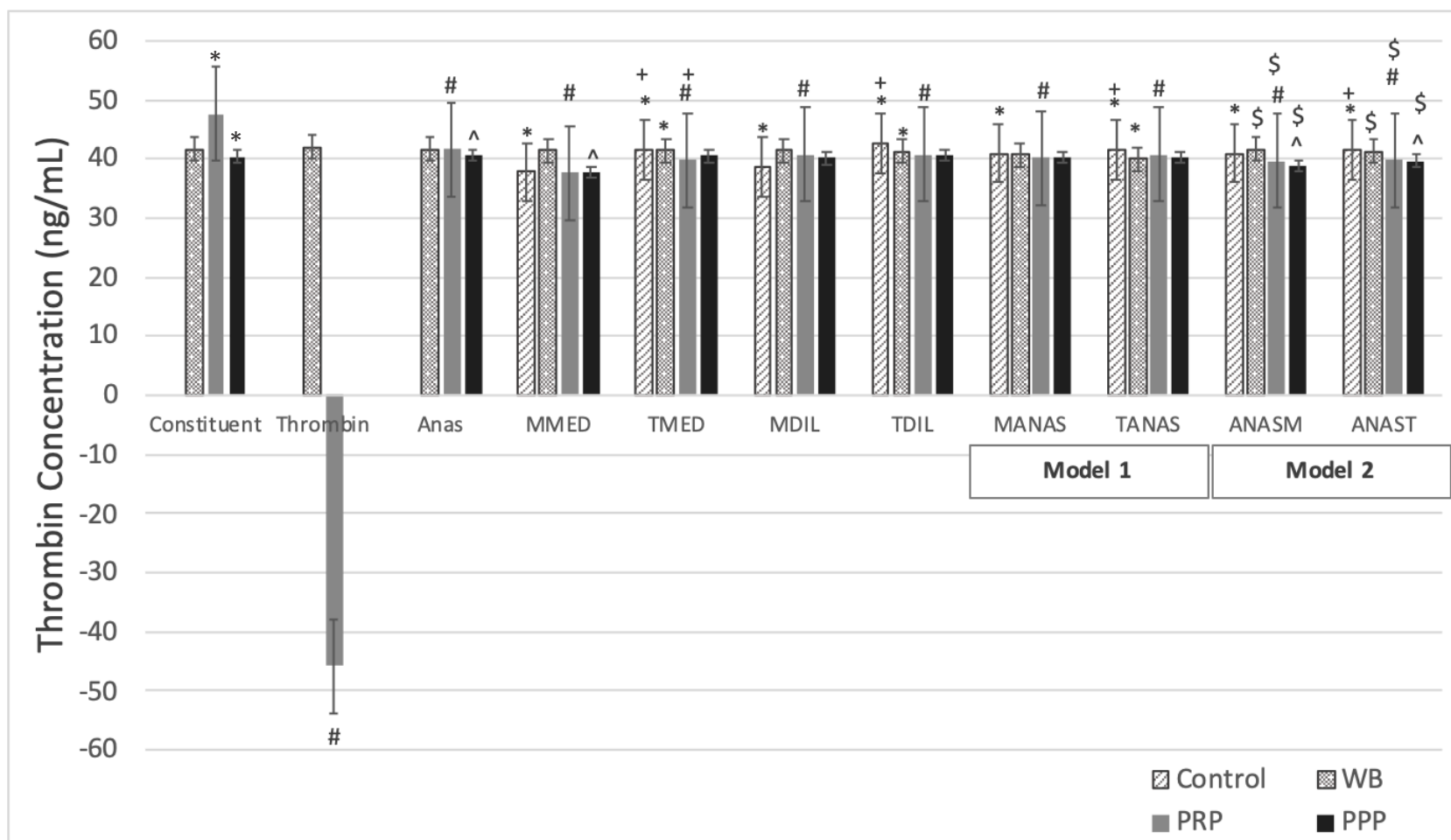


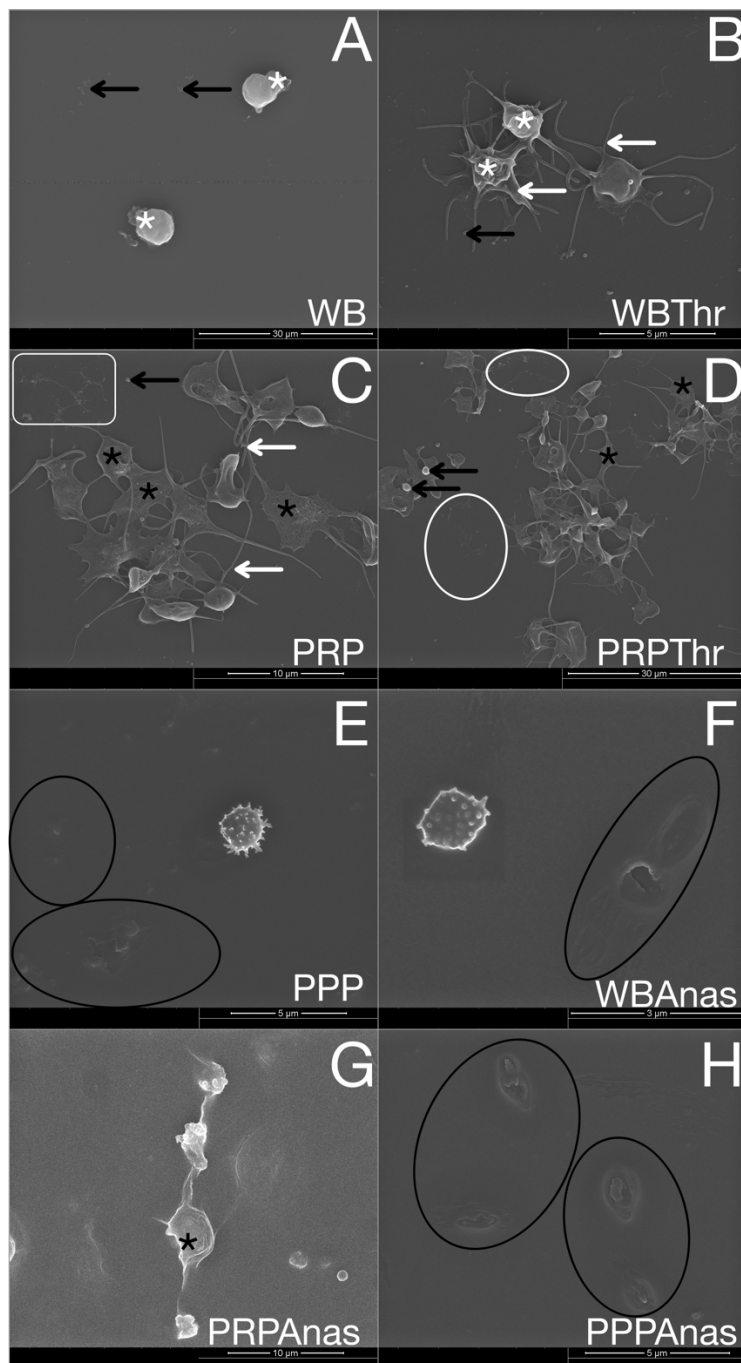
Figure 4.2: Bar-graph showing thrombin generation in MCF7 and T47D cells co-incubated with blood constituent samples, treated with cumulative (Model 1) or circulatory Anastrozole-therapies (Model 2). Constituent: blood constituent controls; WB, PRP and PPP. Thrombin: positive controls, with WB and PRP incubated with 0.1 U/mL of thrombin. M/T MED: Conditioned media control of MCF7/T47D cells. M/T DIL: MCF7/T47D cells treated with 0.1% DMSO, denoting the diluent control. ANAS: Anastrozole-treatment of blood constituents. M/T ANAS: Anastrozole-treated MCF7/T47D cells. ANAS M/T: Anastrozole-treated blood constituents exposed to MCF7/T47D cells. $p < 0.05$ indicated * compared to untreated WB; # compared to untreated PRP; ^ compared to untreated PPP; + compared to matched cell line; \$ between Models.

Table 4.2: Index of platelet activation markers CD62P and CD63, as well platelet progression (CD62P/CD63) of Anastrozole-treatment of MCF7 and T47D cells exposed to WB (Model 1) and Anastrozole treatment of WB exposed to MCF7 and T47D cells (Model 2). Overall levels (Q2) and per interval gate levels; CD62P^{low} and CD63^{low} (I1 and I2), CD62P^{medium} and CD63^{medium} (I3), CD62P^{high} and CD63^{high} (I4 and I5) were assessed. Key: WB – untreated whole blood; WBThr: positive control, whole blood incubated with 0.1 U/ml of thrombin. WBANAS: Anastrozole-treated WB. MMEDWB: WB exposed to MCF7 cells. MDILWB: WB exposed to MCF7 cells treated with 0.1% DMSO, denoting the diluent control. MANASWB: WB exposed to Anastrozole-treated MCF7. WBANASM: Anastrozole-treated WB exposed to MCF7. TMEDWB: WB exposed to T47D cells. TDILWB: WB exposed to T47D cells treated with 0.1% DMSO, denoting the diluent control. TANASWB: WB exposed to Anastrozole-treated T47D. WBANAST: Anastrozole-treated WB exposed to T47D. Bold indicates samples which were significantly ($p < 0.05$) different from untreated WB and (*) indicates samples significantly different to matched media cell lines (MMEDWB or TMEDWB). Italics refers to samples significantly different between cell lines

Marker of Activation	Controls							Model 1		Model 2	
	WB	WBThr	WBANas	MMEDWB	TMEDWB	MDILWB	TDILWB	MANASWB	TANASWB	WBANASM	WBANAST
Q2											
CD62P	3,93E+05	4,99E+05	5,10E+05	6,33E+05	<i>4,31E+05</i>	5,48E+05	3,63E+05	6,18E+05	6,87E+05*	8,85E+05*	<i>4,52E+05</i>
CD63	2,92E+06	3,35E+06	2,94E+06	3,65E+06	4,08E+06	3,15E+06	2,80E+06	3,64E+06	4,14E+06	4,20E+06*	<i>2,78E+06*</i>
Ratio (CD62P/CD63)	1,35E-01	1,49E-01	1,74E-01	1,74E-01	1,06E-01	1,74E-01	1,30E-01	1,70E-01	1,66E-01	2,11E-01	1,63E-01
I1											
CD62P	5,71E+04	4,78E+04	1,12E+05	1,67E+05	1,02E+05	1,19E+05	1,16E+05	1,80E+05	1,48E+05	1,74E+05	1,71E+05
CD63	1,78E+05	2,80E+05	5,17E+05	6,59E+05	4,53E+05	4,58E+05	4,38E+05	7,76E+05	5,01E+05	6,18E+05	5,26E+05
I2											
CD62P	5,71E+04	2,28E+05	2,79E+05	3,41E+05	<i>1,74E+05</i>	2,93E+05	1,73E+05	3,59E+05	3,94E+05*	3,89E+05	2,68E+05
CD63	7,23E+05	1,07E+06	1,55E+06	1,70E+06	1,17E+06	1,09E+06	1,01E+06	1,85E+06	1,50E+06	1,75E+06	1,16E+06
I3											
CD62P	1,69E+05	1,87E+05	1,95E+05	2,40E+05	2,23E+05	2,15E+05	1,44E+05	2,32E+05	1,48E+05	5,14E+05*	1,52E+05
CD63	1,22E+06	2,40E+06	1,35E+06	2,32E+06	2,02E+06	1,80E+06	1,24E+06	2,28E+06	2,46E+06	2,17E+06	1,57E+06
I4											
CD62P	3,52E+04	2,30E+05	2,66E+04	3,59E+04	5,79E+04	4,59E+04	<i>1,97E+04</i>	1,16E+04*	2,67E+04	4,60E+04	1,69E+04
CD63	1,07E+06	1,22E+06	4,66E+05	7,76E+05	<i>1,25E+06</i>	6,61E+05	5,58E+05	7,25E+05	7,26E+05*	4,49E+05*	3,15E+05*
I5											
CD62P	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00
CD63	0,00E+00	0,00E+00	0,00E+00	4,27E+04	4,26E+04	0,00E+00	5,97E+04	5,16E+04	1,39E+05*	2,08E+04*	2,03E+04

Table 4.3: Index of platelet activation markers CD62P and CD63, as well platelet progression (CD62P/CD63) of Anastrozole treatment of MCF7 and T47D cells exposed to PRP (Model 1) and Anastrozole treatment of PRP exposed to MCF7 and T47D cells (Model 2). Overall levels (Q2) and per interval gate levels; CD62P^{low} and CD63^{low} (I1 and I2), CD62P^{medium} and CD63^{medium} (I3), CD62P^{high} and CD63^{high} (I4 and I5) were assessed. Key: PRP – untreated Platelet-rich plasma; PRPThr: positive control, platelet-rich plasma incubated with 0.1 U/ml of thrombin. PRPANAS – Anastrozole-treated PRP. MMEDPRP: PRP exposed to MCF7 cells. MDILPRP: PRP exposed to MCF7 cells treated with 0.1% DMSO, denoting the diluent control. MANASPRP: PRP exposed to Anastrozole-treated MCF7. PRPANASM: Anastrozole-treated PRP exposed to MCF7. TMEDPRP: PRP exposed to T47D cells. TDILPRP: PRP exposed to T47D cells treated with 0.1% DMSO, denoting the diluent control. TANASPRP: PRP exposed to Anastrozole-treated T47D. PRPANAST: Anastrozole-treated PRP exposed to T47D. Bold indicates samples which were significantly ($p < 0.05$) different from untreated PRP and (*) indicates samples significantly different to matched media cell lines (MMEDPRP or TMEDPRP). Italics refers to samples significantly different between cell lines.

Marker of Activation	Controls								Model 1		Model 2	
	PRP	PRPThr	PRPANas	PPP	MMEDPRP	TMEDPRP	MDILPRP	TDILPRP	MANASPRP	TANASPRP	PRPANASM	PRPANAST
Q2												
CD62P	1,35E+06	7,60E+05*	9,56E+05	1,44E+05	1,20E+06	1,20E+06	7,07E+05*	<i>1,76E+06</i>	1,09E+06	1,55E+06	9,16E+05*	<i>1,13E+06</i>
CD63	6,99E+06	8,41E+06*	1,23E+07	5,35E+06	1,25E+07	8,39E+06	7,93E+06*	1,14E+07	8,49E+06*	1,34E+07*	7,13E+06*	1,04E+07*
Ratio (CD62P/CD63)	1,93E-01	9,04E-02*	7,78E-02	2,69E-02	9,62E-02	1,43E-01	8,92E-02	<i>1,54E-01</i>	1,28E-01*	1,15E-01	1,28E-01*	1,08E-01
I1												
CD62P	3,20E+05	2,27E+05	7,27E+05	2,37E+05	4,94E+05	4,14E+05	3,06E+05	4,46E+05	3,23E+05	<i>5,09E+05</i>	5,36E+05	6,98E+05*
CD63	1,16E+06	1,10E+06	4,50E+06	3,19E+06	1,47E+06	1,49E+06	9,75E+05*	1,70E+06	1,31E+06	1,84E+06	2,55E+06	3,53E+06*
I2												
CD62P	4,49E+05	3,79E+05	2,66E+05	1,07E+05	8,43E+05	8,82E+05	4,94E+05*	1,24E+06	6,20E+05	1,09E+06*	3,33E+05*	5,50E+05
CD63	4,62E+06	3,38E+06	6,52E+06	2,19E+06	8,34E+06	5,30E+06	5,32E+06	5,57E+06	6,03E+06	7,82E+06*	4,13E+06*	6,00E+06
I3												
CD62P	4,67E+04	1,66E+05	3,17E+04	1,30E+04	1,77E+05	1,16E+05	4,68E+04	1,44E+05	4,52E+04	8,05E+04*	2,75E+04*	4,58E+04*
CD63	2,90E+06	4,65E+06	1,50E+06	8,28E+05	5,59E+06	3,85E+06	4,26E+06	6,37E+06	3,48E+06	6,20E+06*	1,67E+06*	2,60E+06
I4												
CD62P	7,93E+03	9,29E+03	1,75E+03	0,00E+00	4,91E+03	5,06E+03	0,00E+00	0,00E+00	0,00E+00	0,00E+00	3,73E+03	0,00E+00
CD63	1,20E+05	1,02E+06*	1,46E+05	1,11E+05	2,39E+05	1,06E+05	1,47E+05	2,88E+05	1,05E+05	1,77E+05	1,86E+05	3,97E+05*
I5												
CD62P	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00	0,00E+00
CD63	0,00E+00	3,57E+04*	0,00E+00	0,00E+00	4,02E+04	2,09E+04	0,00E+00	0,00E+00	0,00E+00	0,00E+00	2,08E+04	0,00E+00



Electron Micrographs A, B, C, D and E are from Figure 3.8 and are repeated here for reference purposes.

Figure 4.3: Control Blood Constituent Samples. Platelet ultrastructural alterations. A: lysed WB showing inactive platelets, with a smooth membrane (*) and few surface folds. Note microparticles (black arrow). B: Positive control, addition of thrombin at 0.1U/ml to WB, showed platelets with greater membrane folds (*) and extending filipodia (white arrows) from the platelet body. Note few microparticles (black arrows). C: PRP showed platelets with hyalomere spread and extending filipodia and lamellipodia (white arrows). Note the presence of pores (*) and fibrin (white circle). D: Positive control; addition of thrombin to PRP induced platelet aggregation with platelets displaying hyalomere spread (*), filipodia extension (white arrow). Note microparticles (black arrow) and fibrin formation (white circle). E: untreated PPP showed crenated red blood cells, remnants from fully spread platelets and fibrin clots (black circle). F: Anastrozole treated WB samples showing fibrin plaque formation (black circle). G: Anastrozole-treated PRP sample showing platelets (*) and the presence of microparticles (black circle).

4.3.2 Breast cancer cell induced hypercoagulation

4.3.2.1 Breast Cancer Cell Line Thrombin Generation

Thrombin can be generated and secreted by breast cancer cells themselves. Control, untreated MCF7 cells (Figure 4.2), secreted thrombin at 37.77 ng/mL, significantly lower than that detected in the WB control (41.66 ng/mL) and the positive thrombin control (41.91 ng/mL). T47D breast cancer cells secreted levels of thrombin slightly lower and comparable to that of the WB control at 41.61 ng/mL. These secreted levels were also noted to be lower than that of the PRP control sample; significantly so from that of MCF7 cells. It was thus noted that the T47D cell line was able to induce thrombin generation significantly greater than that of MCF7 cells, correlating with Chapter 3 results indicating the hypercoagulatory nature of the T47D cell line. Establishing this level of thrombin secreted by breast cancer cells allowed for assessment of thrombin when blood constituents were then exposed to breast cancer cells (Figure 4.2).

4.3.2.2 Thrombin Generation by Breast Cancer Cells altered by exposure to blood constituents

MCF7 cells on exposure to WB increased the level of detectable thrombin significantly to 41.48 ng/mL when compared to untreated MCF7 cells. However, when PRP and PPP were exposed to MCF7 breast cancer cells, slight decreases in detectable thrombin levels were noted, with PRP exposure at 37.59 ng/mL and PPP exposure at 37.72 ng/mL when compared to the MCF7 control; which was significantly lower than that of untreated PRP and PPP, respectively. In WB exposed to T47D breast cancer cells, detectable thrombin levels were lower than that secreted levels by the cells themselves (41.61 ng/mL), as well as lower than their matched blood constituent controls. Control, diluent samples (0.1% DMSO) were assessed in comparison to their matched untreated cellular controls; no significant differences were noted and as such hormone-therapy treated samples were compared to their matched untreated cell lines. Furthermore, the exposure of PRP (39.82 ng/mL) and PPP (40.44 ng/mL) to T47D cells significantly heightened levels of thrombin generation compared to PRP (37.58 ng/mL) and PPP (37.72 ng/mL) exposed to MCF7 cells; further indicating the hypercoagulatory nature of the T47D cell line (Figure 4.2).

4.3.2.3 Breast Cancer Cell Line Induced Platelet activation

Baseline levels of platelet activation were determined by assessing IPA in the untreated control samples of WB and PRP. Both early stage (CD62P IPA) and late stage (CD63 IPA) assessment showed low IPA in the negative WB control, while concentrated PRP showed a significant increase in IPA compared to WB (Table 4.2 & 4.3).

Overall IPA and Platelet Ultrastructure

MCF7 breast cancer cells induced heightened levels of activation in platelets from WB, compared to that of both the untreated WB control, as well as the positive control (Table 4.2). CD62P IPA, was significantly greater than that of the control, while CD63 IPA was only slightly higher. The ratio of activation markers was only slightly lower than that of the WB control, indicating the majority of platelets remained at earlier stages of activation. This was confirmed ultrastructurally, with platelets displaying a smooth membrane with many filipodia extensions from the platelet body (Figure 3.9(1)A). A few microparticles were evident extracellularly. Comparatively, diluent-treated MCF7 cells induced a similar response, with IPA remaining at comparable levels to that of untreated MCF7 cells. Ultrastructurally, platelets were aggregated with fibrin present (Figure 3.9(1)B).

PRP exposed to MCF7 cells (Table 4.3) induced an increase in both CD62P IPA and CD63 IPA ($p < 0.05$), with a significant decrease in the ratio of activation markers compared to untreated PRP, indicating a shift to later stages of activation. Ultrastructurally, platelets exhibited hyalomere spreading and extending filipodia (Figure 3.9(1)C), with evidence of aggregation. While lower levels of platelet activation and progression are noted in PRP samples exposed to diluent treated MCF7 cells, compared to MCF7 cells alone. Ultrastructurally, platelets exhibited a folded membrane and microparticles were evident (Figure 3.9.4(1)D).

Induced levels of platelet activation in WB samples exposed to untreated T47D cells was higher than that of untreated WB, as well as the positive control (Table 4.3). Platelet progression was also noted to be only slightly lower than that of the WB control, indicating activation remaining at earlier stages. Ultrastructurally, platelets from WB exposed to T47D cells (Figure 3.10(1)A) displayed a smooth membrane with many lamellipodia and filipodia

extensions from the platelet body, with the presence of microparticles; as opposed to untreated WB which showed platelets with only a few membrane folds and no extensions.

PRP exposed to T47D cells (Table 4.3) showed a decrease in CD62P IPA and a significant increase in CD63 IPA, with a slight decrease in levels of platelet progression compared to untreated PRP. This was apparent in ultrastructural assessment, with platelets from PRP exposed to T47D cells (Figure 3.10(1)C) exhibiting a folded membrane surface and hyalomere spread and extending filipodia, indicating a later stage of platelet activation. It was further noted that the T47D cell line exposed to WB induced ($p>0.05$) a slight reduction in CD62P IPA and an increase in CD63 IPA compared to that induced by MCF7 cells. This phenomenon was noted in PRP samples as well (Table 4.2 and 4.3). Ultrastructural assessment indicated heightened levels of activation from WB and PRP exposed to T47D cells (Figure 3.10(1) A & C), with platelets having a more spread appearance than that from samples exposed to MCF7 cells.

IPA per interval gate

Platelet activation levels in the CD62P^{low} interval gates (I1 and I2) indicated an IPA significantly higher in WB samples exposed to MCF7 cells when compared to that of the untreated control. CD63 IPA, however, was noted to be lower ($p>0.05$) in the CD63^{low} interval gates (Table 4.2). However, on assessment of both CD62P and CD63 medium (I3) and high (I4 and I5) interval gates, WB exposed to MCF7 cells showed an increase in both CD62P and CD63 IPA. Conversely, PRP exposed to MCF7 cells in the CD62P^{high} interval gate indicated a reduction in CD62P IPA, with a significant increase in CD63 IPA in the CD63^{high} interval gates.

T47D breast cancer cells exposed to WB induced platelet activation levels in CD62P^{low} and CD63^{low} interval gates (I1 and I2) higher than that of untreated control WB; with CD63 IPA indicating a significant increase (Table 4.2). In CD62P^{high} and CD63^{high} interval gates, WB exposed to T47D cells induced an increase in both CD62P and CD63 IPA with I5, indicating a significant increase in CD63 IPA when compared to the untreated WB. These results are further heightened in PRP samples exposed to T47D cells (Table 4.3).

Figure 3.9 is repeated here for reference purposes

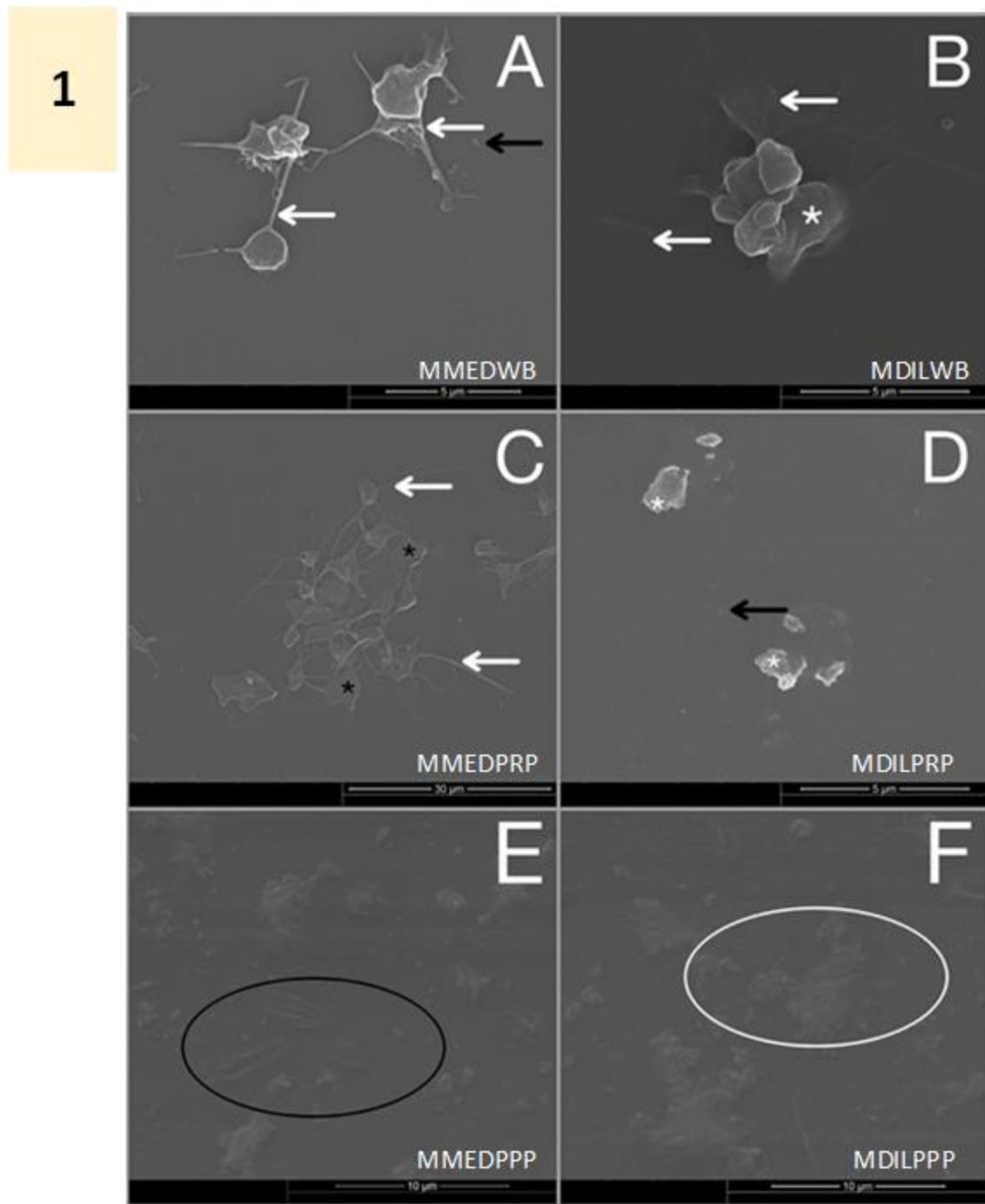


Figure 4.9: **Effects of blood constituent incubation with MCF7 cells - controls.** Key: MMED- media-treated MCF7 cells; MMEDWB: WB exposed to media-treated MCF-7 cells; MMEDPRP – PRP exposed to media-treated MCF-7 cells; MMEDPPP – PPP exposed to media-treated MCF-7 cells; MDIL- diluent-treated MCF7 cells; MDILWB: WB exposed to diluent-treated MCF-7 cells; MDILPRP – PRP exposed to diluent-treated MCF-7 cells; MDILPPP – PPP exposed to diluent-treated MCF-7 cells. **(1)** Platelet ultrastructural alterations. Key: white * - membrane folds, black * - hyalomere spread, white arrow - extending filopodia, black arrow – microparticles, white circle -fibrin, black circle - fibrin pores A: MMEDWB - active platelets, smooth membrane, extending filopodia and microparticles. B: MDILWB – aggregated platelets with fibrin. C: MMEDPRP – spread platelets and filopodia. D: MDILPRP – platelets with membrane folds and microparticles. E: MMEDPPP and F: MDILPPP - remnants of platelets and fibrinogen deposits.

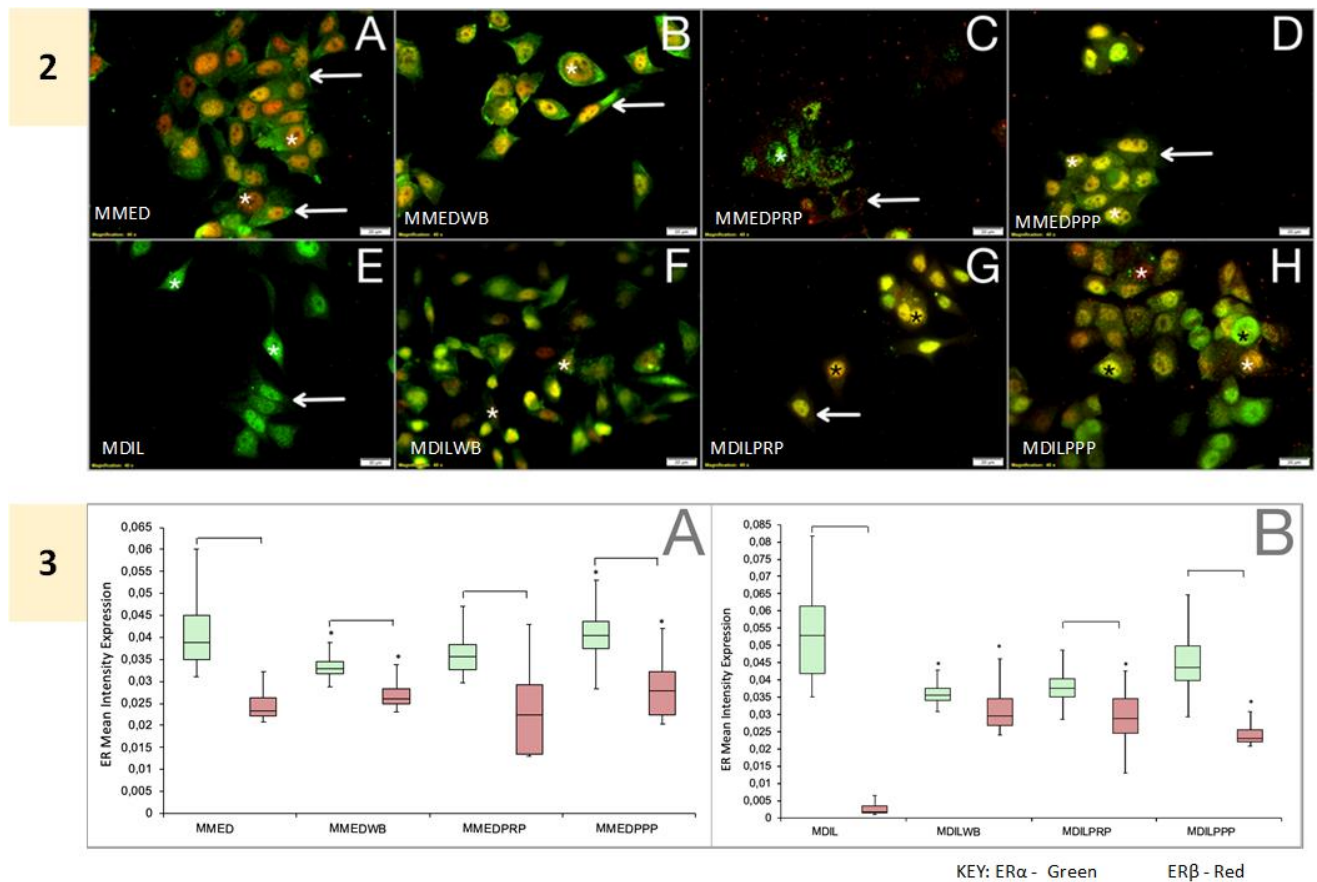


Figure 4.9: Effects of blood constituent incubation with MCF7 cells - controls. *Key:* MMED- media-treated MCF7 cells; MMEDWB: WB exposed to media-treated MCF-7 cells; MMEDPRP – PRP exposed to media-treated MCF-7 cells; MMEDPPP – PPP exposed to media-treated MCF-7 cells; MDIL- diluent-treated MCF7 cells; MDILWB: WB exposed to diluent-treated MCF-7 cells; MDILPRP – PRP exposed to diluent-treated MCF-7 cells; MDILPPP – PPP exposed to diluent-treated MCF-7 cells. **(2)** Co-localisation of ER α (green) and ER β (red) in MCF7 cells, following exposure to blood constituents. Yellow - ER α dominating; Orange - ER β dominating A: MMED – cytoplasmic (white arrow) ER α and nuclear (*) ER β . B: MMEDWB - cellular processes extend, cytoplasmic ER α and nuclear ER β . C: MMEDPRP – diffuse ER α and ER β . D: MMEDPPP- cytoplasmic ER α and nuclear ER β . E: MDIL – nuclear and cytoplasmic ER α , nuclear ER β . F: MDILWB -nuclear and cytoplasmic ER α , nuclear ER β . G: MDILPRP and H: MDILPPP – ER α and ER β nuclear expression. **(3)** Box and whisker plots showing quantitative analysis of ER α (green) and ER β (red) expression in MCF7 cells, following exposure to blood constituents. A: Media-treated. B: Diluent-treated (0.1% DMSO). [$p < 0.05$ between ER α and ER β within the treatment group. * $p < 0.05$ between matched ERs compared to untreated MCF7.

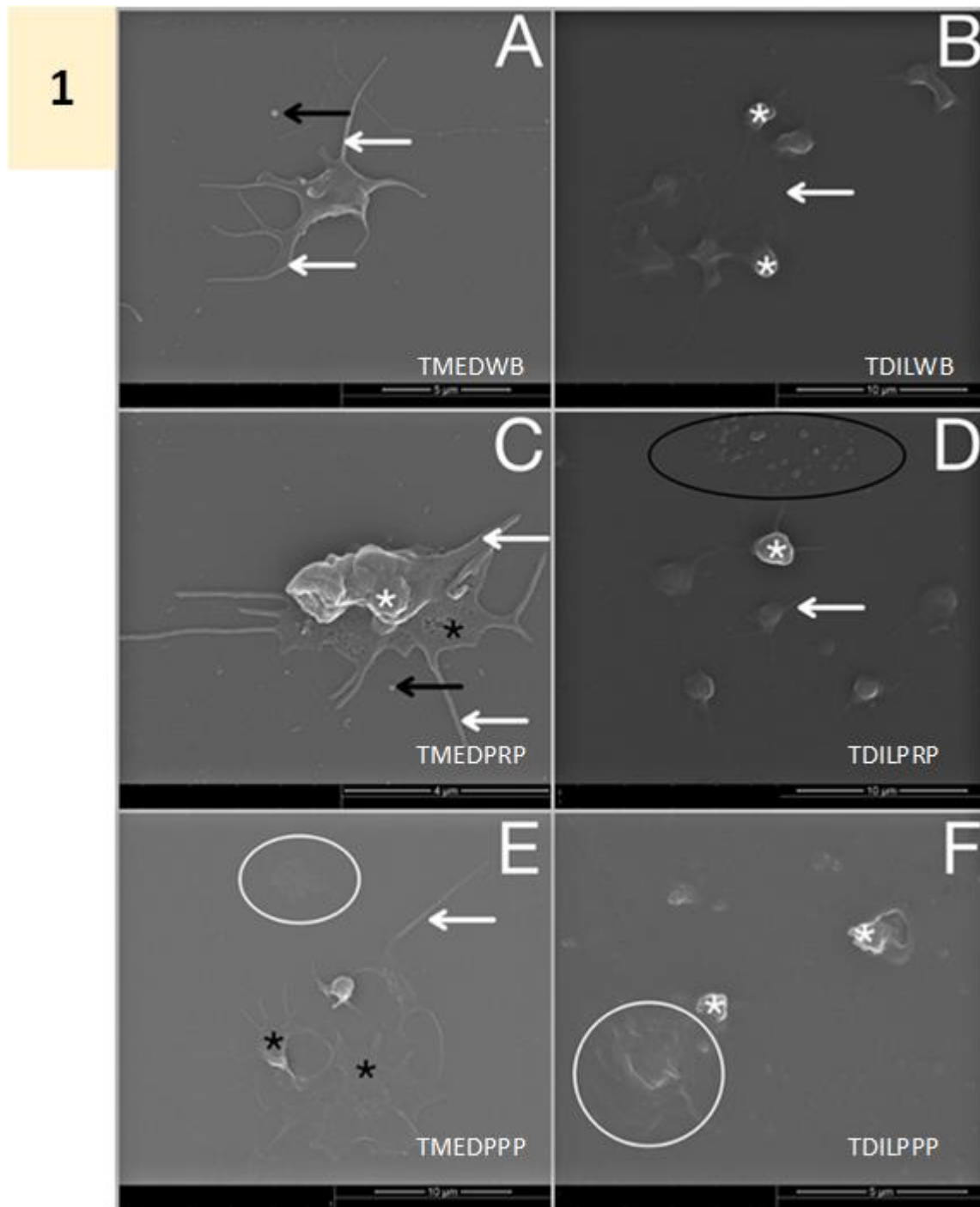


Figure 4.10: **Effects of blood constituent incubation with T47D cells - controls.** *Key:* TMED- media-treated T47D cells; TMEDWB: WB co-incubated with media-treated T47D cells; TMEDPRP – PRP co-incubated with media-treated T47D cells; TMEDPPP – PPP co-incubated with media-treated T47D cells; TDIL- diluent-treated T47D cells; TDILWB: WB co-incubated with diluent-treated T47D cells; TDILPRP – PRP co-incubated with diluent-treated T47D cells; TDILPPP – PPP co-incubated with diluent-treated T47D. **(1).** Platelet ultrastructural alterations. *Key:* white * - membrane folds, black * - platelet hyalomere spread, white arrow - extending filipodia, black arrow – microparticles, white circle -fibrin, black circle - fibrin deposits. A: TMEDWB – slightly active platelet with filipodia and microparticles; B: TDILWB – mostly resting platelets; C: TMEDPRP – spread platelet, filipodia and microparticles. D: TDILPRP – platelets with smooth membrane and extending filipodia, with microparticles. E: TMEDPPP: fully spread platelets with fibrin deposits. F: TDILPPP: fibrin plaque formation.

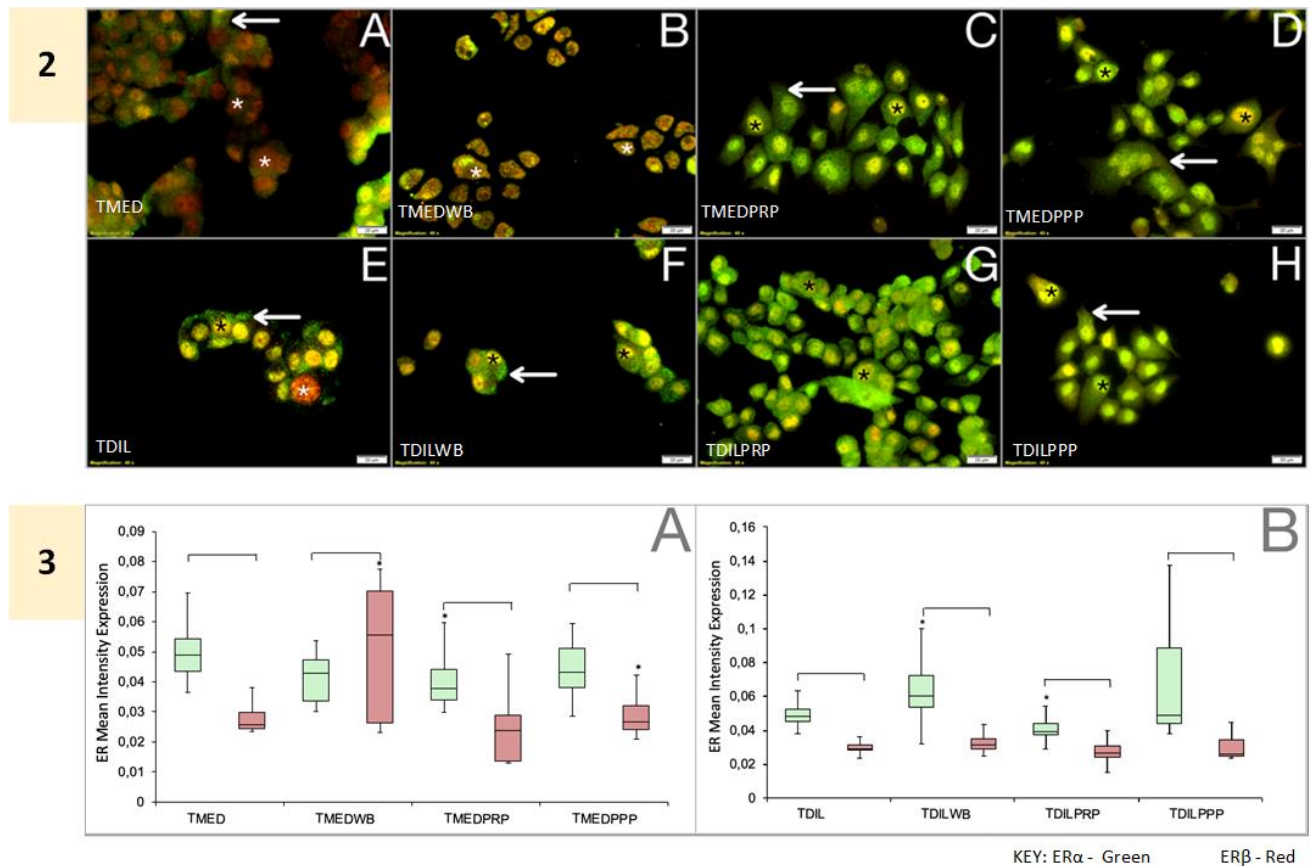


Figure 4.10: Effects of blood constituent incubation with T47D cells - controls. Key: TMED- media-treated T47D cells; TMEDWB: WB co-incubated with media-treated T47D cells; TMEDPRP – PRP co-incubated with media-treated T47D cells; TMEDPPP – PPP co-incubated with media-treated T47D cells; TDIL- diluent-treated T47D cells; TDILWB: WB co-incubated with diluent-treated T47D cells; TDILPRP – PRP co-incubated with diluent-treated T47D cells; TDILPPP – PPP co-incubated with diluent-treated T47D. **(2)** Co-localisation of ERα (green) and ERβ (red) in T47D cells, following exposure to blood constituents. Yellow - ERα dominating; Orange - ERβ dominating. A: TMED: primarily cytoplasmic ERα (white arrow) and nuclear ERβ expression (*). B: TMEDWB: Nuclear ERα and ERβ expression (*). C: TMEDPRP: cytoplasmic and nuclear ERα expression (arrow) and nuclear ERβ (*). D: TMEDPPP: high cytoplasmic ERα (white arrow) and nuclear ERβ (*). E: TDIL – diffuse cytoplasmic and nuclear ERα (white arrow), nuclear ERβ (*). F: TDILWB – cytoplasmic and nuclear ERα (white arrow), higher nuclear ERβ (*). G: TDILPRP – cytoplasmic and nuclear ERα and nuclear ERβ (*). H: TDILPPP – high nuclear and cytoplasmic ERα (black *) and higher nuclear ERβ (white *). **(3)** Box and whisker plots showing quantitative analysis of ERα (green) and ERβ (red) expression in T47D cells, following exposure to blood constituents. A: Conditioned media-treated T47D cells. B: Diluent-treated T47D cells (0.1% DMSO). [indicates significant differences between ERα and ERβ within the treatment group. * indicates significant ($p < 0.05$) differences between matched ERs compared to untreated T47D.

4.3.3 Anastrozole-treatment Alters Breast Cancer Cell-Induced Hypercoagulation.

Previously presented results (Chapter 3 and summarised above), indicated baseline levels of thrombin availability (Figure 4.2) and platelet activation (Table 4.2 & 4.3; Figure 4.3). This section presents Anastrozole-treated samples which will be compared to baseline numbers. For Model 1; MCF7 and T47D breast cancer cells were pre-treated with Anastrozole and exposed to blood constituent. For Model 2; WB was treated with Anastrozole and separated into its' components; PRP and PPP and exposed to untreated MCF7 and T47D cells and analysed.

4.3.3.1 Anastrozole-Treatment Alters Breast Cancer Cell-Induced Thrombin Detection

Model 1

Anastrozole-treatment of MCF7 breast cancer cells, induced significantly greater secretion of thrombin, at 40.93 ng/mL when compared to the conditioned media (untreated cell line control) (Figure 4.2). When Anastrozole-treated MCF7 cells were exposed to WB, a significant decrease in thrombin generation to 40.70 ng/mL was induced, compared to that detected in Anastrozole-treated MCF7 cells (40.93 ng/mL); untreated WB control (41.66 ng/mL) and untreated MCF7 cells exposed to WB (41.48 ng/mL). Anastrozole-treated MCF7 cells exposed to both PRP (40.20 ng/mL) and PPP (40.15 ng/mL) induced thrombin generation significantly higher than that of pre-treated MCF7 cells alone. This significant increase in thrombin generation in Anastrozole-treated MCF7 cells exposed to both PRP and PPP could be due to an increase in both the number of platelets and fibrinogen within the PRP and PPP increasing thrombin binding capacity. It should further be noted that all Model 1 samples; untreated cells, Anastrozole-treated cells and Anastrozole-treated cells exposed to blood constituents, generated thrombin within 40 – 40.93 ng/mL.

Conversely, T47D breast cancer cells treated with Anastrozole induced only a 0.02 ng/mL increase in thrombin generation at 41.63 ng/mL compared to the untreated control at 41.61 ng/mL (Figure 4.2). However, when exposed to blood constituents this was reduced by almost 1 ng/mL regardless of which blood constituent the pre-treated cells were exposed to. WB exposure to Anastrozole-treated T47D cells reduced detectable thrombin to 40.02 ng/mL, while PRP exposure reduced this to 40.70 ng/mL and PPP reduced detectable thrombin to 40.29 ng/mL. When compared to matched untreated T47D cells exposed to WB and PPP, respectively, a significant decrease in thrombin generation is noted within Anastrozole-

treated T47D cells exposed to WB and PRP, comparatively. While Anastrozole-treatment of T47D cells exposed to PRP, however, induced a significant increase in thrombin generation when compared to matched untreated T47D cells exposed to PRP.

Model 2

Pre-treatment of blood constituents with Anastrozole induced varying levels of thrombin generation (Figure 4.2). Anastrozole-treated WB exposed to MCF7 cells induced a slightly higher level of thrombin generation (41.57 ng/mL), compared to untreated WB. Conversely, Anastrozole-treated PRP (39.60 ng/mL) and PPP (38.75 ng/mL) induced significantly lower thrombin generation, compared to matched blood constituent controls, PRP and PPP, respectively. When compared to matched Anastrozole-treated blood constituents; Anastrozole-treated WB exposed to MCF7 cells induced only a slight decrease in thrombin availability, while both PRP and PPP induced a significant reduction (Figure 4.2). These reductions in thrombin generation by MCF7 breast cancer cells in samples exposed to Anastrozole-treated blood constituents may also be due to the binding of thrombin to platelets and aiding in the conversion of fibrinogen to fibrin within the blood constituents itself, heightened by the exposure to MCF7 cells, as visualised ultrastructurally (Figure 4.4 (1)B, D, F).

Anastrozole-treatment of blood constituents induced levels of thrombin generation similar to that of T47D cells exposed to WB, PRP and PPP, respectively. On comparison WB and Anastrozole-treated WB, Anastrozole-treated WB exposed to T47D cells induced thrombin generation that was only slightly lower at 41.25 ng/mL. Conversely, Anastrozole-treated PRP (39.82 ng/mL) and Anastrozole-treated PPP (39.65 ng/mL) had a significantly reduced level of thrombin generation, when compared to untreated PRP and PPP and Anastrozole-treated PPP, respectively (Figure 4.2). As with MCF7 cells, a reduction in detectable thrombin is noticed in Anastrozole-treated blood constituent samples exposed to T47D cells. This could be attributed to endogenous thrombin binding within the platelets and fibrinogen within the blood constituents themselves.

Thrombin Generation Alters between Models

On assessment of induction of thrombin generation between Models; Anastrozole-treatment of WB (Model 2) induced significantly heightened levels of thrombin generation by MCF7 cells compared to that of Model 1 (Anastrozole-treatment of MCF7 cells exposed to WB) (Figure 4.2). Anastrozole-treatment of both PRP and PPP (Model 2) significantly reduced the ability of MCF7 cells to generate thrombin compared to matched Model 1 samples. This was further noted in the generation of thrombin by T47D cells, with Anastrozole-treatment of blood constituents; WB, PRP and PPP inducing significantly lower levels of thrombin than that of matched Model 1 samples, Anastrozole-treated T47D cells exposed to blood constituents. This significant reduction of detectable thrombin noted in both cell lines, altered by blood constituent indicates the ability of circulating Anastrozole (Model 2) to reduce thrombin generation by cell lines. Furthermore, contrasting and indicating the ability of cumulative Anastrozole treatment to heighten thrombin generation by cell lines, not inhibited by the addition of blood constituent; WB, PRP and PPP.

4.3.3.2 Anastrozole Induced Hypercoagulation determined by Platelet Activation

Model 1: Overall IPA and Platelet Ultrastructure

Cumulative effects of Anastrozole (Model 1) were assessed in WB samples exposed to Anastrozole-treated MCF7 cells (Table 4.2). These samples showed an increase in both CD62P IPA and CD63 IPA, when compared to untreated WB, similar but not significantly different to that of WB exposed to untreated MCF7 cells. The ratio of activation markers was also noted to have only decreased slightly, yet still indicating that platelets within the sample remained at earlier stages of activation. Assessment of ultrastructural alterations of platelets from WB exposed to Anastrozole-treated MCF7 cells (Model 1) showed a folded membrane surface indicating the beginning of platelet activation (Figure 4.4(1)A).

Similarly, PRP exposed to Anastrozole-treated MCF7 cells induced a decrease in CD62P IPA with a significant increase in CD63 IPA; however, an increase in the ratio of activation markers is noted when compared to that of untreated PRP, indicating that platelets within this sample have shifted towards a later stage of activation (Table 4.3). This was confirmed ultrastructurally, showing platelets from PRP exposed to Anastrozole-treated cells (Model 1) (Figure 4.4(1)C) with a hyalomere spread and filipodia extension.

Cumulative effects of Anastrozole were assessed in WB samples exposed to Anastrozole-treated T47D cells (Table 4.2). These samples showed a significant increase in both CD62P IPA and CD63 IPA when compared to untreated WB; with CD62P IPA being significantly higher than that of WB exposed to T47D cells alone. The ratio of activation markers was also noted to have only decreased slightly, therefore indicating platelets remained at earlier stages of activation. Ultrastructurally, samples from WB exposed to Anastrozole-treated T47D cells (Model 1) indicated platelet aggregations, in addition to the presence of thick fibrin fibres, forming dense plaques or fibrin sheets with fibrin pores present (Figure 4.5(1)A).

PRP exposed to Anastrozole-treated T47D cells induced an increase ($p>0.05$) in CD62P IPA with a significant increase ($p<0.05$) in CD63 IPA (Table 4.3). This was confirmed ultrastructurally with platelets from PRP exposed to Anastrozole-treated cells (Figure 4.5(1)C) showed platelet aggregations with the presence of fibrin deposits.

Model 1: IPA per Interval Gate

On assessment of IPA per interval gate, Anastrozole-treatment of MCF7 cells (Model 1) indicated an increase in CD62P^{low} and a significant increase in CD63^{low} interval gates (I1 and I2) compared to untreated WB; this increase was also noted when compared to WB exposed to untreated MCF7 cells, however, this was not significant (Table 4.2). In the CD62P^{medium} and CD63^{medium} interval gate (I3), significant increases in both CD62P and CD63 IPA was noted compared to untreated WB; on comparison to WB exposed to untreated MCF7 cells slight decreases in both CD62P and CD63 IPA was noted. The greatest difference was noted within the CD62P^{high} and CD63^{high} interval gates (I4 and I5) with a significant increase in CD63 IPA in I5, and on comparison to WB exposed to MCF7 cells, this was noted to be only slightly higher (Table 4.2).

PRP exposed to Anastrozole-treated MCF7 cells (Model 1) showed an increase of both CD62P and CD63 IPA's in CD62P^{low} and CD63^{low} interval gates (I1 and I2) (Table 4.3). The CD62P^{medium} and CD63^{medium} interval gate (I3) showed only a significant increase in CD63 IPA, with a slight decrease ($p>0.05$) in CD62P IPA when compared to untreated PRP; on comparison to PRP exposed to MCF7 cells, platelet markers decreased slightly. In the CD62P^{high} interval gates (I4

and I5) a significant loss is noted, compared to both untreated PRP and PRP exposed to untreated MCF7 cells. This loss, however, could be due to platelets being highly active; as noted within the ultrastructural analysis (Figure 4.4(1)C&E). In the CD63^{high} interval gate, persistent CD63 IPA is noted and was seen to be higher than that of untreated PRP, but lower than that of PRP exposed to MCF7 cells.

On assessment of IPA per interval gate, Anastrozole-treatment of T47D cells (Model 1) indicated a significant increase in CD62P^{low} and CD63^{low} interval gates compared to untreated WB. A decrease in CD62P^{medium} interval gate is noted, compared to untreated WB; while a significant increase is noted in the CD63^{medium} interval gate. The greatest difference can be noted within the CD62P^{high} and CD63^{high} interval gates with a significant increase in CD63 IPA greater than that of WB exposed to T47D cells. PRP exposed to Anastrozole-treated T47D cells showed an increase in CD62P^{low} and CD63^{low} interval gates. Significant increases are noted within the CD62P^{medium} and CD63^{medium} interval gates compared to untreated PRP. The CD62P^{high} and CD63^{high} interval gates, however, showed a decrease in CD62P IPA in I4; this decrease, however, could be attributed to a loss of markers through microparticles and the formation of platelet aggregated as noted ultrastructurally (Figure 4.5(1)C).

Model 2: Overall IPA and Platelet Ultrastructure

Circulating effects of Anastrozole (Model 2) were assessed in Anastrozole-treated WB samples exposed to MCF7 cells (Table 4.3). CD62P and CD63 IPA were higher when compared to untreated WB and was also seen to be higher than that of WB exposed to cells alone; however, this was not significant. The ratio of activation markers, while slightly low, reflected an early stage of platelet activation. However, samples from Anastrozole-treated WB exposed to MCF7 cells (Model 2) (Figure 4.5(1)B) showed the formation of fibrin plaque with many fibrin pores indicating a hypercoagulatory environment. Despite marker expression evident in IPA, platelets were obscured by the pores and plaque.

Anastrozole-treated PRP exposed to MCF7 cells (Model 2) induced a significant decrease in CD62P IPA overall, and only a slight increase in CD63 IPA; thus, the ratio of activation markers remained at comparable levels to that of untreated PRP, indicating platelets remaining at earlier stages of activation (Table 4.3). On comparison to PRP exposed to untreated MCF7

cells, Anastrozole-treated PRP induced significantly lower IPA of both CD62P and CD63, with the ratio of these markers indicating a shift to earlier stages of platelet activation. Ultrastructurally, however, platelets from Anastrozole-treated PRP exposed to MCF7 cells (Figure 4.4(1)D) showed platelets with many extending filipodia and exhibiting aggregation indicating that this loss of marker expression may not necessarily represent platelets at earlier stages of activation. It should be noted that both PPP exposed to Anastrozole-treated MCF7 cells (Model 1) (Figure 4.4(1)E) and Anastrozole-treated PPP exposed to MCF7 cells (Model 2) (Figure 4.4(1)F) induced heightened fibrinogen and fibrin pore formation, respectively.

Circulating effects of Anastrozole (Model 2) was assessed in Anastrozole-treated WB samples exposed to T47D cells (Model 2) (Table 4.2). CD62P IPA was noted to be higher ($p>0.05$), while CD63 IPA was lower when compared to untreated WB and also behaved similarly when compared to WB exposed to T47D cells alone. The ratio of activation markers, while slightly lower indicating a shift, remained at an early stage. Ultrastuctural assessment indicated platelets from Anastrozole-treated WB exposed to T47D cells (Model 2) (Figure 4.5(1)B) were obscured with samples exhibiting the formation of fibrin plaque.

Anastrozole-treated PRP exposed to T47D cells (Model 2) induced a significant decrease in CD62P IPA overall, and a significant increase in CD63 IPA; allowing for the ratio of activation markers to remain at comparable levels to that of untreated PRP (Table 4.3). On comparison to PRP exposed to T47D cells, Anastrozole-treated PRP exposed to T47D induced significantly higher overall CD63 IPA. Hypercoagulation was viewed ultrastructurally (Figure 4.5(1)D, with samples exhibiting the formation of fibrin plaques, obscuring platelets within the samples, contradicting the ratio of markers and indicating later stages of platelet activation. Similarly, PPP exposed to Anastrozole-treated cells in Model 1 (Figure 4.5(1)E) showed greater levels of fibrin network formation, while Anastrozole-treated PPP exposed to cells (Model 2) (Figure 4.5(1)F) also induced fibrin plaque formation with some active platelets being visible.

Model 2: IPA per interval gate

Anastrozole-treated WB exposed to MCF7 cells indicated significant increases in both CD62P IPA and CD63 IPA in CD62P^{low} and CD63^{low} interval gates (I1 and I2) (Table 4.2). In the CD62P^{medium} and CD63^{medium} interval gate (I3), Anastrozole-treatment of WB exposed to MCF7

cells induced a significant increase in both CD62P and CD63 IPA, compared to untreated WB. On comparison to WB exposed to MCF7 cells, a significant increase is noted within the CD62P^{medium} interval gate, which was also higher than that of Model 1. On assessment of the CD62P^{high} and CD63^{high} interval gates, both CD62P and CD63 IPA persisted, with CD63 IPA significantly higher than untreated WB; but significantly lower than that of Model 1 (Table 4.2).

This was similarly noted for Anastrozole-treated PRP samples exposed to MCF7 cells (Model 2) (Table 4.3). CD62P^{low} and CD63^{low} interval gates indicated a significant increase in both CD62P and CD63 IPA, compared to untreated PRP. The CD62P^{medium} interval gate (I3), indicated a significantly lower CD62P IPA when compared to that of the untreated PRP. The CD62P^{high} and CD63^{high} interval gates indicated a further reduction in CD62P IPA and a significant increase in CD63 IPA, when compared to untreated PRP; as well as higher IPA compared to PRP exposed to MCF7 cells.

Anastrozole-treatment of WB exposed to T47D cells (Model 2) showed significant increases in both CD62P^{low} and CD63^{low}. In the CD62P^{medium} and CD63^{medium}, Anastrozole-treated WB exposed to T47D cells induced only slight increases in both CD62P and CD63 IPA ($p>0.05$). On assessment of CD62P^{high} and CD63^{high} interval gates, CD63 IPA persisted, significantly higher than untreated WB. This was similarly noted for Anastrozole-treated PRP exposed to T47D cells (Table 4.3), CD62P^{low} and CD63^{low} interval gates indicated a significant increase in both CD62P and CD63 IPA. The CD62P^{high} and CD63^{high} interval gates indicated a further reduction in CD62P IPA and an increase in CD63 IPA ($p>0.05$), the loss of CD62P and persistence of CD63 in this interval is further substantiated by ultrastructural assessment in which aggregated platelets are noted with the presence of microparticles (Figure 4.5(1)D)

Comparison between Cumulative (Model 1) and Circulating (Model 2) effects of Anastrozole on Platelet Activation.
Assessment between Models indicated no significant difference between cumulative (Model 1) and circulating effects (Model 2) of Anastrozole in the MCF7 cell line on assessment of CD62P and CD63 IPA in the WB component (Table 4.2), however, a significant reduction in CD62P IPA is noted in Anastrozole-treated PRP exposed to MCF7 cells compared to

Anastrozole-treated MCF7 cells exposed to PRP. This substantiates results noted in thrombin generation and indicates the ability of circulating Anastrozole (Model 2) to reduce thrombin generation by MCF7 cells. On assessment of marker expression in the T47D cell line exposed to Anastrozole-treated blood constituents (Model 2) indicated a significant reduction in CD62P and CD63 IPA overall, with the ratio indicating platelets remained at an earlier stage of activation (Table 4.2). Furthermore, on assessment per interval gate, a significant reduction in CD63 IPA in the CD63^{high} is noted in Anastrozole-treated WB exposed to T47D cells. These results were further reflected in PRP samples, with Anastrozole-treated PRP exposed to T47D cells inducing a reduction in both CD62P and CD63 IPA. However, on assessment of ultrastructural alterations (Figure 4.5(1)D) it is noted that Anastrozole-treatment of blood constituents exposed to T47D cells induced the formation of platelet aggregates and fibrin plaque indicating that the reduction of both thrombin generation and platelet activation markers due to the later stages of platelet activation. Anastrozole-treatment heightened the capability of the T47D cell line to induce a hypercoagulatory environment compared to that of the MCF7 cell line for both Models (Table 4.3). Furthermore, due to the loss of markers at later stages of activation, substantiated by electron micrographs indicating the formation of fibrin networks and plaques, it was noted that the ratio of activation markers which indicated platelets remained at earlier stages of activation, may not be the most accurate calculation and that marker expression needs to be assessed in a cohort with ultrastructural assessment.

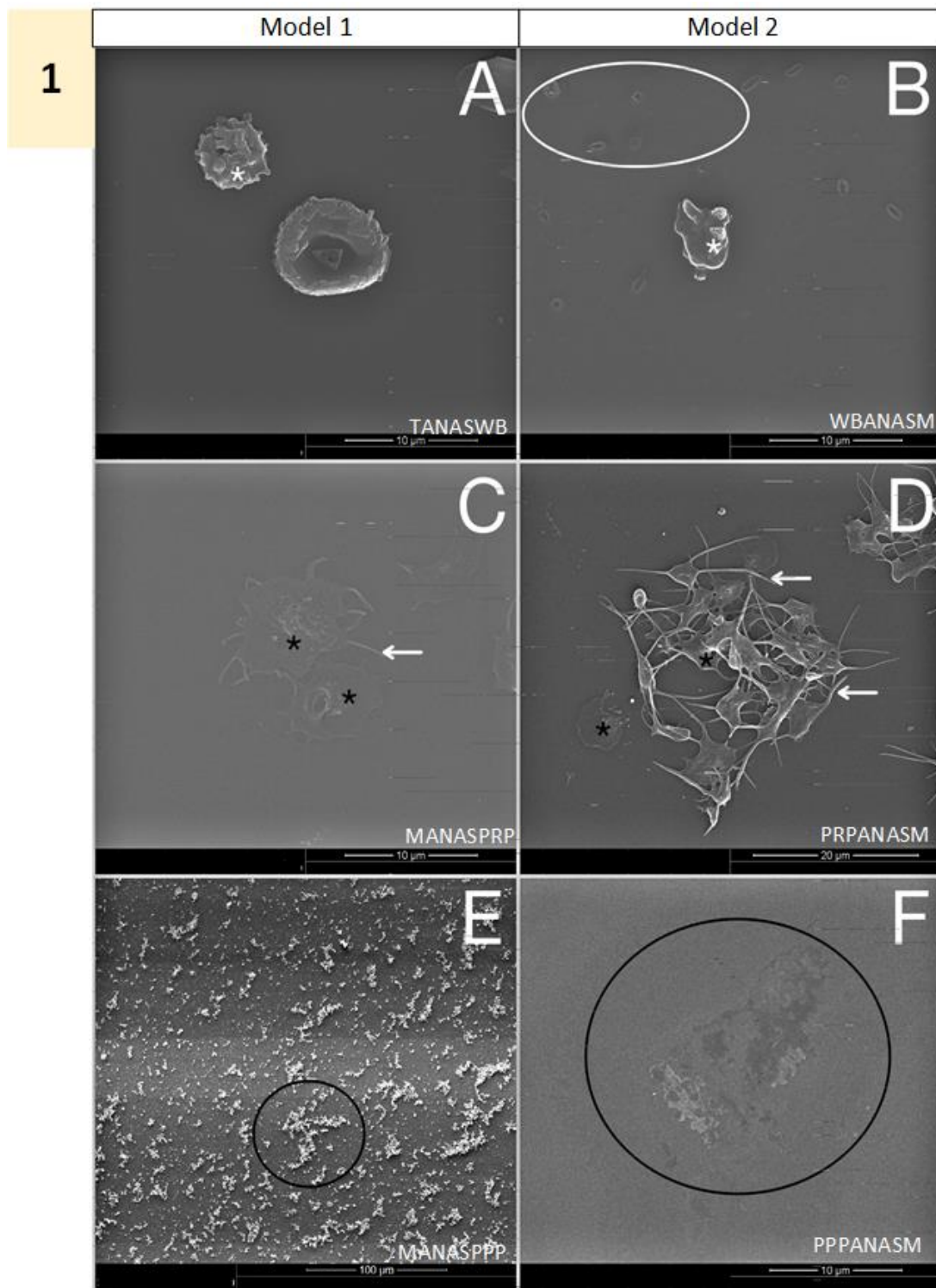
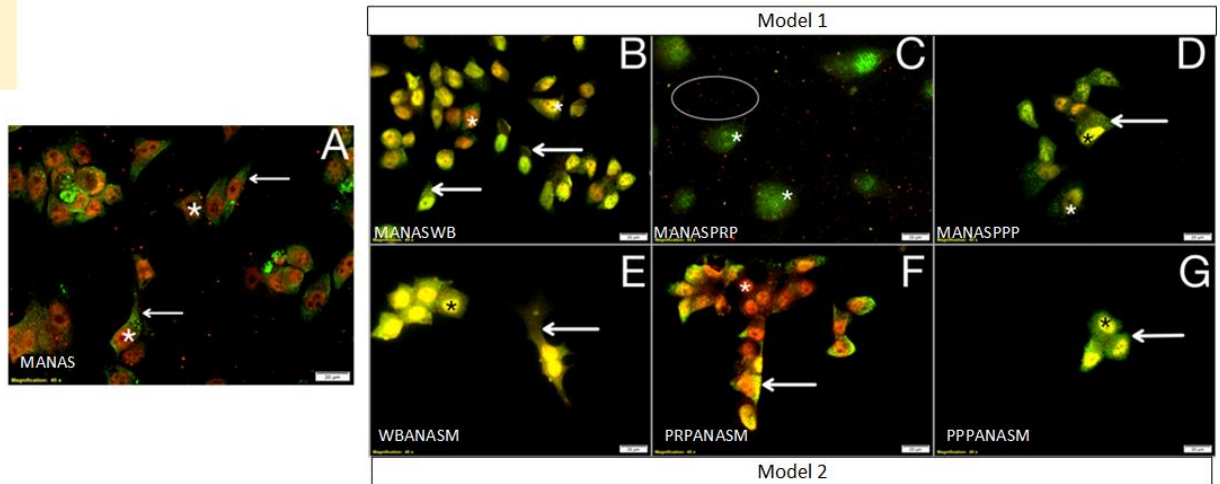


Figure 4.4: Effects of Anastrozole-treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with MCF7 cells. **Key:** MANAS: Anastrozole-treated MCF7 cells. MANASWB: Anastrozole-treated MCF7 cells with WB. MANASPRP: Anastrozole-treated MCF7 cells with PRP. MANASPPP: Anastrozole-treated MCF7 cells with PPP. WBANASM: Anastrozole-treated WB with MCF7 cells. PRPANASM: Anastrozole-treated PRP with MCF7 cells. PPPANASM: Anastrozole-treated PPP with MCF7 cells. **(1)** Platelet ultrastructural alterations. A: MANASWB: active platelets with a folded membrane (*). B: WBANASM: outward projections of fibrin (*) with the presence of fibrin pores (white circle). C: MANASPRP: platelets with a hyalomere spread (*) with extending filipodia (white arrow). D: PRPANASM: platelets with a hyalomere spread (*) and extending filipodia (white arrow) in early stages of aggregation. E: MANASPPP: fibrinogen deposits (black circle). F: PPPANASM: remnants of platelets with fibrinogen deposits (black circle).

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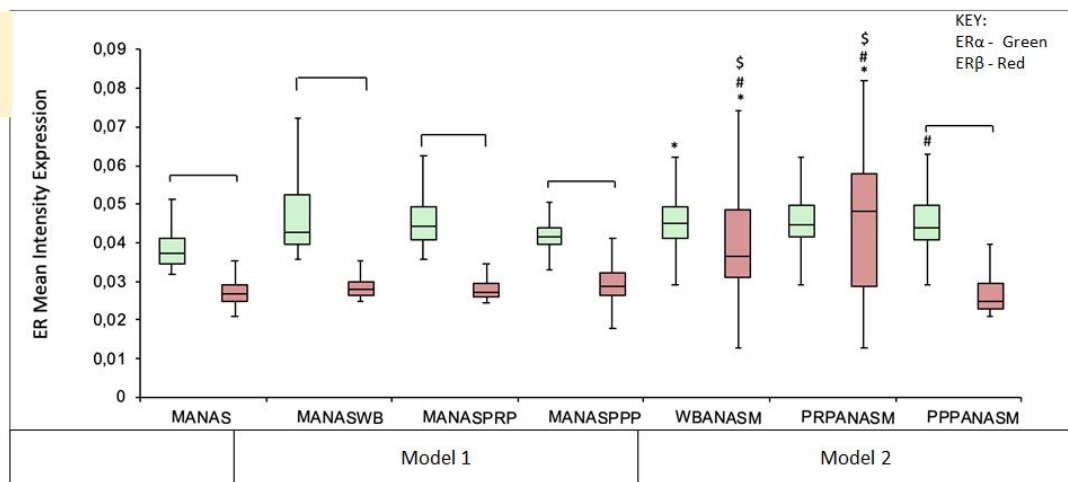


Figure 4.4: **Effects of Anastrozole-treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with MCF7 cells.** **Key:** MANAS: Anastrozole-treated MCF7 cells. MANASWB: Anastrozole-treated MCF7 cells with WB. MANASPRP: Anastrozole-treated MCF7 cells with PRP. MANASPPP: Anastrozole-treated MCF7 cells with PPP. WBANASM: Anastrozole-treated WB with MCF7 cells. PRPANASM: Anastrozole-treated PRP with MCF7 cells. PPPANASM: Anastrozole-treated PPP with MCF7 cells. **(2)** Co-localisation of ER α (green) and ER β (red) in MCF7 cells. A: MANAS: cytoplasmic ER α (arrow) and nuclear ER β (*). B: MANASWB: nuclear and cytoplasmic ER α (arrow) and nuclear ER β (*). C: MANASPRP: nuclear and cytoplasmic ER α (*) and extracellular ER β expression (circle). D: MANASPPP: cytoplasmic and nuclear ER α (arrow) with nuclear expression of ER β (*). E: WBANASM: nucleus and cytoplasmic ER α (arrow) and nuclear ER β (*). F: PRPANASM: cytoplasmic ER α (arrow) with nuclear expression of ER β (*). G: PPPANASM: cytoplasmic and nuclear expression of ER α (arrow) with more nuclear expression of ER β (*). **(3)** Box and whisker plots representing quantitative analysis of ER α (green) and ER β (red) expression in MCF7 cells for Model 1 and 2. Yellow - ER α dominating; Orange - ER β dominating. [$p < 0.05$ between ER α and ER β within the treatment group. # $p < 0.05$ between matched ERs compared to untreated MCF7. * $p < 0.05$ between matched ERs compared to Anastrozole- treated MCF7. \$ $p < 0.05$ between matched ERs and blood constituent groups compared between Models.

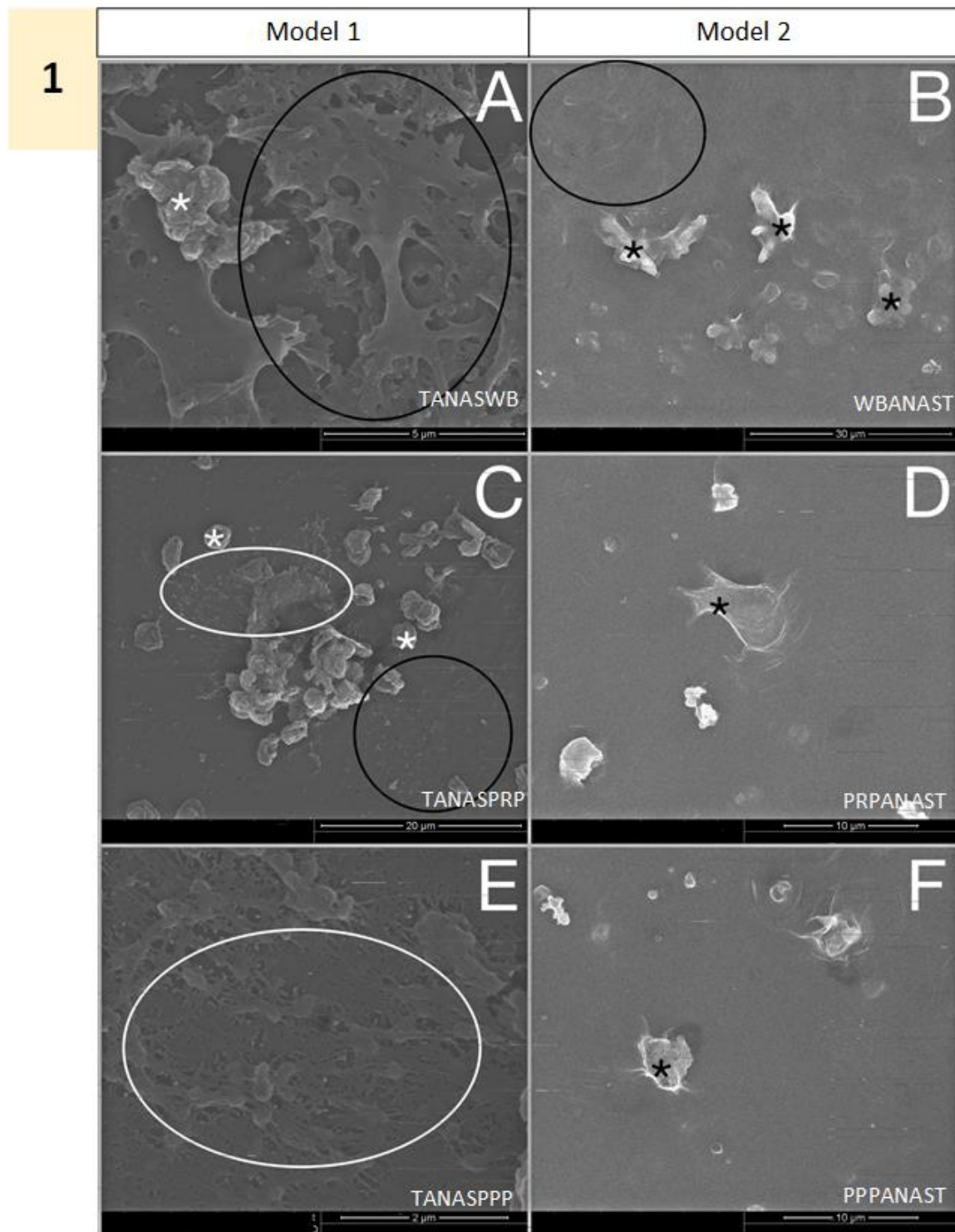


Figure 4.5: **Effects of Anastrozole treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with T47D cells.** **Key:** TANAS: Anastrozole-treated T47D cells. TANASWB: Anastrozole-treated T47D cells with WB. TANASPRP: Anastrozole-treated T47D cells with PRP. TANASPPP: Anastrozole-treated T47D cells with PPP. WBANAST: Anastrozole-treated WB with T47D cells. PRPANAST: Anastrozole-treated PRP with T47D cells. PPPANAST: Anastrozole-treated PPP with T47D cells. **(1) Platelet ultrastructural alterations.** **Key:** white * - membrane folds, black * - platelet hyalomere spread, white arrow - extending filipodia, black arrow - microparticles, white circle - fibrin, black circle - fibrin deposits. **A:** TANASWB: aggregated platelets (*), the formation of fibrin networks (black circle). **B:** WBANAST: outward projections of fibrin (*) with fibrin plaque (white circle). **C:** TANASPRP: aggregated platelets (*) with fibrin deposits (white circle) and fibrin pores (black circle). **D:** PRPANAST: fibrin plaque formation with outward projections (*). **E:** TANASPPP: fibrin meshwork with underlying platelets (white circle). **F:** PPPANAST: fibrin plaque formation with outward projections (*).

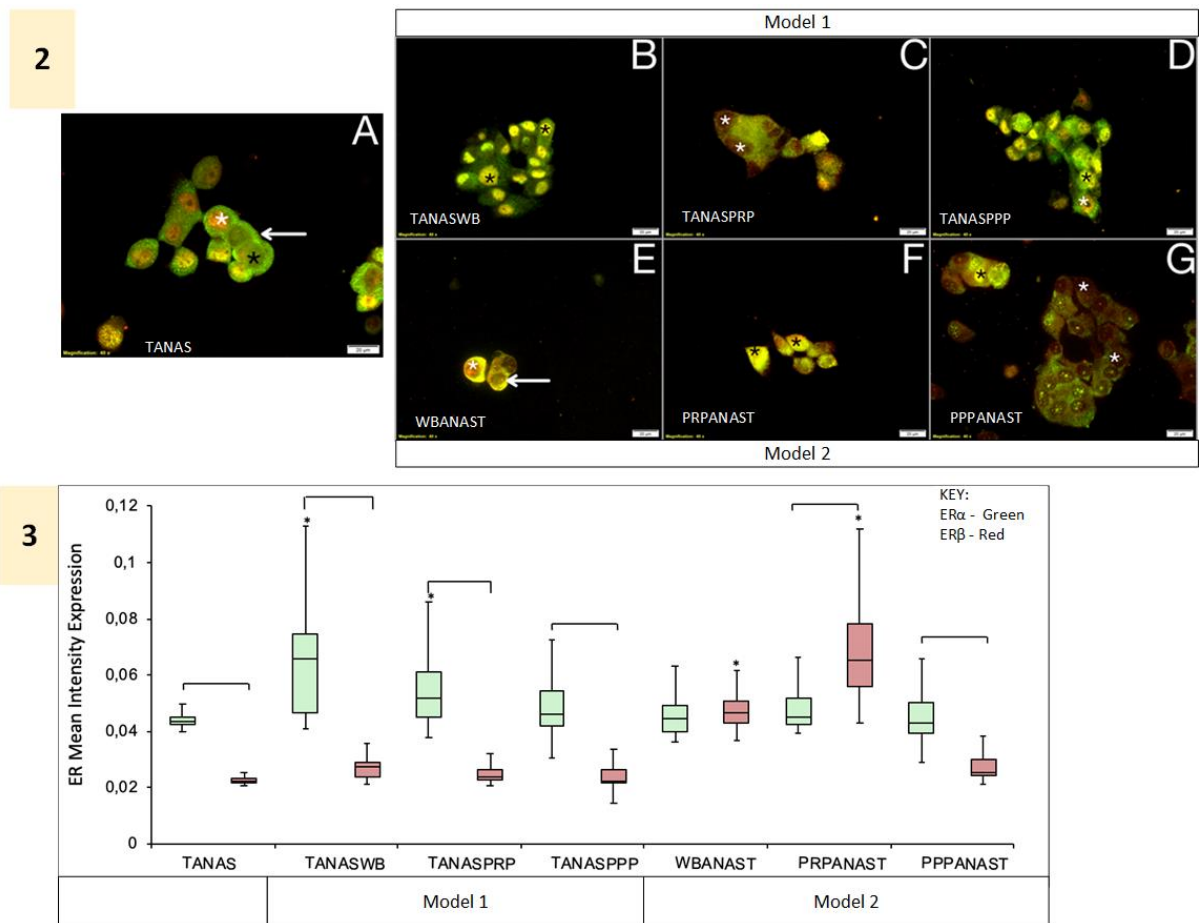


Figure 4.5: Effects of Anastrozole treatment, cumulative (Model 1) and circulatory (Model 2) following co-incubation with T47D cells. Key: TANAS: Anastrozole-treated T47D cells. TANASWB: Anastrozole-treated T47D cells with WB. TANASPRP: Anastrozole-treated T47D cells with PRP. TANASPPP: Anastrozole-treated T47D cells with PPP. WBANAST: Anastrozole-treated WB with T47D cells. PRPANAST: Anastrozole-treated PRP with T47D cells. PPPANAST: Anastrozole-treated PPP with T47D cells. **(2)** Co-localisation of ERα (green) and ERβ (red) in T47D cells. A: TANAS: cytoplasmic and nuclear ERα (white arrow), and nuclear ERβ expression (*). B: TANASWB: nuclear and cytoplasmic ERα and nuclear ERβ (*). C: TANASPRP: cytoplasmic ERα and nuclear ERβ (*). D: TANASPPP: cytoplasmic and nuclear ERα with nuclear ERβ (*). E: WBANAST: cytoplasmic ERα (white arrow), with cytoplasmic and nuclear ERβ (*). F: PRPANAST: cytoplasmic ERα and nuclear ERβ (*). G: Diluent-treated T47D cells exposed to PRP expressing ERα in the nucleus and cytoplasm, and ERβ expression in the nucleus (*). H: PPPANAST: cytoplasmic and nuclear ERα (*), and nuclear ERβ (arrow). **(3)** Box and whisker plots representing quantitative analysis of ERα (green) and ERβ (red) expression in T47D cells for Model 1 and 2. Yellow - ERα dominating; Orange - ERβ dominating. [$p < 0.05$ between ERα and ERβ within the treatment group. # $p < 0.05$ between matched ERs compared to untreated T47D. * $p < 0.05$ between matched ERs compared to Anastrozole-treated T47D. \$ $p < 0.05$ between matched ERs and blood constituent groups compared between Models.

4.3.4 Tumour invasion profiles marked ER responses

4.3.4.1 Control Breast Cancer Cell Expression of ER

Control MCF7 breast cancer cells indicated immunofluorescence of ER α concentrated within the cytoplasm and along the edges of the cell, with nuclear expression of ER β (Figure 3.9(2)A). On exposure to WB greater ER α expression is noted in the perinuclear cytoplasmic region and cytoplasm; while ER β persisted within the nucleus (Figure 3.9(2)B); this increase in ER β was significantly higher than that of the untreated MCF7 cells. PRP (Figure 3.9(2)C), altered ER expression considerably: ER α was diffusely spread within cytoplasm and ER β more dispersed with significantly higher intensity when compared to that of untreated MCF7 cells (Figure 3.9(3)A). PPP exposure (Figure 3.9(2)D), induced greater nuclear expression of ER β which was significantly higher than the control (Figure 3.9(3)A). The diluent (Figure 3.9(2)E), 0.1% DMSO, induced greater ER α expression in the nucleus with diffuse cytoplasmic expression, and ER β was minimally expressed within the nucleus, correlating with quantitative assessment (Figure 3.9(3)B). On exposure to blood constituents, WB induced greater nuclear expression of ER β (Figure 3.9(3)B), while PRP heightened ER β nuclear expression significantly; and PPP (Figure 3.9(2)H) also induced greater ER β nuclear expression compared to diluent-treated MCF7 cells.

T47D breast cancer cells were noted to express ER β consistently higher compared to that of MCF7 cells. T47D breast cancer cells expressed ER α diffusely within the cytoplasm while ER β is noted within the nucleus (Figure 3.10(2)A). On exposure to WB, ER α and ER β present within the nucleus, with ER β being expressed much higher than that in T47D cells alone (Figure 3.10(2)B). Quantitative assessment showed a reduction in ER α mean intensity while a significant increase in ER β was noted (Figure 3.10(3)). However, when T47D cells were exposed to PRP (Figure 3.10(2)C), ER α was localised in both the nucleus and cytoplasm, with ER β expression remaining in the nucleus. Mean intensity expression of markers, however, was noted to be lower than untreated T47D cells (Figure 3.10(3)). PPP exposed to T47D cells (Figure 3.10(2)D), induced similar expression to PRP, with higher ER α and ER β mean intensity expression observed (Figure 3.10(3)). The diluent (Figure 3.10(2)E) induced greater ER α expression in the nucleus and cytoplasm, ER β expressed in the nucleus. On exposure to blood constituents; WB (Figure 3.10(2)F) induced greater cytoplasmic expression of ER α , with ER β expressed within the nucleus. Both PRP (Figure 3.10(2)G) and PPP (Figure 3.10(2)H) exposure;

induced greater ER α expression in the cytoplasm with some nuclear expression, while ER β was localised to the nucleus. Quantitative assessment shows an increase in ER α mean intensity expression on exposure to PPP, with greater variability of the data indicating variable expression intensity sites within the cells themselves in response to PPP (Figure 3.10(3)).

4.3.4.2 Anastrozole induced ER alterations

Model 1

Anastrozole-treatment of MCF7 cells (Model 1) induced translocation of ER α from the nucleus into the cytoplasm allowing for greater expression to be noted within the cellular processes of the cells, while ER β was noted to be highly expressed in the nucleus with what seems to be the nucleolus void of expression (Figure 4.4(2)A) (Solakidi, Psarra and Sekeris, 2005). Furthermore, ER α and ER β mean intensity was noted to have increased (Figure 4.4(3)) compared to the untreated MCF7 control (Figure 3.9(2)A). On exposure to WB (Figure 4.4(2)B), Anastrozole-treated MCF7 cells expressed greater ER α staining both in the cytoplasmic regions, perinuclear region and now within the nucleus, while ER β expression remained concentrated in the nucleus. This was noted in the mean intensity assessment of both markers (Figure 4.4(3)). PRP exposure (Figure 4.4(2)C), induced greater alterations in expression of both proteins, with ER α dispersed throughout the cytoplasm with high intensity, while ER β was dispersed throughout the cell and extracellular area, indicating lysing of the cell, and quantitative assessment which did not include debris indicated low intensity (Figure 4.4(3)). PPP exposure (Figure 4.4(2)D) induced diffuse cytoplasmic expression of ER α with heightened nuclear ER β expression (Figure 4.4(3)), compared to Anastrozole-treated MCF7 cells.

Anastrozole treatment of T47D cells (Model 1) induced greater diffuse ER α expression within the cytoplasm, with ER β primarily expressed in the nuclear region (Figure 4.5(2)A). On exposure to WB (Figure 4.5(2)B), Anastrozole-treated T47D cells expressed ER α both in the nucleus and cytoplasm. Quantitatively, WB induced significantly greater ER α mean intensity expression than the untreated control (Figure 4.5(3)). PRP exposure (Figure 4.5(2)C) induced greater ER α cytoplasmic expression, with no expression noted in the nucleus. PRP exposure also caused a reduction of nuclear expression of ER β , with some cytoplasmic expression, this was shown quantitatively (Figure 4.5(3)). On exposure to PPP (Figure 4.5(2)D), Anastrozole-

treated T47D cells expressed greater levels of ER α both within the cytoplasm and nucleus of the cells, while ER β was primarily concentrated in the nucleus; quantitatively ER α mean intensity was increased while ER β was only comparable to that of Anastrozole-treated T47D cells (Figure 4.5(3)).

Model 2

Anastrozole-treated WB (Model 2) exposed MCF7 cells (Figure 4.4(2)E) induced heightened ER α and ER β expression, in both nuclear and cytoplasmic regions, with greater ER β expression concentrated within the nucleus, significantly higher than that of Anastrozole-treated MCF7 cells (Model 1) (Figure 4.4(3)B). Anastrozole-treated PRP (Figure 4.4(2)F), showed perinuclear ER α expression with persisting ER β expression heightened both in the cytoplasm and nuclear regions, compared to Anastrozole-treated MCF7 cells (Model 1). Quantitative assessment indicated ER α mean intensity at levels similar to that of Anastrozole-treated MCF7 cells, with ER β mean intensity noted to be significantly higher (Figure 4.4(3)). Anastrozole-treated PPP (Figure 4.4(2)G) induced ER α expression similar to that of Anastrozole-treated WB, however, with a reduction in ER β expression, this was further substantiated quantitatively compared to Anastrozole-treated cells (Model 1) (Figure 4.4(3)A).

Anastrozole-treated blood components (Model 2) induced greater ER β expression overall, compared to untreated T47D cells exposed to blood constituents. Specifically, Anastrozole-treated WB exposed to T47D cells (Figure 4.5(2)E) induced heightened nuclear and cytoplasm ER β , significantly higher when assessing mean intensity expression (Figure 4.5(3)), with ER α expression concentrated primarily in the cytoplasm. Similar expression can be noted in T47D cells exposed to Anastrozole-treated PRP (Figure 4.5(2)F), with ER α concentrated within the cytoplasm and some nuclear expression and heightened ER β expression in both the nucleus and cytoplasm, substantiated by a significant increase in ER β mean intensity (Figure 4.5(3)). T47D cells exposed to Anastrozole-treated PPP (Figure 4.5(2)G), altered this response: while ER β was still present in both the nucleus and cytoplasm, ER α persisted in the cytoplasm with an increase in expression in the nucleolus, indicating its' role in protein construction. Quantitatively, the mean intensity of both ERs were seen to be comparable to that of Anastrozole-treated T47D cells (Figure 4.5(3)).

Alterations in ER expression between Cumulative (Model 1) and Circulating (Model 2) Anastrozole-treatment.

On assessment of ER expression between models, it was noted that the circulating effects of Anastrozole (Model 2) specifically, Anastrozole-treated WB and PRP exposed to MCF7 cells induced significantly heightened ER β expression compared to matched Anastrozole-treated MCF7 cells representing cumulative therapy (Model 1) exposed to WB and PRP, respectively (Figure 4.4 (2) & (3)). This was similarly noted for Anastrozole-treated WB and PRP exposed to T47D cells, in which ER β expression was significantly higher than that of matched Model 1 samples (Figure 4.5(3); reflected in photomicrographs (Figure 4.5(2)).

4.3.5 The correlation between thrombin detection, ER expression and platelet activation.

The non-parametric Spearman's correlation test showed a significant ($p < 0.05$), positive ($r > 0.5$) correlation between CD62P and CD63 IPA (Appendix), with no direct correlation between IPA and thrombin detection, overall (Table 4.5). On analysis per treatment group a significant ($p < 0.05$) positive ($r > 0.5$) correlation between thrombin detection and CD62P IPA is noted within Anastrozole-treated T47D cells exposed to PRP (Model 1). In the same treatment group, a significant ($p < 0.05$) positive ($r > 0.5$) correlation between ER α and thrombin detection was also noted. Furthermore, while significant ($p < 0.05$) negative ($r < 0.5$) correlation between ER β and thrombin detection is noted overall, the association was weak; indicating an opposing action of ER β to that of ER α . The significant strong correlations to thrombin detection noted remain within Model 1, particularly ER α and CD62P IPA in Anastrozole-treated T47D cells exposed to PRP, indicating a role for cumulative Anastrozole treatment in the induction of a hypercoagulatory environment dependent on Model and subphenotype.

Table 4.4: Tabulation showing *r* values obtained from the nonparametric Spearman's correlation between CD62P, CD63 IPA and ER α and ER β , with thrombin detection for MCF7 and T47D cells. Key: MMEDWB: MCF7 cells exposed to WB. MMEDPRP: MCF7 cells exposed to PRP. MANASWB: Anastrozole-treated MCF7 cells exposed to WB. MANASPRP: Anastrozole-treated MCF7 cells exposed to PRP. WBANASM: MCF7 cells exposed to Anastrozole-treated WB. PRPANASM: MCF7 cells exposed to Anastrozole-treated PRP, TMEDWB: T47D cells exposed to WB. TMEDPRP: T47D cells exposed to PRP. TANASWB: Anastrozole-treated T47D cells exposed to WB. TANASPRP: Anastrozole-treated T47D cells exposed to PRP. WBANAST: T47D cells exposed to Anastrozole-treated WB. PRPANAST: T47D cells exposed to Anastrozole-treated PRP. Red *p*<0.05 compared to thrombin detection, italics *p*<0.05 compared to CD62P IPA, bold *p*<0.05 compared to CD63 IPA. Controls: MMEDWB, MMEDPRP, TMEDWB, TMEDPRP, are for reference purposes.

Spearman Rank Order Correlations																	
Highlighted correlations are significant at <i>p</i> <0.05. <i>r</i> =0.6																	
	Overall	Control				Model 1						Model 2					
		MME DWB	MME DPRP	TMED WB	TMED PRP	MCF7 Overall	T47D Overall	MANA SWB	MANA SPRP	TANA SWB	TANAS PRP	MCF7 Overall	T47D Overall	WBAN ASM	PRPAN ASM	WBAN AST	PRPA NAST
Thrombin Generation																	
CD62P IPA	-0,123	-0,314	0,086	-0,029	0,257	-0,23258	0,07232 5	-0,486	0,257	-0,086	0,600	-0,27676	- 0,07553 7	0,543	0,029	-0,257	0,100
CD63 IPA	<i>-0,091</i>	-0,314	<i>0,029</i>	<i>0,314</i>	<i>0,429</i>	-0,18836	0,08592 3	-0,486	0,257	-0,029	0,486	-0,24654	- 0,04452 8	-0,143	-0,657	-0,314	0,100
ER- Alpha	0,020	-0,714	0,029	0,257	0,257	0,077686	0,01736 5	-0,600	0,257	0,029	0,771	0,13707	0,00912 06	0,314	0,486	0,429	0,100
ER- Beta	-0,154	-0,200	-0,265	-0,257	0,143	-0,1047	- 0,07875 2	-0,486	-0,486	0,200	0,714	-0,18243	- 0,02870 6	-0,657	-0,543	0,771	0,000

4.4 Discussion

Platelets are known to play a key role in tumour invasion and metastasis, and through this facilitation cause an increase in both tumour markers and coagulatory mechanisms leading to thromboembolic complications (Rugo, 2016). In addition to being exposed to hormone-therapy within the circulation, platelets have been found in the extravascular tumour microenvironment where hormone-therapies target tumour cells (Li *et al.*, 2014; Plantureux *et al.*, 2020). Anastrozole is a targeted hormone-therapy used as part of standard adjuvant treatment regimens, however, it has been associated with an increase in patients risk for suffering a thromboembolic event compared to that of healthy individuals (Miller *et al.*, 2005; Fabian., 2007; Masakazu *et al.*, 2009). Furthermore, Anastrozole may be given in combination with other hormone-therapies as evidenced by the ATAC randomized trial, which indicated a role for Anastrozole in reducing breast cancer recurrence rates (Hillner, 2004; Howell *et al.*, 2005). The molecular relationship between reduced E2 conversion and thus reduced binding to ERs through aromatase inhibition, thrombin release and resulting platelet activation is not fully understood. Thus, the aim of this chapter was to determine the effect of cumulative and circulating Anastrozole on ER expression and their relationship to hypercoagulation *in vitro*.

Cancer metastasis is the leading cause of cancer mortality, with thrombotic complications due to hypercoagulation following closely behind (Caine, 2002). A hypercoagulable state, also known as a prothrombotic state, in malignant cancers occurs when tumour cells are able to activate the coagulation system and cause thrombi; formed by intravascular platelet aggregates (Caine, 2002; Bauer, 2019). By separating and identifying blood constituents we were able to isolate baseline levels of coagulation in these samples; however, methodologically, soft centrifugation to obtain PRP and PPP resulted in heightened levels of platelet activation, though this did not prevent induction of activation further by the addition of thrombin (Augustine *et al.*, 2016). Our results indicated platelets from PRP being more active with heightened levels of CD62P and CD63 IPA, confirmed ultrastructurally; and indicated greater levels of detectable thrombin, compared to that of WB. PRP exposed to both breast cancer cells and Anastrozole-treatment (both Models) also indicated the greatest levels of CD62P and CD63 marker expression as well as heightened levels of detectable thrombin.

Cumulative and circulating Anastrozole effects were assessed by mimicking the physiological environment of breast cancer and hormone-therapy treatment. By employing these Models, this allowed us to investigate specific responses of not only the breast cancer cells but the platelets as well, with reduced interference in a more controlled environment. However, it should be noted that this approach may have technical and methodological implications potentially affecting the outcomes. Anastrozole is commonly used as a second-line therapy in adjuvant treatment strategies in postmenopausal women (Mokbel, 2002; Campos, 2004; Brueggemeier, Hackett and Diaz-Cruz, 2005; Hole *et al.*, 2015); however, its associated cytotoxicity through ADP induction (Hassan *et al.*, 2017), still plays a role in facilitating platelet involvement. Studies have shown that hormone-therapies, including Anastrozole, induce heightened sensitivity to thrombin by upregulating the PAR-1 receptors, further increasing a women's risk of suffering a thromboembolic event (Jick *et al.*, 2000). Additionally, breast cancer cells are able to secrete and induce the release of procoagulant factors, such as thrombin and TF (Reddel., 2019). Thrombin plays a key role in the coagulation cascade and is a potent inducer of platelet activation (Herkert *et al.*, 2001). Our results indicate the ability of breast cancer cells to secrete thrombin, dependent on phenotype and exposure of blood constituent; with T47D cells secreting significantly higher levels of thrombin compared to that of MCF7 cells. This was further reiterated in Anastrozole-treated samples, which indicated an increase in thrombin generation and binding in breast cancer cells treated with Anastrozole (Model 1); however, this was dependent on both the cell line employed and blood constituent evaluated, with T47D cells under Anastrozole treatment (Model 1) decreasing thrombin generation compared to Model 2 (blood constituents treated with Anastrozole). However, this did not hinder Anastrozoles effect on breast cancer cell induction of a hypercoagulatory environment which still remained high compared to T47D cells alone. Furthermore, Anastrozole-treated T47D cells induced later stages of activation as evidenced by ultrastructural assessment, where fibrin networks and plaques were formed; however, this reflected as a loss in CD62P IPA and persistence of CD63 IPA which suggested hypercoagulation. The formation of fibrin was noted in samples that had not had exogenous thrombin added to it; however, the levels of available thrombin was heightened and indicates induction of a highly hypercoagulatory state (Pate and Mankowski, 2011); substantiating studies which indicate Anastrozole treatment and its' clinical association with high endogenous thrombin potential as well as greater numbers of CD62P positive microparticles

(Trappenburg *et al.*, 2009). Furthermore, activated platelets undergo morphological changes including release of granules; CD62P initially followed by CD63, at full activation platelets release membrane granules through budding facilitating loss of the granule, specifically CD62P from the α -granules (Augustine *et al.*, 2016; Smith *et al.*, 2018). CD63, a component of platelet lysosomes which is also released from platelet dense granules during this process, is involved in preventing degradation of the plasma membrane and mediates later events including fibrinolysis (Nishibori *et al.*, 1993; Marquardt *et al.*, 2002). Furthermore, upon achievement of full activation, fibrin networks and meshwork are formed obscuring aggregated platelets and creating a thrombus (Nash *et al.*, 2002; Pretorius *et al.*, 2007, 2011; Falanga, Russo and Verzeroli, 2013; Mitrugno *et al.*, 2014) allowing for a decrease in membrane expression of markers of activation (Swieringa *et al.*, 2018).

Clinically, studies have indicated an increase in platelet number in breast cancer patients compared to those of healthy individuals (Carpenter *et al.*, 2006). Platelets play a role in facilitating tumour metastasis contributing to steps in cancer metastasis, including facilitating tumour cell migration, invasion and arrest within the vasculature (Felding-Habermann *et al.*, 2001; Li *et al.* 2014; Qi *et al.* 2015; Mezouar *et al.* 2016; Michael *et al.* 2017; Schlesinger 2018). Studies have shown, that both activated platelet membranes and platelet release increase the invasion capacity of cancer cells (Holmes *et al.*, 2009). Hormone-dependent breast cancer cells synthesize inflammatory cytokines which mediate the recruitment of leukocytes, such as platelets and stimulate the production of thrombin *in vivo* (Chen and Geng, 2006). An increase in thrombin allows for an increase in platelet activation and the formation of aggregates, as noted in our results. Furthermore, levels of platelet activation and platelet progression were noted to be different between MCF7 and T47D cells; with platelet activation associated with T47D cells being at later stages. Both MCF7 and T47D breast cancer cells are implicated in the secretion of PAF under thrombin stimulation (Bussolati *et al.*, 2000; da Silva Junior *et al.*, 2018). Platelet-activating factor, an inflammatory phospholipid acts through PAF-R which mediates platelet aggregation and degranulation (Zimmerman *et al.*, 2002), supporting the increase in platelet activation markers seen in our results. Furthermore, the T47D breast cancer cell line is said to be more aggressive than that of MCF7 cells due to the presence of proteins associated with tumourigenesis (Adjo Aka and Lin, 2012). Additionally, Anastrozole treatment, regardless of Model heightened the capacity of T47D breast cancer

cells to induce platelet activation and the formation of fibrin, further indicating its' association with an aggressive phenotype. Aggressive tumours recruit platelets even before they enter the circulatory system, activated platelets secrete proteolytic enzymes, including heparanase and MMPs, aiding in the process of fibrinolysis which is a key component in metastasis and is essential for the release of tumour cells from their primary site (Cesarman-Maus and Hajjar, 2005; Mosesson, 2005; Chapin and Hajjar, 2015).

Malignant invasive tumour cells are able to enter the circulation even before the tumour has been diagnosed (Massagué and Obenauf, 2016). Research on metastatic colonization and invasion capacity of cells is not well understood. To further classify differences noted within cell lines, we assessed ERs as a marker of invasion and progression and subphenotypic alterations. ER α has been extensively studied with its association with breast cancer well known; Anastrozole was designed to affect oestradiol conversion through aromatase enzyme inhibition within the body (Carey *et al.*, 2006). ER β , however, is not well studied but is said to oppose the proliferative and pro-tumourigenic effects of ER α (Carey *et al.*, 2006); our results highlight a switched response in different hormone-dependent cell lines, specifically MCF7 and T47D cells, with T47D cells exhibiting a higher ER β receptor profile regardless of treatment or blood constituent co-incubation. Our results indicated greater ER β expression in T47D cells, further highlighting its propensity as a more invasive phenotype (Ogba *et al.*, 2014; Mota *et al.*, 2017). Furthermore, T47D cells treated with Anastrozole; of both models indicated an increase in ER β expression, compounded by WB, PRP and PPP, correlating not only with *ESR2* gene expression in Chapter 2; but with clinical studies which indicate the upregulation of ER β expression in response to Anastrozole treatment, with significant decrease noted in the expression of ER α (Torrise *et al.*, 2007). This indicates a role for ER β in tumour progression, correlating with other studies through the regulation of WISP-2 a pro-tumourigenic gene (Tee *et al.*, 2004; Chai *et al.*, 2019). This ability of breast cancer cells to alter their ER profiles, particularly ER α downregulation, in response treatment strategies, blood constituents and models highlight their metastatic potential and invasive capacity. ER α downregulation is characteristically associated with the transition to a more aggressive phenotype that does not respond to therapy (Begam, Jubie and Nanjan, 2017), and is further noted in breast cancer cells during the metastatic process (Aktas *et al.*, 2011).

Our results indicate that Anastrozole is able heighten levels of induced hypercoagulation, dependent on the cell line. This level of induced hypercoagulation was not hampered by the model employed, with both circulatory and cumulative models inducing comparative levels of activation in the T47D cell line. The heightened level of hypercoagulation is noted by the formation of fibrin networks, the formation while indicating a highly inflammatory state, also plays a role in tumour cell migration by providing a matrix framework (Kwaan and Lindholm, 2019). These results further indicate a correlation between thrombin detection and ER α and thrombin detection and CD62P IPA in Anastrozole-treated T47D cells exposed to PRP (Model 1); indicating a role for cumulative Anastrozole treatment in the induction of a hypercoagulatory environment dependent on Model and subphenotype and substantiate clinical studies, which indicate that patients with breast cancer are more likely to suffer from thromboembolic events (Scully *et al.*, 2007); and studies which indicate an increase in the metastatic potential of breast cancers (Reinert *et al.*, 2017). However, this study is limited in that it did not assess markers of tumour invasion; which could be included for future studies with respect to the expression of ER β and its potential as a pro-tumourigenic marker as well. These results further substantiate results obtained in both Chapter 2 and 3, indicating the procoagulatory effects of breast cancer, dependent on phenotype with the T47D breast cancer cell line representing cells of a more invasive phenotype. Furthermore, these results provide much needed evidence on Anastrozole effects on ERs and hypercoagulation, the research on which is lacking, as well as highlights the need to assess hormone-receptor profile subphenotypes prior to Anastrozole treatment clinically; as a receptor profile similar to that of T47D cells, may induce levels of thrombosis at alarmingly fast rates.

5 CHAPTER 5: Discussion

5.1 Introduction

Breast cancer has become the most commonly diagnosed cancer in women worldwide (Akram *et al.*, 2017), with incidence rates in developing countries steadily increasing (Jemal *et al.*, 2011; Youlden *et al.*, 2012). South Africa, in particular, has one of the highest incidence rates of female breast cancer with lifetime risks of developing breast cancer ranging from 1 in 81 African women to 1 in 13 Caucasian women (Vorobiof *et al.* 2001, Herbst 2013; Singh *et al.* 2015; Schonfeld *et al.* 2016). Furthermore, the risk of development of breast cancer is increased in women over the age of 40 and menopausal women, further heightened by hormone replacement therapy (Kumle, 2008; Kotsopoulos, 2019). Treatment strategies for women presenting with breast cancer are defined by the molecular subtype and stage of the disease (Carey *et al.*, 2006; Dai *et al.*, 2017); with luminal breast cancers commonly treated using hormone-therapies which target oestrogen-induced proliferation itself (Bonnetterre *et al.*, 2000; Carey *et al.*, 2006; Burstein *et al.*, 2019).

5.2 Breast Cancer Induced Hypercoagulation

Breast cancer itself is associated with an increased risk for thrombotic complications, with hormone-therapeutic approaches increasing this risk further (Meier and Jick, 1998; Decensi *et al.*, 2005; Howell *et al.*, 2005; Falanga, Russo and Verzeroli, 2013). Studies have shown that cancers of the lung, breast and prostate increase a patients' risk of suffering from a venous thromboembolic event (Scully *et al.*, 2007). The link between thrombosis and cancer is further indicated with 5-10% of cardiovascular disease patients presenting with VTE being at a higher risk of a cancer diagnosis at least two years after their initial presentation (Nash *et al.*, 2002; Sandén, Svensson and Sjölander, 2017). While an increase in age is a proposed factor in the association between VTE and initial cancer diagnosis (Sandén, Svensson and Sjölander, 2017), studies indicate the diagnosis of unprovoked VTE to be the first manifestation of an undiagnosed cancer (Carrier *et al.*, 2016; Ihaddadene *et al.*, 2016; Khan *et al.*, 2017). Furthermore, while diagnosed VTEs are treated using antiplatelet therapies, these do not necessarily reduce platelet number but rather act to reduce platelet aggregation (Flumignan, Flumignan and Baptista-Silva, 2016). This allows for an increase in platelet number within the vasculature, which increases the number of platelets able to extravasate into the tumour

microenvironment, facilitating tumour cell engagement and reciprocal platelet activation (Li *et al.*, 2014; Plantureux *et al.*, 2020). Furthermore, this reciprocal interaction between tumour cells and platelets, as well as other immune cells via cytokine secretion in the tumour microenvironment could be regarded as a local pro-inflammatory response, potentially progressing to systemic inflammation through metastasis (Li *et al.*, 2010; Plantureux *et al.*, 2018). However, luminal phenotype breast cancer is not considered a disease of inflammation, and therefore the treatment of pro-inflammatory responses does not form part of the standard management regimen clinically.

Thromboembolic complications have been identified as the second leading cause of death in breast cancer patients, implicating the interactions between tumour cells and platelets in the induction and facilitation of metastasis (Karimi and Cohan, 2010; Bambace and Holmes, 2011; Mezouar *et al.*, 2014). In the present study, in Chapter 2 which represented the MSc.Med portion of this study prior to conversion to PhD, results firstly indicated an increase in platelet activation, as identified by CD62P in WB samples induced by breast cancer cells alone, substantiating clinical studies (Onitilo *et al.* 2012; Zarà *et al.* 2018; Kedzierska *et al.* 2013). CD62P is a surface antigen expressed on the cell membrane of α -granules and also facilitates platelet adhesion (Chen and Geng, 2006; McNicol and Israels, 2008; Yun *et al.*, 2016); as noted in this study CD62P was lost during the latter part of the activation process, as denoted by a decrease in IPA corresponding with samples visualised (ultrastructurally). During platelet activation, platelets undergo morphological changes and release granular contents (Haemmerle *et al.*, 2018; Smith *et al.*, 2018). This loss of CD62P was therefore due to the process of platelet activation itself in which microparticles would be shed from the platelet membrane in order to facilitate further progression of platelet activation, including recruitment for aggregation (Yun *et al.*, 2016) and substantiates clinical studies which indicate an increase in microparticles in patients with cancer (Trappenburg *et al.*, 2009). Chapter 3 and 4 present the expanded scope of this study, in which we included the use of blood constituents (WB, PRP and PPP) and introduced an additional platelet activation marker, CD63 as well as assessed detectable levels of the agonist, thrombin within samples. CD63 is released from platelet dense granules following activation and plays a role in mediating later events (Nishibori *et al.*, 1993; Marquardt *et al.*, 2002); however, our results indicated a continued expression of CD63 throughout the process of activation and, in some cases,

aggregation as well. This indicates that sustained CD63 expression may play a greater role in the recruitment and facilitation of platelet aggregation, fibrin formation and fibrinolysis, which is shown to play a role in cell migration by providing a matrix and by interactions with adhesion molecules (Kwaan *et al.*, 2019). Furthermore, by making use of the IPA we present a calculation to assess CD62P or CD63 marker expression in relation to the number of platelets expressing the respective marker, at earlier stages of activation preceding aggregation which may impact tumour processes (Leytin *et al.*, 2000; Augustine *et al.*, 2016; Pather *et al.*, 2019), different to that of aggregometry which remains the gold standard for platelet aggregation in disease states (Ling *et al.*, 2017). Additionally, while we had proposed using the ratio of activation markers (CD62P/CD63) as an indicator for progression of platelet activation through the early through to later stages, results obtained indicated its inefficiency when analysed with corresponding morphology. This indicated later stages of activation are not corroborated by the ratio but is most likely due to the sustained presentation of CD63, however, even though IPA remains meaningful, this aspect provides a limitation of this study which requires further investigation.

Platelets play a crucial role in tumour metastasis (Massagué and Obenauf, 2016), facilitating tumour cell migration, invasion and arrest within the vasculature (Felding-Habermann *et al.*, 2001; Li *et al.* 2014; Qi *et al.* 2015; Mezouar *et al.* 2016; Michael *et al.* 2017; Schlesinger 2018). Furthermore, activated platelets release proteolytic enzymes, such as heparanase and MMPs, aiding in the breakdown of the basement membrane increasing this metastatic potential (Huang *et al.*, 2002). Our results indicate an increase in not only platelet activation but thrombin detection, induced by breast cancer cells, dependent on subphenotype. Furthermore, breast cancer cells were able to generate thrombin comparative to that of WB alone, however, this was dependent on phenotype with T47D cells able to induce greater levels of thrombin generation. The ability of breast cancer cells to secrete and generate thrombin is an important mechanism of not only of the activation process, but of TCIPA, and is thus involved in stimulating tumour growth and aiding metastatic cell survival in circulation (Palacios-Acedo *et al.*, 2019). Breast cancer cells are also able to synthesise inflammatory cytokines which mediate the recruitment of leukocytes, platelets and further stimulates the production of thrombin (Chen and Geng, 2006; Falanga *et al.*, 2013; Bauer, 2019). Furthermore, both MCF7 and T47D cell lines are able to secrete PAF under thrombin

stimulation, mediating platelet aggregation and degranulation (Bussolati et al., 2000; Zimmerman et al., 2002; da Silva Junior et al., 2018), which supports the increase in platelet activation noted within our results.

In the vascular system, platelet interaction with tumour cells contributes to metastasis through formation of heterotypic aggregates, shielding tumour cells from natural killer cell identification, endothelial attachment and the release of angiogenic and growth factors assisting tumour cell invasion; however, their role in the microenvironment is not well defined (Yan et al., 2016; Li et al., 2014; Planteureux et al., 2020). Platelets are also able to extravasate into the tumour microenvironment across the vasculature and initiate direct contact with tumour cells through secreted cytokines, indicating a local pro-inflammatory response (Li et al., 2014; Yan and Jurasz, 2016; Planteureux et al., 2020). Breast cancer cell line studies have shown an increase in cellular invasiveness following exposure to activated platelets and their granules (Labelle et al., 2011; Lal et al., 2013). TF and thrombin constitute major components of the coagulation cascade which contribute to breast cancer progression, with TF mediated thrombin generation noted to contribute to the more invasive and procoagulant phenotype seen in breast cancer patients (Bluff et al., 2008; Lal et al., 2013). Our results indicate an increase in the detectable levels of thrombin, and hypercoagulation, induced by breast cancer cells following exposure to blood constituents; WB, PRP and PPP; dependent on subphenotype with T47D cells inducing the greatest levels. These results substantiate studies indicating primary tumour cell engagement facilitating metastatic processes through the induction of hypercoagulatory processes (Li et al., 2014; Planteureux et al., 2020). Furthermore, this indicates a role for platelets in tumour growth through their activation by tumour cells and secretion of TGF- β , VEGF and platelet derived growth factor (PDGF); which are further involved in angiogenesis and tumour vascularisation (Palacios-Acedo et al., 2019). The results further indicate that while MCF7 and T47D cells represent luminal phenotype breast cancers, that their capacity to induce variable levels of hypercoagulation point to subphenotypes that would clinically, be disregarded. Our results further stress altered responses by these subphenotypes to hormone-therapy.

5.3 Hormone-therapy compounds pro-coagulatory effects of breast cancer

Anastrozole and Tamoxifen are targeted hormone-therapies used as standard adjuvant treatment for post and pre-menopausal women, respectively (Carey *et al.*, 2006; Ogba *et al.*, 2014; Turashvili and Brogi, 2017). Anastrozole, an AI, binds the aromatase enzyme and prevents the conversion of androgens to oestrogens, thereby decreasing hormone-dependent breast cancer proliferation (Miller *et al.*, 2005). Anastrozole has also been associated with lower thrombotic risk in patients compared to the risk associated with breast cancer itself (Fennerty, 2006; Fabian, 2007; Gonnelli and Petrioli, 2008). Tamoxifen, a SERM, is widely used and is associated with an increased risk for thrombosis (Decensi *et al.*, 2005; Howell *et al.*, 2005; Dobryднева *et al.*, 2007; Lin *et al.*, 2018). Initial results obtained in the present study indicated heightened levels of early stage platelet activation, identified by CD62P marker expression, induced by breast cancer cells and compounded by Anastrozole and Tamoxifen pre-treatment. This was again, dependent on the cell line employed. Anastrozole pre-treatment of T47D cells induced the greatest levels of platelet activation as evidenced by the loss of CD62P expression as well as the formation of thick fibrin fibres obscuring platelets, ultrastructurally. As presented by our lab previously and discussed above, CD62P marker expression was lost during the activation process due to microparticle shedding, therefore marker expression analysis was coupled with ultrastructural analysis to elucidate clearer results (Augustine *et al.*, 2016; van der Spuy and Augustine, 2016; Pather *et al.*, 2019). The formation of fibrin, without the addition of exogenous agonists, indicates induction of a highly hypercoagulatory and possibly, inflammatory, state associated with Anastrozole and more aggressive subphenotypes of luminal breast cancer (Pate and Mankowski, 2011). These initial results correlated with clinical studies indicating that hormone-therapies, particularly Tamoxifen, are associated with a greater risk for developing VTE's (Vitseva *et al.*, 2005; Fennerty, 2006; Walker *et al.*, 2016); and they are contradictory to laboratory-based studies indicating inhibition of platelet activation by Tamoxifen (Chang *et al.*, 2011; Johnson *et al.*, 2017). We had postulated the difference to the aforementioned laboratory studies to be attributed to methodological differences, including differences in Tamoxifen concentration used, platelet washing and not having directly treated the platelet component with Tamoxifen. We thus expanded the scope of this study to include the use of more defined blood constituents (WB, PRP and PPP) and employed models to mimic the physiological environment; Model 1 involved the cumulative effects of hormone-therapy,

where they would accumulate within the tumour microenvironment with the introduction of blood constituents, and Model 2 involved the circulatory effects of hormone-therapies in which blood constituents were treated directly and exposed to breast cancer cells to assess the impact of different *in vitro* models on outcomes. Isolating blood components allowed for assessment of the specific effects of high platelet numbers and plasma to better understand hormone-therapy effects on platelet activation, platelet number as well as thrombin potential.

Modelling the physiological environment allows for an investigation of cellular responses in a more controlled environment; however, this also poses a limitation to this study, in which outcomes will be limited due to methodologies, including generation of PRP and PPP through soft centrifugation resulting in, albeit low, platelet activation and subsequent thrombin availability. Our results, however, still investigated these baseline levels of platelet activation in blood constituents as identified by CD62P and CD63 IPA. Platelet activation and thrombin availability within the PRP component was higher than that of WB, as expected due to granular release from platelets as part of the methodology; however, the soft centrifugation did not hinder the ability of platelets to react to exogenous agonists (Haemmerle *et al.*, 2018). Furthermore, the induced granular release in platelets within PRP, as well as greater levels of available thrombin was expected to further compound breast cancer-cell induced hypercoagulation. This was noted within our results, with heightened levels of hypercoagulation induced when treated with hormone-therapy. Additionally, for this study blood was collected from healthy female volunteers between days 1 – 10 of the menstrual cycle due to the lower levels of oestrogen and progesterone present (Reed and Carr, 2000) to limit the action of these hormones. In this way we limited the effects of platelet responses to changes in the menstrual cycle; however, this nevertheless could be a limiting factor in this study (Chen *et al.*, 2014).

Hormone-therapies are able to induce heightened sensitivity to thrombin by upregulating the PAR-1 receptors (Jick *et al.*, 2000), facilitating platelet activation, thereby inducing a hypercoagulatory environment and increasing the risk of developing thromboembolic complications. The results obtained in this study indicate an increase in levels of induced hypercoagulation, defined by levels of detectable thrombin and platelet activation, in breast

cancer cells treated with either Tamoxifen or Anastrozole. This level of induced hypercoagulation was not hampered by the model employed, with both circulatory and cumulative models inducing comparative levels of activation. However, as noted in our initial study, Anastrozole heightened induced levels of hypercoagulation by T47D cells with IPA indicating a loss of CD62P IPA and sustained CD63 IPA regardless of blood constituent exposure, with ultrastructural analysis indicating the formation of fibrin networks and aggregated platelets; indicating an irreversible process of platelet plug formation (Wagner and Burger, 2003; Gresele and Page, 2002; Kuwahara *et al.*, 2002; Pretorius *et al.*, 2007, 2011 Tomaiuolo *et al.*, 2018). However, it should be noted that during data acquisition the FEI 600 Nova SEM had broken with the FEI 400 SEM taking its' place, as such image quality between remaining samples can be seen to be reduced.

Tamoxifen induced greater levels of hypercoagulation in the T47D cell line as well, compared to that of the MCF7 cell line, with the presence of fibrin networks and detectable thrombin noted to be higher than induction by cells alone. These results substantiate clinical studies indicating that patients with breast cancer are more likely to suffer from thromboembolic complications, heightened by hormone-therapy (Nash *et al.*, 2002; Cesarman-Maus and Hajjar, 2005; Chapin and Hajjar, 2015). This further substantiates our initial study with both Model 2, where the treatment of blood constituents directly, did not hinder the capacity of hormone-therapy to heighten breast cancer cell-induction of a hypercoagulatory environment. Thus, we propose differences noted within contradictory studies (Chang *et al.*, 2011; Johnson *et al.*, 2017) to be due to more intricate methodologies, particularly the process of washing, commonly used in the assessment of platelet function. Both studies involved platelet washing and indicated Tamoxifen exerted potent activity in inhibiting platelet aggregation when stimulated by collagen (Chang *et al.*, 2011), and also showed that pre-treatment of washed platelets with Tamoxifen led to significant inhibition of platelet activation (Johnson *et al.*, 2017). While our expanded study involved the pre-treatment of blood constituents, this did not hinder the induction of a hypercoagulatory environment; however, we did not employ the process of pre-washing platelets. A fundamental concern is that during the washing process, the loss of plasma which consists of proteins including fibrinogen is noted and prevents the capacity for tumour-cell induced platelet activation and subsequent aggregation (Zarà *et al.*, 2018). As such, in addition to the use of a range of

concentrations of Tamoxifen (3 μ M – 20 μ M), the studies conducted by Chang *et al.*, and Johnson *et al.*, (Chang *et al.*, 2011; Johnson *et al.*, 2017) may have found conflicting results to ours and Zara (Zarà *et al.*, 2018), primarily due to the use of washed platelets.

5.4 Breast Cancer Heterogeneity and Oestrogen Receptors

Breast cancers of the luminal phenotype are treated holistically as ER-positive due to their overexpression of ERs in 75% of breast cancers (Pal, Childs and Pegram, 2011; Williams and Lin, 2013; Hashmi *et al.*, 2018); with ER α and ER β not readily identified (Haldosén, Zhao and Dahlman-wright, 2014). ER α has been extensively studied and indicates a positive prognostic outcome in patients treated with hormone-therapies (Giacinti *et al.* 2006; Altundag & Ibrahim 2006; Bahreini *et al.* 2017; Begam *et al.*, 2017); while ER β has not been as extensively studied and has no clearly identified clinical links to breast cancer prognosis (Haldosén, Zhao and Dahlman-Wright, 2014). Studies indicate, however, that ER β which is expressed in both benign and malignant breast tissue but at lower levels in the majority of breast cancer cells, can oppose the actions of ER α (C Palmieri *et al.*, 2002; Girgert, Emons and Gründker, 2019). While contradictory evidence exists, indicating a role for ER β in tumour suppression through its regulation of genes *p53* and *PTEN* (Dey *et al.*, 2013); and a role in tumour progression through the regulation of *WISP-2* (Tee *et al.*, 2004; Chai *et al.*, 2019), the actual role of ER β remains ill-defined.

Breast cancer heterogeneity, typically defined by selected marker expression denoting the major subtypes (e.g. luminal A, luminal B, luminal B-like, HER2 positive basal-like and normal-like tumours), plays a key role in diagnosis and treatment (Carey *et al.*, 2006; Dai *et al.*, 2017). However, intertumoral heterogeneity exists between similar types of tumours of the same origin in numerous patients, indicating more specific subphenotypes; while intratumoral heterogeneity also exists and consists of phenotypically different cell populations within the same tumour (Januškevičienė and Petrikaitė, 2019). These forms of heterogeneity indicate the ability of the tumour cells to adapt to the ever-changing microenvironment; however, this is not taken into account clinically and biopsied samples from primary tumours may not be accurately representative of late tumour stages or of how the tumour responds to treatment. During tumour progression cells acquire characteristics or heighten expression of markers

including TGF- β , promoting tumour growth and indicating cells of a more invasive phenotype, which further impacts the efficacy and response to treatment (Visvader, 2011; Januškevičienė and Petrikaitė, 2019). Due to clinical breast cancer heterogeneity, multiple cell line models of breast cancer subtypes are required for *in vitro* studies.

Luminal A breast cancers are modelled by the MCF7 and T47D cell lines; however, while T47D cells express greater PR and lower ERs compared to MCF7 cells; in a clinical scenario, tumours presenting these biomarkers would be diagnosed and treated the same (Ogba *et al.*, 2014; Mota *et al.*, 2017). The initial study, as presented in Chapter 2, assessed ER encoding genes, *ESR1* and *ESR2*, which encode ER α and ER β , respectively (Strom *et al.*, 2004) to determine profile alterations in response hormone-therapy, and whether these alterations could be associated with early platelet activation. Results indicated holistically, greater *ESR1* expression in both MCF7 and T47D cells compared to *ESR2*, as expected. However, on further assessment it was noted that *ESR2* expression was greater in the T47D cell line than that of the MCF7 line. Furthermore, when exposed to either WB or pre-treated with Tamoxifen and exposed to WB, the MCF7 cell line was able to alter its receptor profile to that of a more *ESR2* positive profile. This was further noted in T47D cells exposed to either WB or pre-treated with Anastrozole. This switched response in *ESR* expression, to that of a more *ESR2* positive profile indicates cell line differences in response to hormone-therapy, with studies suggesting greater *ESR2* expression being associated with a decrease in ER-associated proliferation and invasion (Ghali *et al.* 2018; Strom *et al.* 2004; Roger *et al.* 2001; Lazennec *et al.* 2001). A limitation of the present study is that assessment of those parameters did not form part of our question, with the focus rather on induced hypercoagulation. As such, our results indicate a greater association of a switched profile to *ESR2* positive with heightened platelet activation. This was particularly evident induced by T47D cells under Anastrozole pre-treatment, with greater platelet activation, as noted ultrastructurally; indicating a potential role for *ESR2* in predicting hypercoagulatory states. The results also showed a correlation between *ESR1* expression and early platelet activation in T47D cells exposed to WB; further highlighting the prothrombotic nature of the T47D cell line itself and indicating a role for *ESR1* associated with platelet activation, potentially different to that of *ESR2*. These results indicate an association between ER gene expression and platelet activation dependent on breast

cancer cell subphenotype; however, the role of each ER in platelet activation required more investigation.

We thus expanded the scope of this study to investigate ER α and ER β protein expression in hormone-dependent breast cancer cells and their relationship to hypercoagulation, altered by Anastrozole and Tamoxifen treatment. Furthermore, we had intended to use the MCF10A mammary cell line as a control cell line in the expanded scope of the study to model “normal” mammary tissue. However, discrepancies were found in its ability to not only heighten platelet activation but to not only express but alter its ER α and ER β expression in response to blood constituents and as such, we were unable to use this cell line as a control in this study. These results substantiate studies which show transformation of MCF10A cells to a more tumour-like phenotype and indicate that further research is required into the MCF10A cell line and whether it should be used as a standard for modelling normal breast epithelial tissue (Rhee *et al.*, 2008; Imbalzano *et al.*, 2009; Qu *et al.*, 2015).

Our results indicate greater ER β expression in the T47D cell line coupled with heightened levels of hypercoagulation, particularly under Anastrozole treatment, regardless of Model employed, compared to the MCF7 cell line. This reflects the *ESR* results obtained and substantiates studies implicating ER β in tumour progression (Hartman, Ström and Gustafsson, 2009; Begam, Jubie and Nanjan, 2017). This study identified that T47D cells also secrete thrombin at greater levels than that of MCF7 cells; thus in addition inducing a hypercoagulable microenvironment, the facilitation of platelet activation of platelets could also be accompanied by the release of growth factors including TGF- β , VEGF and PDGF known to induce tumour growth (Palacios-Acedo *et al.*, 2019). We thus propose that our results highlight the aggressive nature of T47D cells; as indicated by its induction of a hypercoagulable state which may facilitate tumour growth. However, while hypercoagulation may be further heightened by the addition of Tamoxifen or Anastrozole, and while ERs can indicate the proliferative capacity of breast cancer cells with up/downregulation facilitating associated outcomes, including tumour progression, our study is limited in that we did not assess markers of invasion or those with EMT, including TGF- β and VEGF; this, however, can be a future avenue for further studies due the associations noted. Thus, further research is

required to determine what the associated long-term effects are on tumour growth and recurrence may be when breast cancer cells are exposed to the vascular system.

Tamoxifen directly targets ERs, as such, breast cancer cells employ a strategy by which they downregulate their receptor in an attempt to reduce binding and is associated with the transition to a more aggressive phenotype that does not respond to therapy (Begam, Jubie and Nanjan, 2017). Our results indicate that Tamoxifen-treatment of breast cancer cells (Model 1) induced a translocation of ER α to the nucleus, and greater ER α expression within the cytoplasm as well; however, circulating effects of Tamoxifen-treated blood constituents (Model 2) induced greater ER β expression in the nucleus of breast cancer cells; particularly Tamoxifen-treated WB exposed to T47D cells. These results indicating a translocation of ER α , further substantiate ER α downregulation in response to ER directed therapies, including Tamoxifen (Begam, Jubie and Nanjan, 2017), with the noted increase in ER β expression suggesting an opposing role or potentially not being targeted by Tamoxifen due to sharing only a similarity of 56% of their ligand binding domains (Williams and Lin, 2013). Furthermore, conflicting results on ER β exist, with recent studies implicating high ER β expression in breast tumour development and Tamoxifen-resistance (Begam, Jubie and Nanjan, 2017) and other studies implicating low ER β expression in Tamoxifen-resistant tumours (Fox et al., 2008) and better prognostic outcomes opposing the actions of ER α (Carey *et al.*, 2006). Our results indicate an association between heightened ER β expression with greater levels of hypercoagulation known to be induced to facilitate tumour progression; indicating a potential role in Tamoxifen resistance. However, our study is limited, and this relationship requires further investigation in growth factors including epidermal growth factor receptor (EGFR), known to be overexpressed in Tamoxifen-resistant tumours (Viedma-Rodríguez *et al.*, 2014); and provides a future direction for this study. Conversely, Anastrozole, regardless of Model and blood constituent exposure indicated an increase in ER β expression in T47D cells, reflecting *ESR2* expression noted initially, and further highlighting its aggressive nature and potential invasive profile associated with hypercoagulation as evidenced by a loss of platelet activation marker expression and the formation of fibrin networks. These results correlate with clinical studies which indicate an increase in ER β expression in response to Anastrozole treatment (Torrise *et al.*, 2007); and substantiate clinical studies which indicate that hormone-

therapies targeted against reducing oestradiol in the body, heighten the possibility of thrombotic events (Jayachandran *et al.*, 2005; Miller *et al.*, 2005; Fabian., 2007; Masakazu *et al.*, 2009). While Anastrozole does not directly target ERs but rather targets the availability of oestrogen (Fabian, 2007), these results indicating an increase in ER β in response to Anastrozole treatment further highlights its upregulation associated with hypercoagulation and may indicate a role for AIs, as evidenced by its consistent induction of hypercoagulation with T47D cells, in the initiation of tumour progression contradicting clinical evidence indicating its role in lower thrombotic risk than that associated with cancer itself (Fabian, 2007). The results further indicated a correlation between thrombin and ER α ; and thrombin and CD62P IPA in Anastrozole-treated T47D cells exposed to PRP (Model 1). Conversely, this was different to that of Tamoxifen in which Model 2; Tamoxifen treatment of PRP exposed to MCF7 cells showed a correlation between both ER's, positive for ER α and negative for ER β and IP. This indicates a potential role for cumulative Anastrozole treatment and circulatory Tamoxifen treatment in the induction of hypercoagulation, dependent cellular subphenotypes.

Overall, results obtained in this study showed both gene and protein ER differences between luminal A cell lines; where the T47D cell line expressed *ESR2* and ER β greater than that of MCF7 cells. However, it should be noted that both cell lines expressed overall greater *ESR1* and ER α expression, as expected in luminal A subtypes (Ali and Coombes, 2000; Thomas and Gustafsson, 2011; Feng *et al.*, 2018). These differences in ER profiles, even of the same luminal phenotype, highlight and substantiate our results indicating greater hypercoagulatory responses and altered responses to hormone-therapy, particularly induced by that of T47D cells. Studies have indicated a change from the Luminal A subtype to Luminal B subtype in metastatic breast cancers (Januškevičienė and Petrikaitė, 2019); with recruitment of platelets and induction of a hypercoagulatory environment through thrombin secretion and fibrin formation aiding tumour progression (Kwaan *et al.*, 2019). Fibrin formation is increased through thrombin enzymatic action on fibrinogen, initiating cleavage; an increase in fibrin and associated networks provides a matrix for tumour cell migration, which is further facilitated through platelet-tumour cell interactions (Kwaan *et al.*, 2019). As such further assessment of

a potential switch from Luminal A to Luminal B phenotype, in light of the hypercoagulatory evidence in this study is warranted.

Furthermore, while comparisons have been made between ESR gene expression and ER protein expression, we had intended to further investigate both the breast cancer and platelet proteome to identify activated proteins, as well as their up/downregulation and networks associated with tumour progression and hypercoagulation. All samples were obtained, and proteins isolated for proteomic analysis on a liquid chromatography-mass spectrometer (LC-MS) at the Council for Scientific and Industrial Research (CSIR). Unfortunately, during data acquisition on the LC-MS in December 2019, the machine stopped working and remains under repair. With no date stipulated for when samples may be run, it was deemed necessary that this aspect would be conducted as a future study and prepared as a manuscript with gene and protein data from this thesis. Data obtained regarding the proteome will elucidate networks and pathways which have been activated in response to hormone-therapies and blood constituents; of particular interest; the VEGF signalling pathway and actin cytoskeleton signalling, which would be upregulated in breast cancer cells denoting tumour progression (Fu-Jun, Shao-Hua and Xiao-Fang, 2012; Lal, Dittus and Holmes, 2013; Stravodimou and Voutsadakis, 2013; Gomes *et al.*, 2017; Palacios-Acedo *et al.*, 2019). VEGF is elevated when tumour cells express interleukin (IL)-6, IL-1 and tumour necrosis factor (TNF) α and plays an important role in angiogenesis and thus tumour progression (Viale, 2005; Li *et al.*, 2014; Plantureux *et al.*, 2018).

5.5 Conclusion

Understanding the regulation of ERs in response to hormone-therapy is essential, particularly since ER-positive tumours have been shown to downregulate ER during the metastatic process (Aktas *et al.*, 2011), which in turn is facilitated by hypercoagulatory processes (Palacios-Acedo *et al.*, 2019). Changes in cytosolic and membrane ER α thus may relate to alterations of the metastatic potential of breast cancer cells, in which prothrombotic cytokines and thrombin would be released, activating platelets to allow for subversion of the haematological system for tumour progression (Falanga, Russo and Verzeroli, 2013). Our results indicate an association between ER profiles; particularly an increase in ER β and the

induction of a hypercoagulatory environment regardless of hormone-therapy involved, dependent on the subphenotype of breast cancer cells; with T47D cells eliciting the highest level of hypercoagulation. Our results substantiate clinical studies indicating that Tamoxifen induces a hypercoagulatory environment mediated by platelet interactions with breast cancer cells (Meier and Jick, 1998; Decensi *et al.*, 2005; Howell *et al.*, 2005; Jonat *et al.*, 2006; Walker *et al.*, 2017; Bray *et al.*, 2018); and further contradict studies indicating Tamoxifen inhibition of platelet activation (Chang *et al.*, 2011; Johnson *et al.*, 2017). Additionally, our findings propose a role for ER β in tumour progression (Viedma-Rodríguez *et al.*, 2014; Begam *et al.*, 2017) through its upregulation and resulting association with heightened hypercoagulation, and as such it may be a potential target for future tailored approaches for hormone-dependent breast cancer (Austin *et al.*, 2018). These results further highlight the procoagulant nature of T47D cells compared to that of MCF7 cells indicating that variation in hormone-receptor profiles and hormone-dependent breast tumour subphenotypes may elicit a differential thrombotic response, which is not usually considered in clinical management and indicate a role for personalised assays to further define subphenotypes, prior to hormone-therapy management. The results also highlight the importance of selecting appropriate cell line models for breast cancer studies and further identify a role for understanding breast cancer heterogeneity in the identification and treatment of the disease. Furthermore, these results provide much needed information about the procoagulant nature of Anastrozole and highlight its pro-thrombotic nature, the data for which is lacking. In conclusion, the present study provides a framework and *ex vivo* models in which to undertake further research, to complement our findings regarding breast tumour subphenotype heterogeneity and to determine other biomarkers that may be predictive of hypercoagulatory risk. In doing so, we hope that this study can be used to improve the diagnosis and management of breast cancer and associated thromboembolic disease.

6 References

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7 Appendix

Appendix A

Original Approved HREC Certificate



R14/49 Miss Kyrntania Pather et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160826

NAME: Miss Kyrntania Pather et al
(Principal Investigator)
DEPARTMENT: School of Anatomical Sciences

PROJECT TITLE: Analysis of Hormone-Dependent Breast Cancer Cells
and Whole Blood Treated with Common Hormone
Therapeutic Drugs

DATE CONSIDERED: 26/08/2016

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr Tanya Augustine and Ms Therese Dix-Peek

APPROVED BY:

A handwritten signature in black ink, appearing to read 'P Cleaton-Jones'.

Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 07/10/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in August and will therefore be due in the month of August each year.

A handwritten signature in black ink, appearing to read 'Kyrntania Pather'.
Principal Investigator Signature

Date

10/10/16

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE
WITWATERSRAND
JOHANNESBURG



R14/49 Miss Kyrntania Pather et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160826

NAME: Miss Kyrntania Pather et al
(Principal Investigator)
DEPARTMENT: School of Anatomical Sciences
The Council for Scientific and Industrial Research (CSIR),
Pretoria
PROJECT TITLE: Hormone-therapy mediated interactions between platelets
and breast cancer cells: an in vitro study
DATE CONSIDERED: 26/08/2016
DECISION: Approved unconditionally
CONDITIONS: Protocol Amendment - Initial Approval 07/10/2016
SUPERVISOR: Dr T. Augustine, Ms T. Dix-Peek and Prof N. Chetty

APPROVED BY:

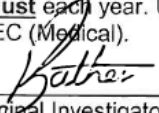

Professor CB Penny, Chairperson, HREC (Medical)

DATE OF APPROVAL: 07/08/2018

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **August** and will therefore be due in the month of **August** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).


Principal Investigator Signature

Date 01/09/2018

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Participant Information Sheet

Title of the study: Analysis of hormone-dependent breast cancer cells and whole blood treated with common hormone therapeutic drugs.

Ethical Clearance Number: M160826

Principal investigator: Kyrta Pather

Co-investigator/s: Dr Tanya Augustine / Ms Therese Dix-Peek / Prof Nanthkumarn Chetty / Dr Stoyan Stoychev / Dr Sindisiwe Buthelezi / Mrs K Xulu

Good day. My name is Kyrta Pather. I am a master's student registered in the School of Anatomical Sciences, University of the Witwatersrand. We are conducting research in order to investigate the relationship between commonly used hormone-therapies, hormone-dependent breast cancer and thrombosis. Our research is experimental and will be conducted in a laboratory. Breast cancer cells will be grown in the laboratory under standard physiological conditions and then treated with hormone-therapy drugs (Anastrozole or Tamoxifen). The treated cells will then be exposed to whole blood (collected from study participants) and the response of to the treatment will be studied by looking at surface receptors which are exposed when platelets are activated and the accompanying change in morphology of activated platelets, furthermore the oestrogen receptors (ER α and ER β) expression in platelets and their corresponding effect on platelet activation and coagulation will be studied. This study will help in understanding the *in vitro* effects of hormone-therapy drugs on platelets and suggest the possible effect of the drugs in thromboembolic disease in hormone dependant breast cancer.

What we are requesting from you

We are asking you to volunteer in the study by giving us permission to take 3 vacutainers of blood (approximately 3 teaspoons) from the vein in your arm. The blood will be taken by a qualified phlebotomist at Charlotte Maxeke Academic Hospital. A needle will be used to prick your arm during blood collection, while this procedure may be uncomfortable with minimal pain, there is no other known risk to the procedure. For the purposes of this study, we will be using whole blood and the blood will be used immediately. Any remaining blood will be stored at -80°C in our laboratory (University of the Witwatersrand, School of Anatomical Sciences) for the duration of this study (a maximum of 5 years).

In order to ensure the accuracy of the experiments to be conducted, peripheral blood will only be collected from seemingly healthy females between the ages of 19-30 years. The following exclusion criteria will be taken into consideration: pregnancy, smoking, use of oral contraceptives or injection, immunodeficiency, autoimmune disease, and consumption of anti-platelet and anti-coagulation medication (e.g. aspirin and warfarin) in previous 72 hours. Therefore, if you meet the above-mentioned exclusion criteria you cannot participate in the study.

The menstrual cycle is a complex process which consists of three phases; follicular phase, luteal phase and menstruation. Oestrogen and progesterone are the two main hormones that control the menstrual cycle. The main function of platelets is blood clotting. The effects of oestrogen and progesterone on platelets are not well understood; however, some studies have shown that the menstrual and early follicular phase is associated with the least amount factors which promote blood clotting. Therefore collecting blood during this period is desirable as there are not many factors that can affect the function of platelets. Therefore blood will only be collected during the follicular phase of the menstrual cycle (between day1-10) because, during this period, oestrogen and progesterone will be at their lowest. In order to ensure that blood is collected within the desired days of the menstrual cycle, we will thus request to know when your last menstrual cycle was.

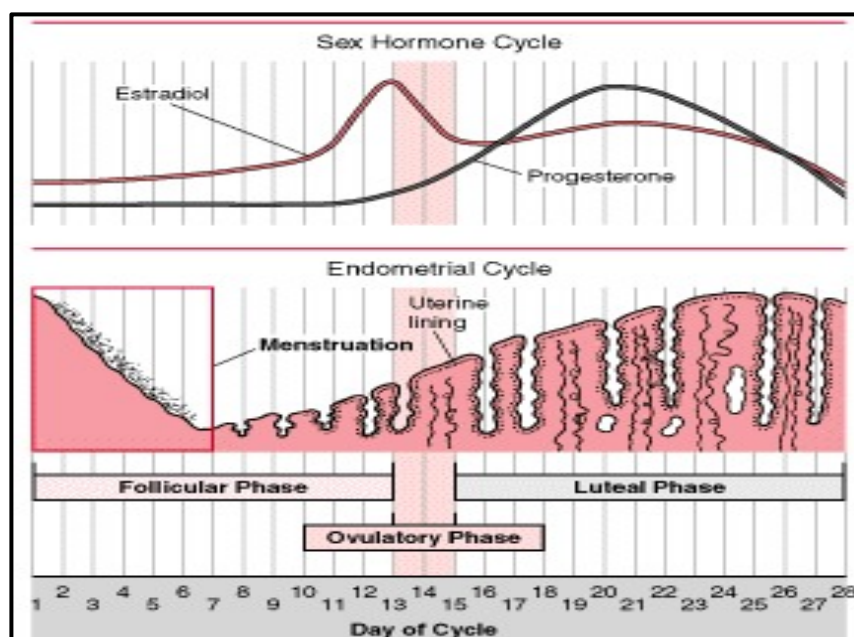


Figure 1: The diagram illustrates the levels of oestradiol and progesterone (top panel) during different phases of the menstrual cycle (bottom panel). As shown, hormone levels are the lowest between day 1 and 10 of the menstrual cycle which are during the menstrual and follicular phase.

Image source: www.menstruation-info-with-doc.com-288X311

Please note the following statements that may help you in making your decision;

1. The blood collected will only be used for this study. We will be investigating the response of platelets to common hormone-therapies in hormone treated breast cancer cell lines.
2. Further approval from the HREC will have to be obtained if any new research other than that described in the protocol is to be done using your blood.
3. We request that any remaining blood be stored at -80°C in our laboratory (University of the Witwatersrand, School of Anatomical Sciences) for the duration of this study (a maximum of 5 years).
4. All residual blood will be destroyed at the end of the storage period (i.e. 5 years).
5. The outcome of this study will not benefit you directly should you agree to participate. However, we hope that the outcome of the study will add new knowledge that will be important in understanding the response of platelets to common hormone-therapy in breast cancer and hopefully be useful in the treatment of cancer related thrombosis.
6. Your personal details will not be used by or disclosed to anyone. We will allocate you a number and will not use any other information which will allow you to be identified. Your participation will be anonymous.
7. The researchers in this study will not benefit financially by your participation in this study.
8. Should you agree to participate in this study, you will at any point be allowed to withdraw your participation should you wish to do so. In that case, all experiments conducted using your blood will be omitted from the final analysis of results and stored samples destroyed.

If you are willing to volunteer to participate in the study we will ask you to sign this document stating that you are volunteering and that you understand that we have explained our study to you.

If you have any questions about the study, please feel free to contact me;

Kyrtania Pather

Email: kyrtania.pather@students.wits.ac.za

Contact number: 0728268179

If you have any questions or complaints as a research participant you may contact the chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC) (Medical) during office hours (8:00am-4:00pm);

Prof P. Cleaton-Jones

Email: peter.cleaton-jones1@wits.ac.za

Contact number: 011717 2301

Ms Z. Ndlovu

Administrative officer (HREC)

Email: Zanele.ndlovu@wits.ac.za

Contact number: 011 717 2700/1234/1252

Appendix B

Solutions Used

Normal MCF7 Media

- 45ml Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 4.5g/L glucose
- 5ml heat inactivated Foetal Bovine Serum (FBS)
- 50ul Penicillin (10mg/ml)/Streptomycin (10mg/ml) (P/S)
- Sterile filter through a 0.2µM filter.
- Stored at 4°C.

Normal T47D Media

- 45ml Roswell Park Memorial Medium
- 0.2 U/ml bovine insulin
- 5ml FBS
- 50ul Penicillin (10mg/ml)/Streptomycin (10mg/ml) (P/S)

Ammonium chloride erythrocyte lysis buffer

- 8.02g NH₄Cl (150mM)
- 0.84g NaHCO₃ (10mM)
- 0.37g EDTA (Ethylenediaminetetraacetic acid) (1mM)
- 1L dH₂O (distilled water)
- Stored at 4°C.

Tyrode's Buffer

- 7.83g NaCl (134mM)
- 0.1g NaHCO₃ (1.2mM)
- 0.22g KCl (2.9mM)
- 0.05g Na₂HPO₄ (0.34mM)
- 0.1g MgCl₂ (1mM)
- 2.38g HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid) (10mM) (Sigma-Aldrich, St. Louis, MO, USA; H4034)
- pH 7.4.

- Stored at 4°C.

1X PBS (1 M)

- 8g NaCl
- 0.2g KCl
- 1.15g Na₂HPO₄
- 0.2g KH₂PO₄
- 1L dH₂O
- pH 7.4. Stored at 4°C.

0.1 M PBS

- 100ml 1M PBS
- 900ml dH₂O

2.5% formaldehyde/glutaraldehyde

- 1ml 25% formaldehyde
- 1ml 25% glutaraldehyde
- 3ml dH₂O
- 5ml 0.1M PBS

Poly-d-lysine

- Dissolve 10mg poly-d-lysine in 1ml distilled water to make 1% stock
- Dilute stock solution with 2ml distilled water to create 1X coating solution
- Coat glass coverslips with 200 ul solution and incubate for 5 minutes at RT
- Remove remaining solution and rinse with 1 M PBS
- Allow to dry and plate cells as protocol

Appendix C

Optimal cell number determination.

Table 1: Tabulation indicating LDH release from MCF7 and T47D cells plated at different concentrations per well. Red indicates the optimal cell number used for ensuing experimentation, and was noted to have the highest level of LDH released.

Number of cells/well	LDH Release	
	MCF7	T47D
1 000 000	0,233338	1,97133333
500 000	0,50400467	2,399
250 000	0,54000467	2,26066667
125 000	0,56367133	2,61966667
100 000	0,92000047	2,62000133
62500	0,91700467	2,15133333
31 250	0,87100467	1,56333333
15 625	0,28167133	1,11733333
7 812	0,602338	0,97533333
3 906	0,730338	0,73666667
1 953	0,48100467	0,58266667

Concentrations and Dilutions of Anastrozole and Tamoxifen

Stock solutions of both Anastrozole (16.16 mg/mL) and Tamoxifen (0.09 mg/mL) were prepared at 1.0mM and diluted in 0.1% DMSO in respective normal media in order to obtain 5 dilution concentrations illustrated in the table below.

Table 2: Tabulation indicating dilution concentrations for Anastrozole and Tamoxifen for ensuing concentration determination experimentations.

Anastrozole (16,16 mg/mL)	Tamoxifen (0.09 mg/mL)
1 µM	0.5 µM
2 µM	1 µM
4 µM	1.5 µM
8 µM	2 µM
10 µM	2.5 µM

Appendix D

Optimisation: RNA Extraction

RNA extraction was first conducted on three samples sets: (1) cells maintained in wells; (2) cells removed from wells with Trypsin EDTA; (3) frozen cells, to determine the most effective way to retain RNA yield, purity and integrity. By making use of a nanodrop to assess RNA purity, it was found that cells maintained in the wells (following 1 M PBS washing after co-incubation) yielded the purest RNA.

cDNA synthesis

cDNA was elucidated from RNA by following the described methodology and the use of “MJ Mini Personal Thermocycler” according to the table below:

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10min	120min	5 sec	-

- set the reaction volume to 20ul
- load the reactions into the thermal cycler
- Start the reverse transcription run
- Store cDNA in microtubes in the freezer (-20)

RNA Integrity (RINs) Values for Samples

Table3: RINs numbers obtained for RNA extracted from samples.

Label	Name	Concentration (ng/uL)	260/280	260/230	RINs Value
M1A	MCF7 Cells Only	25,4	2,09	0,13	10
M1B	MCF7 Cells w Anastrozole	9,5	3,05	0,02	10
M1C	MCF7 Cells w Tamoxifen	22,3	1,77	0,29	9,6
M1D	MCF7 Cells w WB	13,2	2,26	0,13	10
M1E	MCF7 Cells w WB and Anast	6,5	3,71	0,02	5,5
M1F	MCF7 Cells w WB and Tamox	8,7	4,57	0,01	10
M1G	MCF7 Cells w Diluent	9,4	2,62	0,07	10
T1A	T47D Cells Only	24	2,01	0,19	8,3
T1B	T47D Cells w Anastrozole	21,4	2,14	0,09	9
T1C	T47D Cells w Tamoxifen	7,3	2,42	0,04	8,3
T1D	T47D Cells w WB	4,9	3,92	0,01	9,5
T1E	T47D Cells w WB and Anast	9,1	2,71	0,07	9,3
T1F	T47D Cells w WB and Tamox	3,6	3,34	0,08	9
T1G	T47D Cells w Diluent	10,6	1,99	0,09	9,4
M7A	MCF7 Cells Only	310,5	2,08	2,13	8,9
M7B	MCF7 Cells w Anastrozole	380,3	2,09	0,83	8,8
M7C	MCF7 Cells w Tamoxifen	486,3	2,09	1,33	8,9
M7D	MCF7 Cells w WB	423,1	2,06	1,92	8,6
M7E	MCF7 Cells w WB and Anast	393,8	2,08	1,79	8,8
M7F	MCF7 Cells w WB and Tamox	316,2	2,08	1,46	8,4
M7G	MCF7 Cells w Diluent	498,2	2,12	2,24	8,9
T6A	T47D Cells Only	61,4	2,07	1,96	8,3
T6B	T47D Cells w Anastrozole	66,8	2,08	0,42	9
T6C	T47D Cells w Tamoxifen	49,8	2,09	0,27	9,2
T6D	T47D Cells w WB	12	2,6	0,02	8,3
T6E	T47D Cells w WB and Anast	21,6	2,13	0,81	7,8
T6F	T47D Cells w WB and Tamox	31,6	2,03	0,13	10
T6G	T47D Cells w Diluent	19	2,53	0,03	10
M3A	MCF7 Cells Only	21,5	2,16	1	9,2
M3B	MCF7 Cells w Anastrozole	11,2	2,35	0,37	10
M3C	MCF7 Cells w Tamoxifen	5,3	2,16	1,09	9,6
M3D	MCF7 Cells w WB	15,5	2,3	0,28	9,8
M3E	MCF7 Cells w WB and Anast	10,1	2,33	0,31	9,9

M3F	MCF7 Cells w WB and Tamox	6,5	2,66	0,07	10
M3G	MCF7 Cells w Diluent	9,4	2,79	0,21	9,9
T3A	T47D Cells Only	5,1	4,57	0,03	9,9
T3B	T47D Cells w Anastrozole	5,9	4,2	0,01	9
T3C	T47D Cells w Tamoxifen	20,7	2,31	0,05	9,7
T3D	T47D Cells w WB	9,7	2,29	0,05	8,6
T3E	T47D Cells w WB and Anast	12,7	2,23	0,29	8,6
T3F	T47D Cells w WB and Tamox	18,5	2,16	0,09	7
T7G	T47D Cells w Diluent	40,9	2,04	0,77	9,3
M4A	MCF7 Cells Only	15,4	2,2	0,19	9,2
M4B	MCF7 Cells w Anastrozole	13,4	2,22	0,25	8,9
M4C	MCF7 Cells w Tamoxifen	20,5	2,14	0,84	8,5
M4D	MCF7 Cells w WB	15,3	2,28	0,04	9,1
M4E	MCF7 Cells w WB and Anast	15,5	2,28	0,19	9,4
M4F	MCF7 Cells w WB and Tamox	11	2,68	0,14	9,6
M4G	MCF7 Cells w Diluent	12,3	2,53	0,12	9,5
T4A	T47D Cells Only	22,3	2,37	0,32	9,5
T4B	T47D Cells w Anastrozole	24,7	2,74	0,14	9,8
T4C	T47D Cells w Tamoxifen	22	2,81	0,19	9,4
T4D	T47D Cells w WB	20,6	2,63	0,29	9,5
T4E	T47D Cells w WB and Anast	12,7	2,14	0,26	9,7
T4F	T47D Cells w WB and Tamox	19,1	2	0,97	9,1
T4G	T47D Cells w Diluent	27,1	2,11	0,32	9,4
M5A	MCF7 Cells Only	64,1	2,14	0,48	8,1
M5B	MCF7 Cells w Anastrozole	45,4	2,27	0,29	8,9
M5C	MCF7 Cells w Tamoxifen	71,9	2,18	0,53	8,1
M5D	MCF7 Cells w WB	84,2	2,13	0,7	8,7
M5E	MCF7 Cells w WB and Anast	23,3	2,25	0,24	9,1
M5F	MCF7 Cells w WB and Tamox	22,5	2,25	0,31	7,5
M5G	MCF7 Cells w Diluent	8,4	2,68	0,23	10
T5A	T47D Cells Only	22,6	2,74	0,14	9,6
T5B	T47D Cells w Anastrozole	22,9	2,44	0,2	9,5
T5C	T47D Cells w Tamoxifen	20,9	2,12	0,85	9,4
T5D	T47D Cells w WB	8,2	2,55	0,08	9,3
T5E	T47D Cells w WB and Anast	3,2	2,55	1,37	9,2
T5F	T47D Cells w WB and Tamox	6,7	2,35	0,99	9
T5G	T47D Cells w Diluent	33,4	2,12	0,41	9,1

Reference Gene Optimisation

Table 4: Tabulation outlining Reference genes optimised for each cell line. Droplet separation for ddPCR indicated discrimination between Negative and Positive droplets, showing amplification. ACTB was chosen as a reference gene for this study.

Reference Gene	Cell Line	Run No.	Result	Droplet Separation
GAPDH	MCF7	1	No Call	None
GAPDH	T47D	1	1278	None
RPLPO	MCF7	1	781	None
RPLPO	T47D	1	No Call	None
ACTB	MCF7	1	No Call	Clear
ACTB	T47D	1	No Call	Clear
TFRC	MCF7	2	No Call	None
TFRC	T47D	2	No Call	Clear
ACTB	MCF7	2	612	Clear
ACTB	T47D	2	1255	Clear

ESR Gene Optimisation

Table 5: Tabulation outlining ESR genes optimised for each cell line. Droplet separation for ddPCR indicated discrimination between Negative and Positive droplets, showing amplification.

Gene	Cell Line	Run No.	Result	Droplet Separation	Positives	Negatives
ESR1	MCF7	1	502	Clear	3402	6394
ESR1	T47D	1	23	Clear	80	4107
ESR2	MCF7	1	0.28	Clear	3	12528
ESR2	T47D	1	2.7	Clear	24	10616
ESR1	MCF7	2	1386	Clear	8328	3706
ESR1	T47D	2	81	Clear	678	9487
ESR2	MCF7	2	1.4	Clear	13	10758
ESR2	T47D	2	0.4	Clear	3	8431
ESR1	MCF7	3	520	Clear	1357	2442
ESR1	T47D	3	448	Clear	3245	7008
ESR2	MCF7	3	1.4	Clear	2	8311
ESR2	T47D	3	5.7	Clear	39	7969

Appendix E

Antibody Titration

Fluorescence Compensation

Prior to flow cytometry experimentation, compensation for spectral overlap was conducted on the LSR Fortessa (BD Biosciences, South Africa), configured with 4 lasers (blue, red, violet and UV) using FACSDiva software. In order to account for the spectral overlap between the different markers used; single stained samples for each marker were run and using the Diva software. Overlapping channels were subtracted.

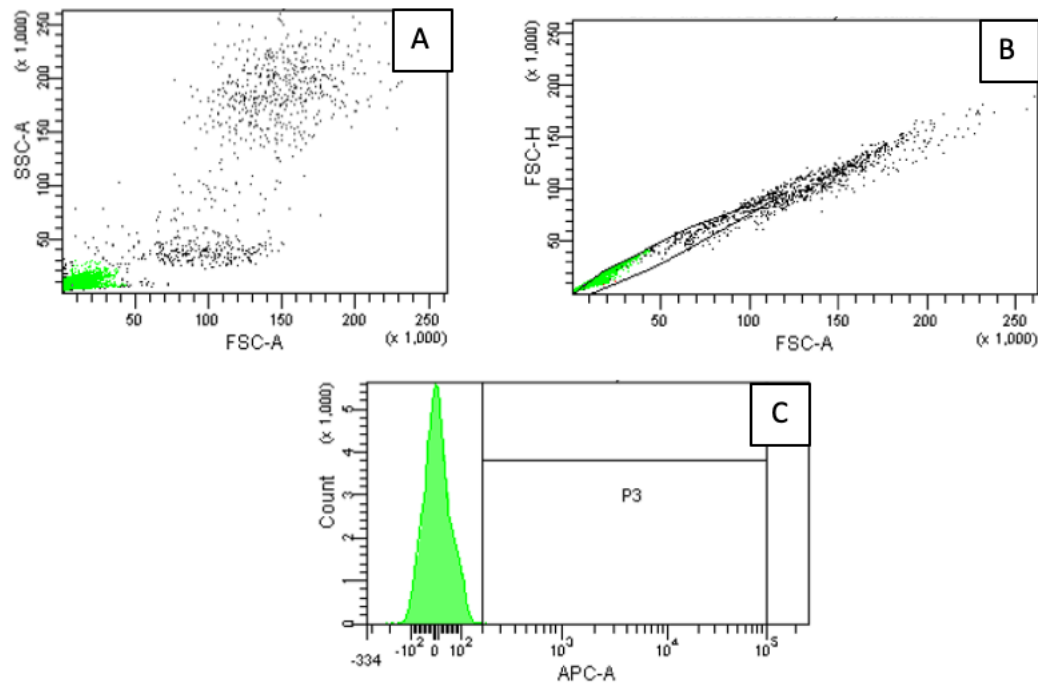


Figure 7.1: Representative Scatterplots and Histogram illustrating unlabelled control WB samples used to determine correct gating of samples. A: FSC vs SSC showing the total cellular populations within the WB sample, the green population indicates the gated platelet population of interest. B: Singlet gated population found within the WB sample. C: Histogram indicating the unstained platelet population (green peak), with gated blank area where samples positive for APC fluorescence (CD41a platelet marker) would be found.

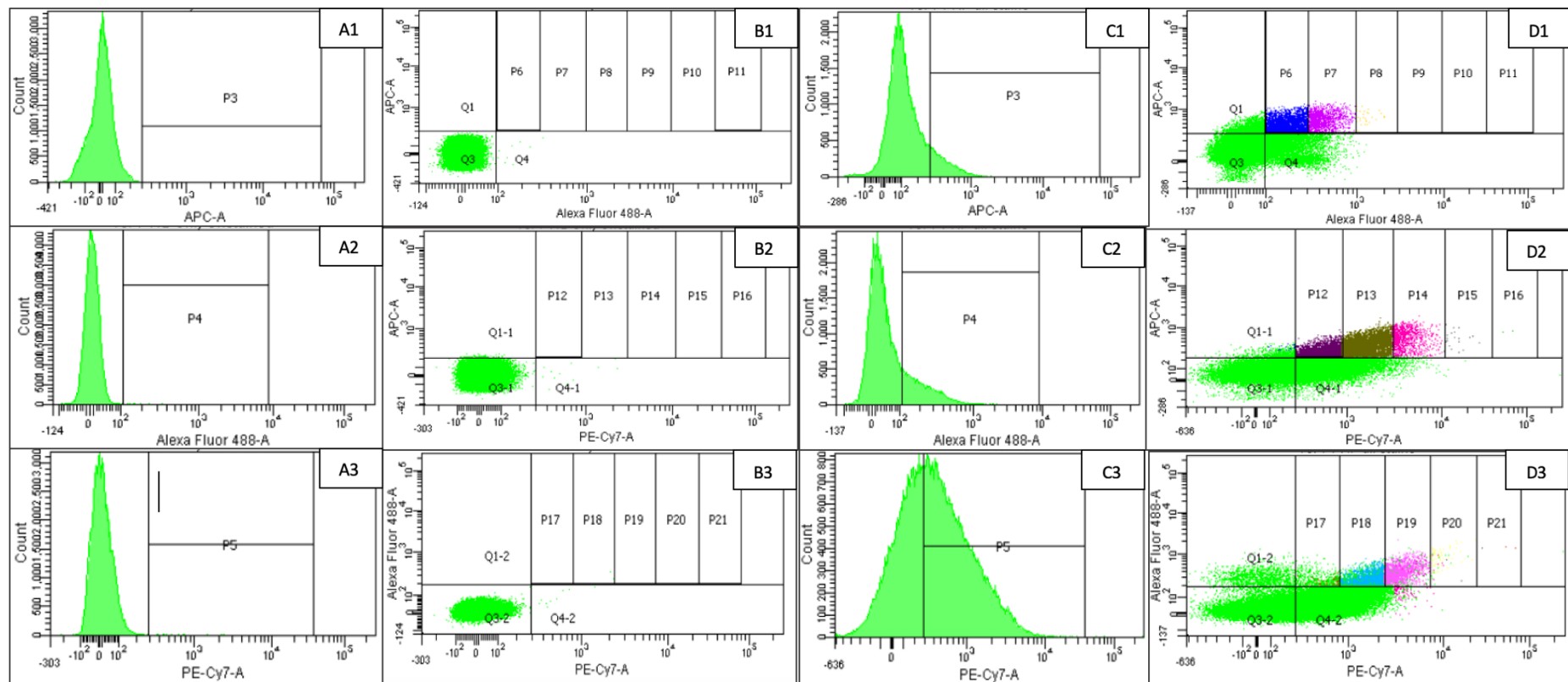


Figure 7.2: Representative histograms and quadrant plots indicating the negative population (A & B) and the positive and active platelet populations (C & D). A: Unstained sample for compensation. B: Unstained sample in Q3. C: Unstained samples indicated as green peak before the gated, positive population. D: representative positive population stained with all markers.

Appendix F

Thrombin Assay Dilution, red indicating chosen dilution at 1/10.

Sample	Thrombin Conc (ng)	Thrombin Activity (ng/mL)
WB dil 1/2	0,934474471	9,344744706
PRP dil 1/2	0,403503369	4,035033686
PPP dil 1/2	0,532505076	5,325050762
Tmed dil 1/2	-0,798105677	-7,981056769
Mmed dil 1/2	-0,517913466	-5,179134661
WB dil 1/5	0,904272995	3,61709198
PRP dil 1/5	0,554391594	2,217566375
PPP dil 1/5	0,542605751	2,170423004
Tmed dil 1/5	-0,097417778	-0,389671111
Mmed dil 1/5	0,249856885	0,99942754
WB dil 1/10	0,910194627	1,820389254
PRP dil 1/10	0,555479439	1,110958878
PPP dil 1/10	0,537477675	1,07495535
Tmed dil 1/10	0,43112579	0,86225158
Mmed dil 1/10	0,476338135	0,95267627

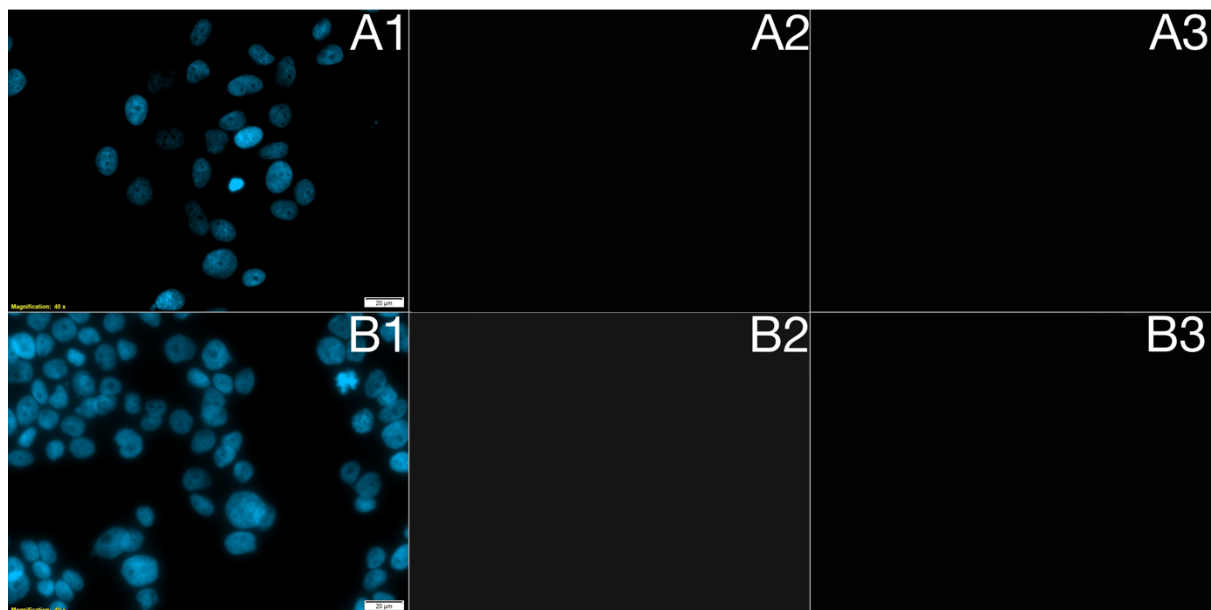
Appendix G

ICC Dilution Concentration

X1° Ab - ERβ	1° Ab - ERβ	X1° Ab - ERβ	1° Ab - ERβ	1° Ab - ERβ	1° Ab - ERβ
X2° Ab – 594	X2° Ab – 594	2° Ab – 594	(1:100)	(1:200)	(1:400)
			2° Ab – 594	2° Ab – 594	2° Ab – 594
			(1:500)	(1:500)	(1:500)
1° Ab - ERβ	1° Ab - ERβ	1° Ab - ERβ	1° Ab - ERβ	1° Ab - ERβ	1° Ab - ERβ
(1:100)	(1:200)	(1:400)	(1:100)	(1:200)	(1:400)
2° Ab – 594	2° Ab – 594	2° Ab – 594	2° Ab – 594	2° Ab – 594	2° Ab – 594
(1:750)	(1:750)	(1:750)	(1:1000)	(1:1000)	(1:1000)

ICC controls

Photomicrographs showing efficacy of immunocytochemistry negative controls. A1: Nuclei stained with DAPI in MCF7 cells. A2: Primary antibody omission. A3: Secondary antibody omission. B1: Nuclei stained with DAPI in T47D cells. B2: Primary antibody omission. B3: Secondary antibody omission.



Immunocytochemistry Plates

Representative photomicrographs showing individual fluorescence of antibodies. Column 1: DAPI nuclear stain; column 2: ER α expression; column 3: ER β expression; column 4: overlay of ER α and ER β expression.

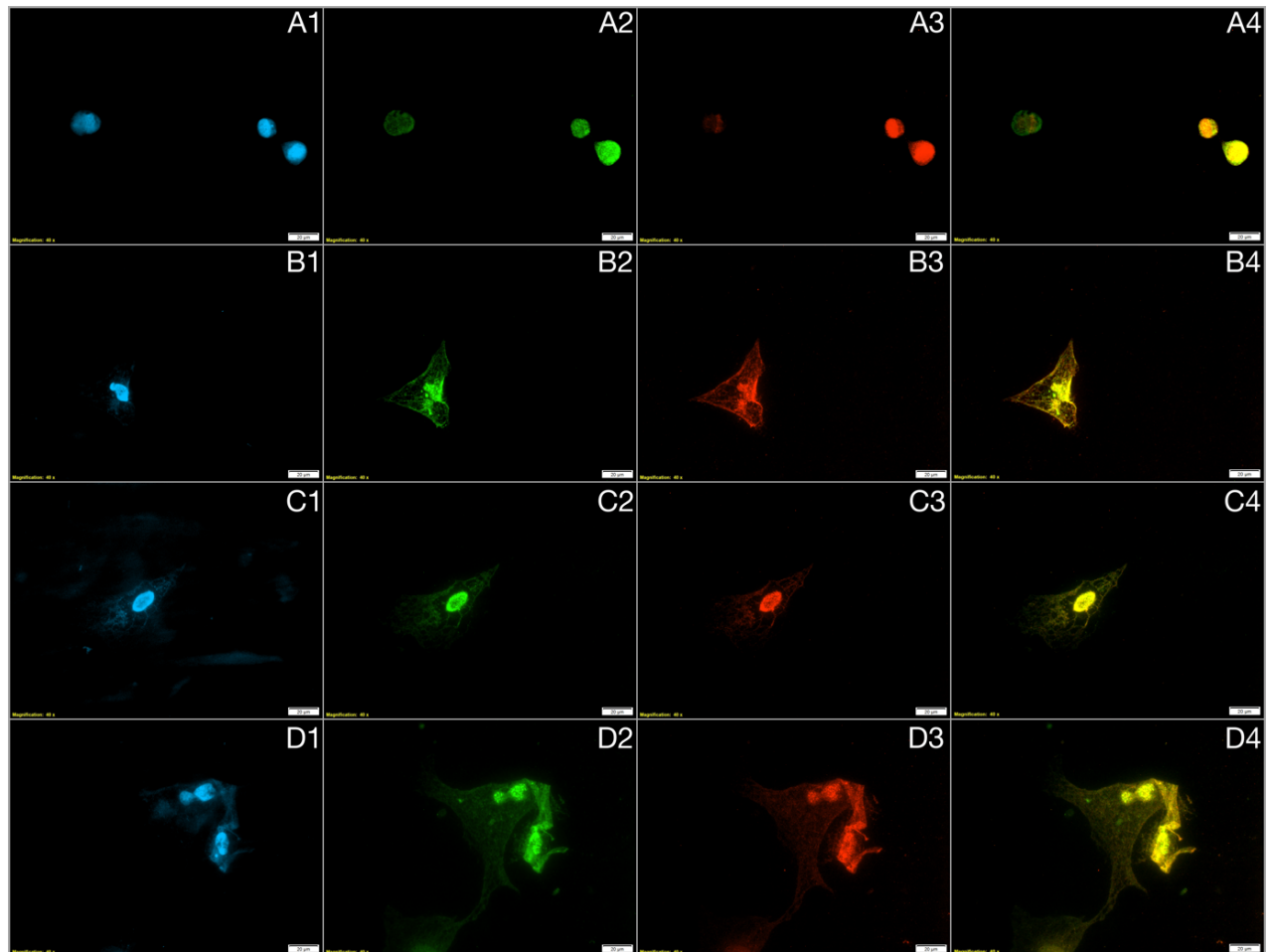


Figure 7.3: MCF10A cells. A: MCF10A cells in media. B: MCF10A cells exposed to WB. C: MCF10A cells exposed to PRP. D: MCF10A cells exposed to PPP.

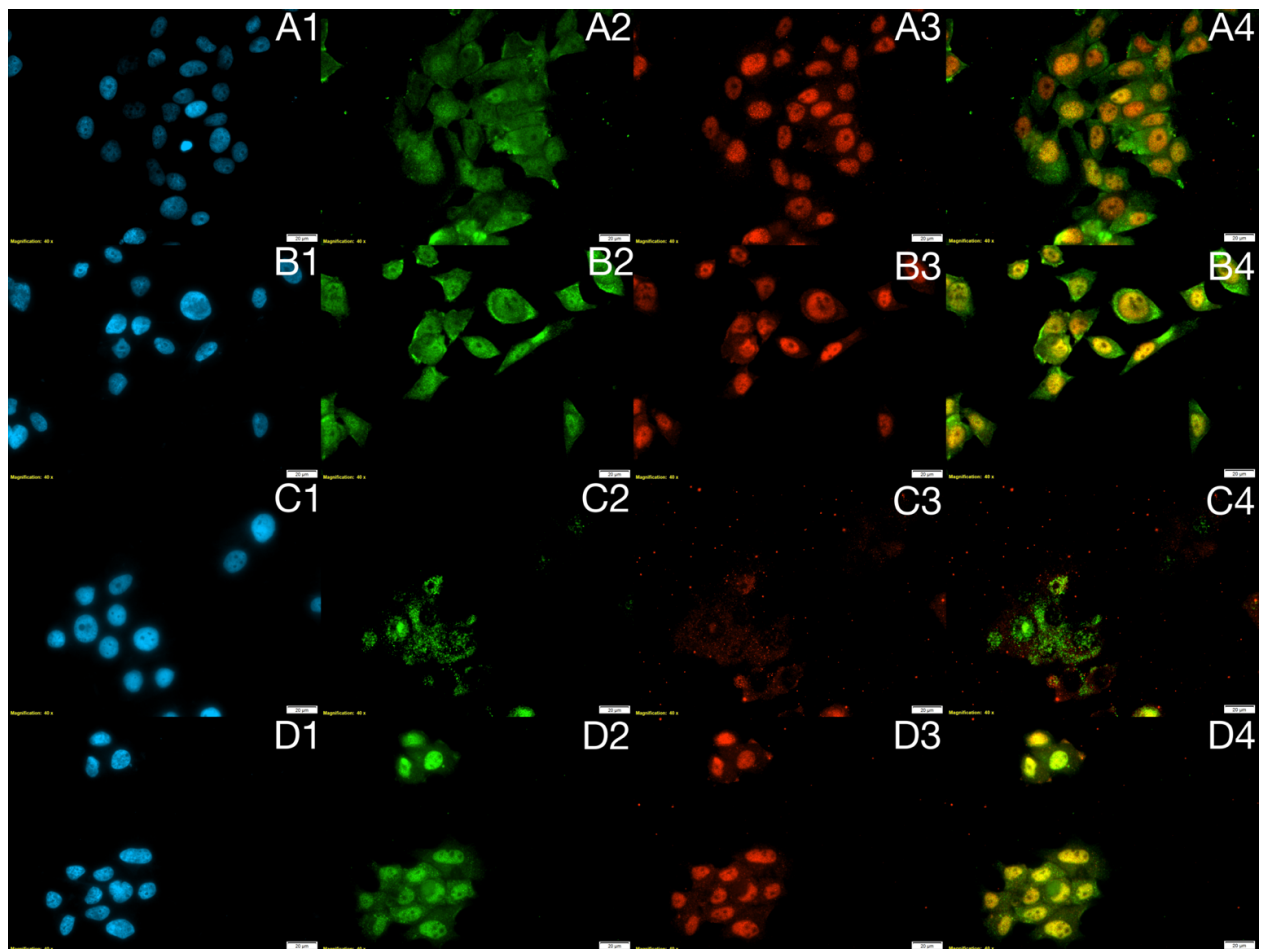


Figure 7.4: MCF7 cells. A: MCF7 cells in media. B: MCF7 cells exposed to WB. C: MCF7 cells exposed to PRP. D: MCF7 cells exposed to PPP.

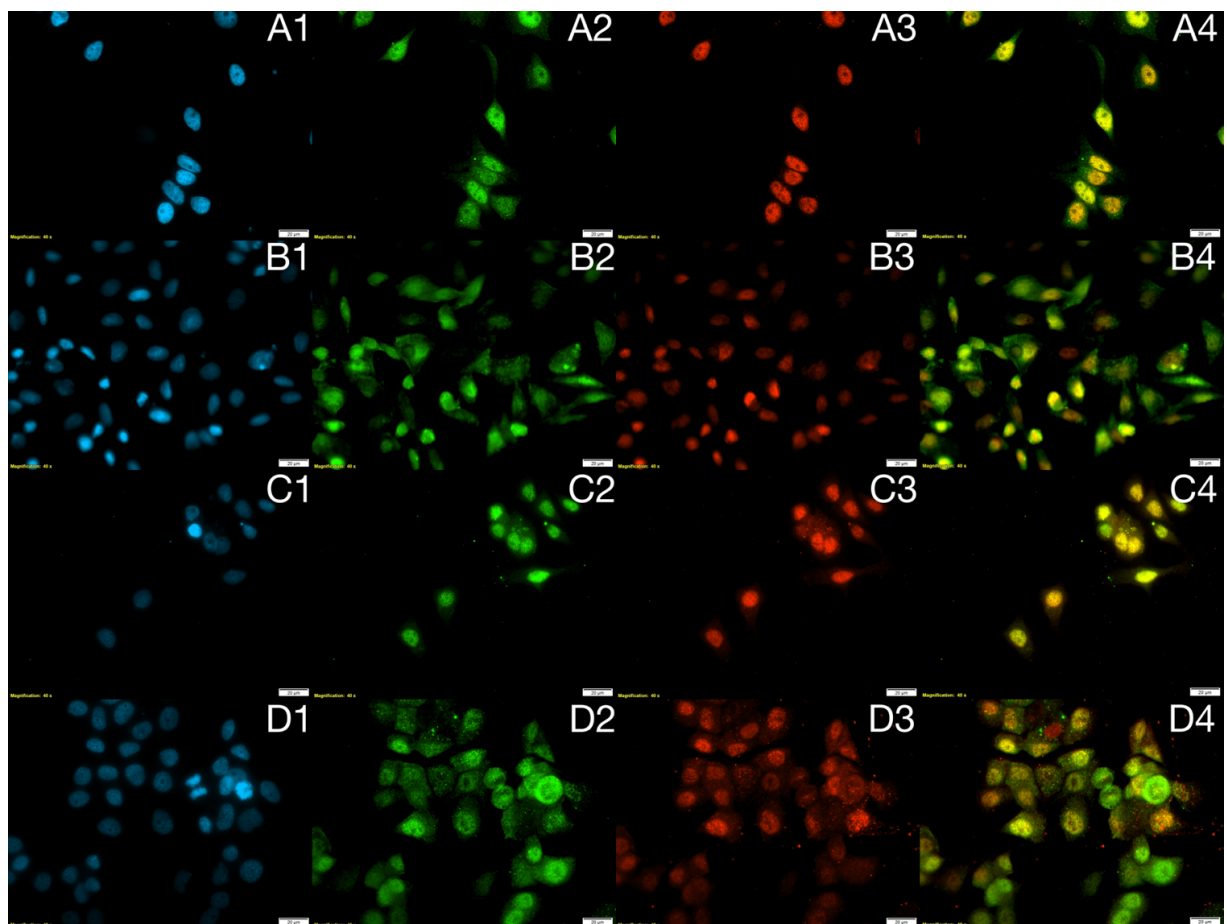


Figure 7.5: MCF7 cells. A: MCF7 cells treated with 0.1% DMSO diluent. B: MCF7 cells treated with 0.1% DMSO diluent exposed to WB. C: MCF7 cells treated with 0.1% DMSO diluent exposed to PRP. D: MCF7 cells treated with 0.1% DMSO diluent exposed to PPP.

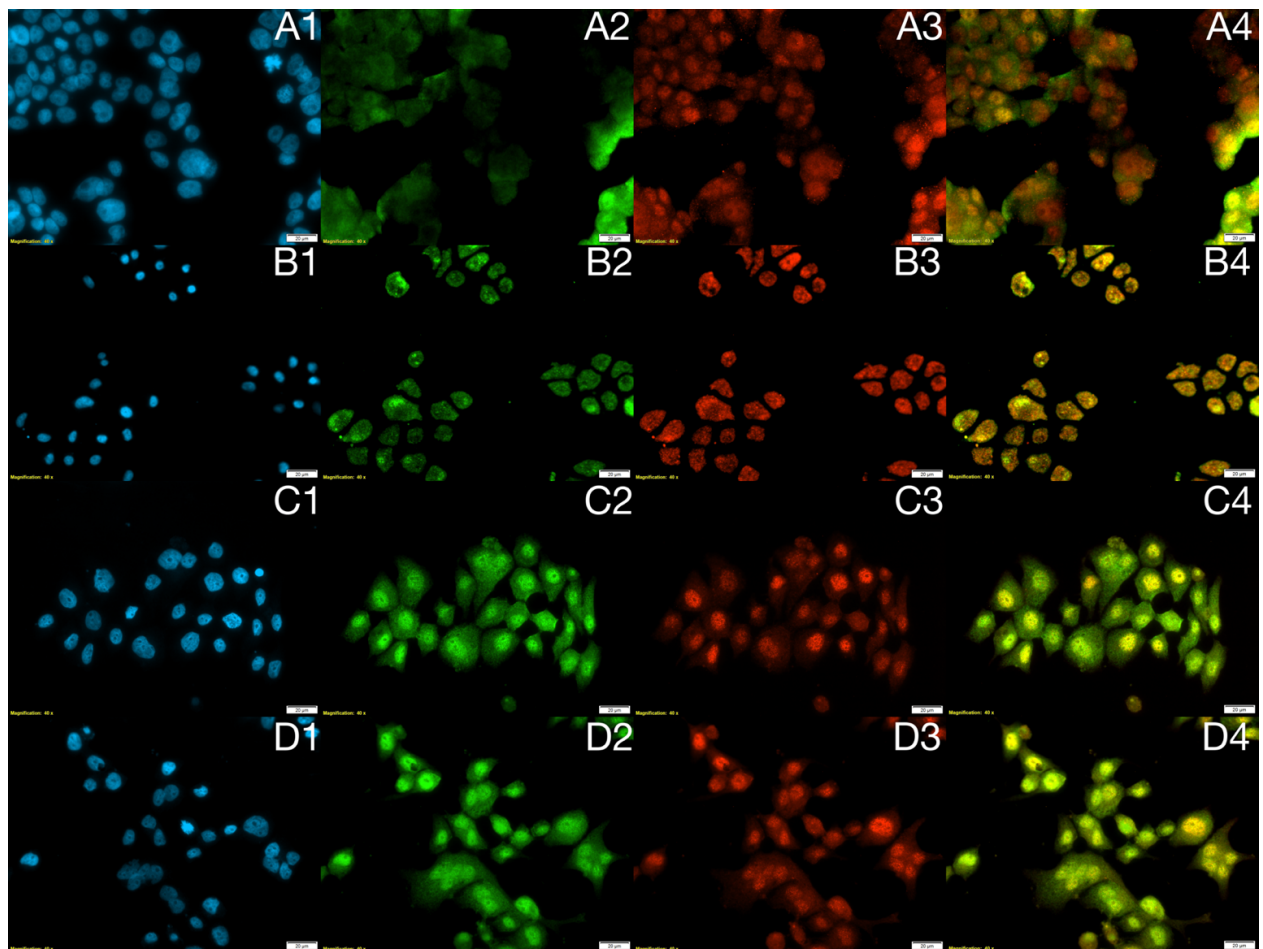


Figure 7.6: T47D cells. A: T47D cells in media. B: T47D cells exposed to WB. C: T47D cells exposed to PRP. D: T47D cells exposed to PPP.

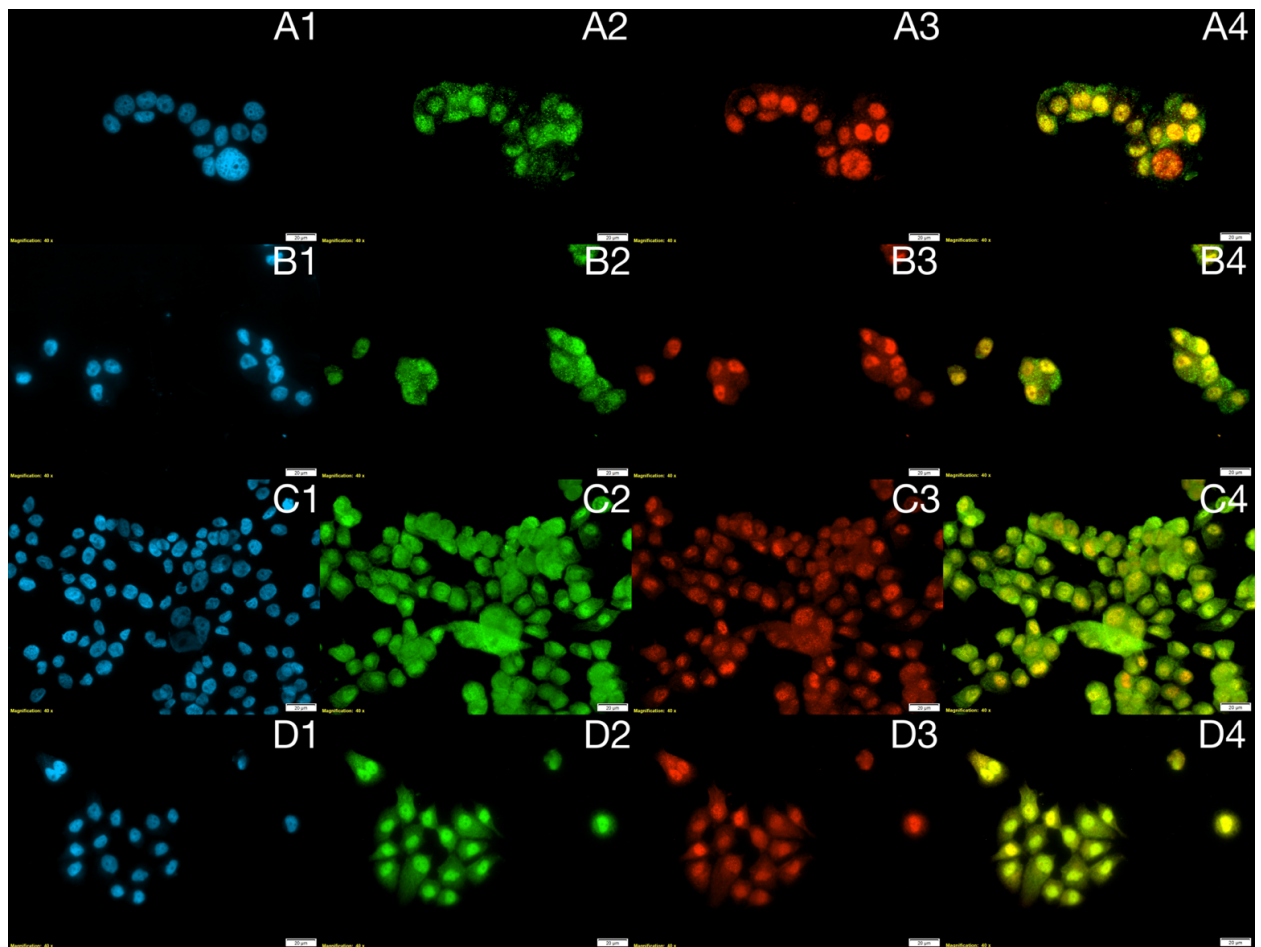


Figure 7.7: T47D cells. A: T47D cells treated with 0.1% DMSO diluent. B: T47D cells treated with 0.1% DMSO diluent exposed to WB. C: T47D cells treated with 0.1% DMSO diluent exposed to PRP. D: T47D cells treated with 0.1% DMSO diluent exposed to PPP.

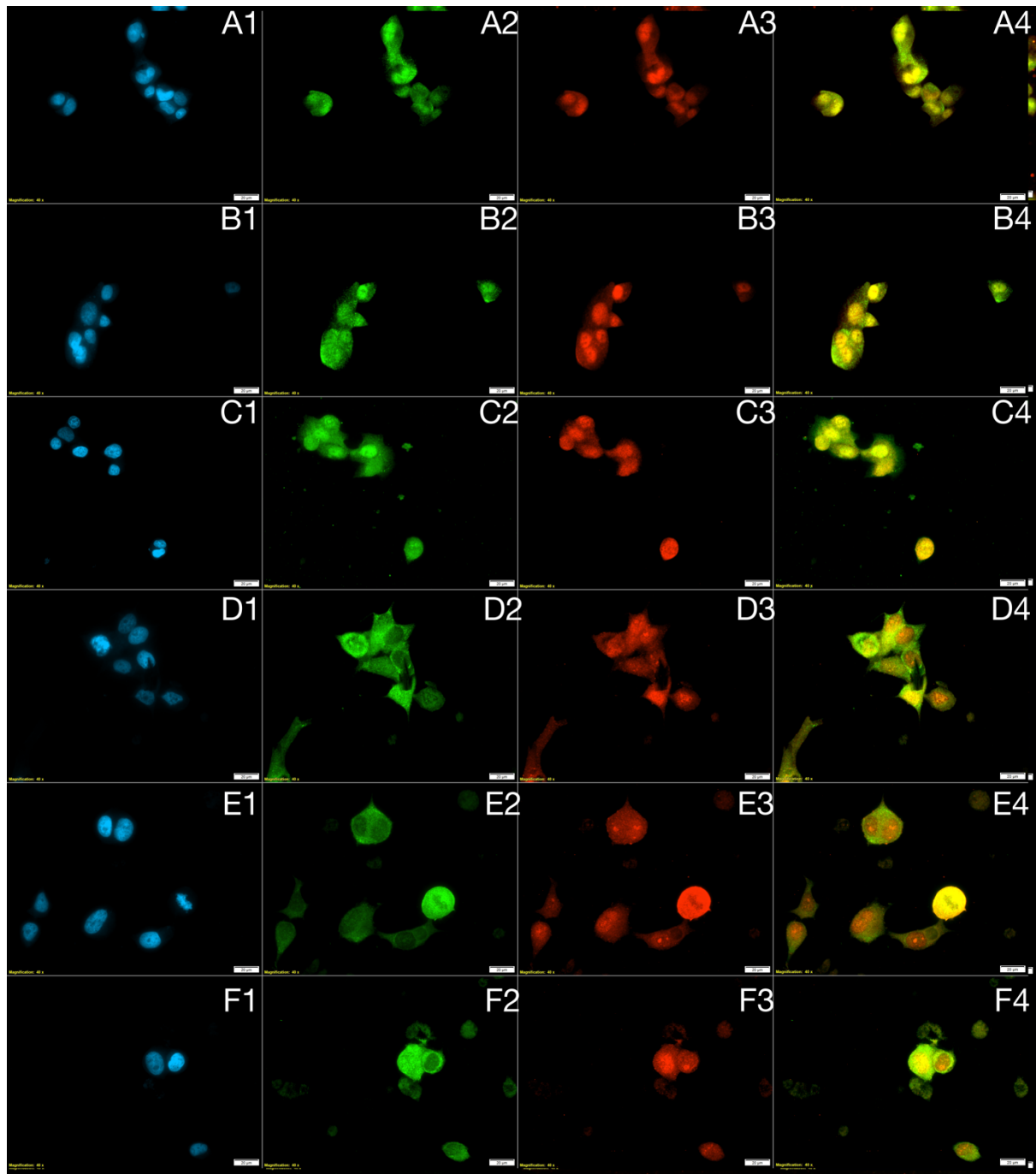


Figure 7.8: MCF7 cells. A: MCF7 cells treated Tamoxifen. B: MCF7 cells treated with Tamoxifen exposed to WB. C: MCF7 cells treated with Tamoxifen exposed to PRP. D: MCF7 cells treated with Tamoxifen exposed to PPP. E: WB treated with Tamoxifen exposed to MCF7 cells. F: PRP treated with Tamoxifen exposed to MCF7 cells. G: PPP treated with Tamoxifen exposed to MCF7 cells.

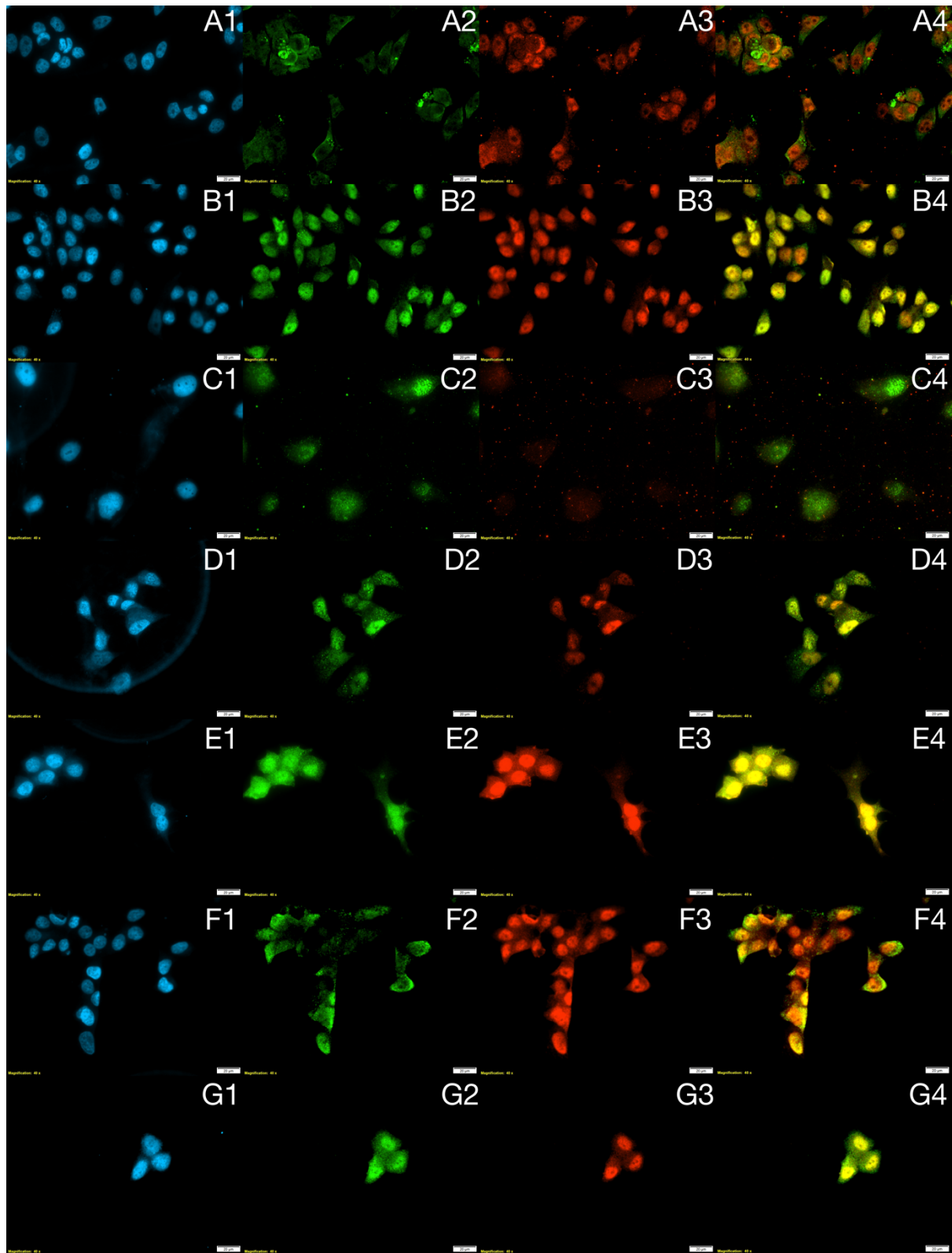


Figure 7.9: MCF7 cells. A: MCF7 cells treated Anastrozole. B: MCF7 cells treated with Anastrozole exposed to WB. C: MCF7 cells treated with Anastrozole exposed to PRP. D: MCF7 cells treated with Anastrozole exposed to PPP. E: WB treated with Anastrozole exposed to MCF7 cells. F: PRP treated with Anastrozole exposed to MCF7 cells. G: PPP treated with Anastrozole exposed to MCF7 cells.

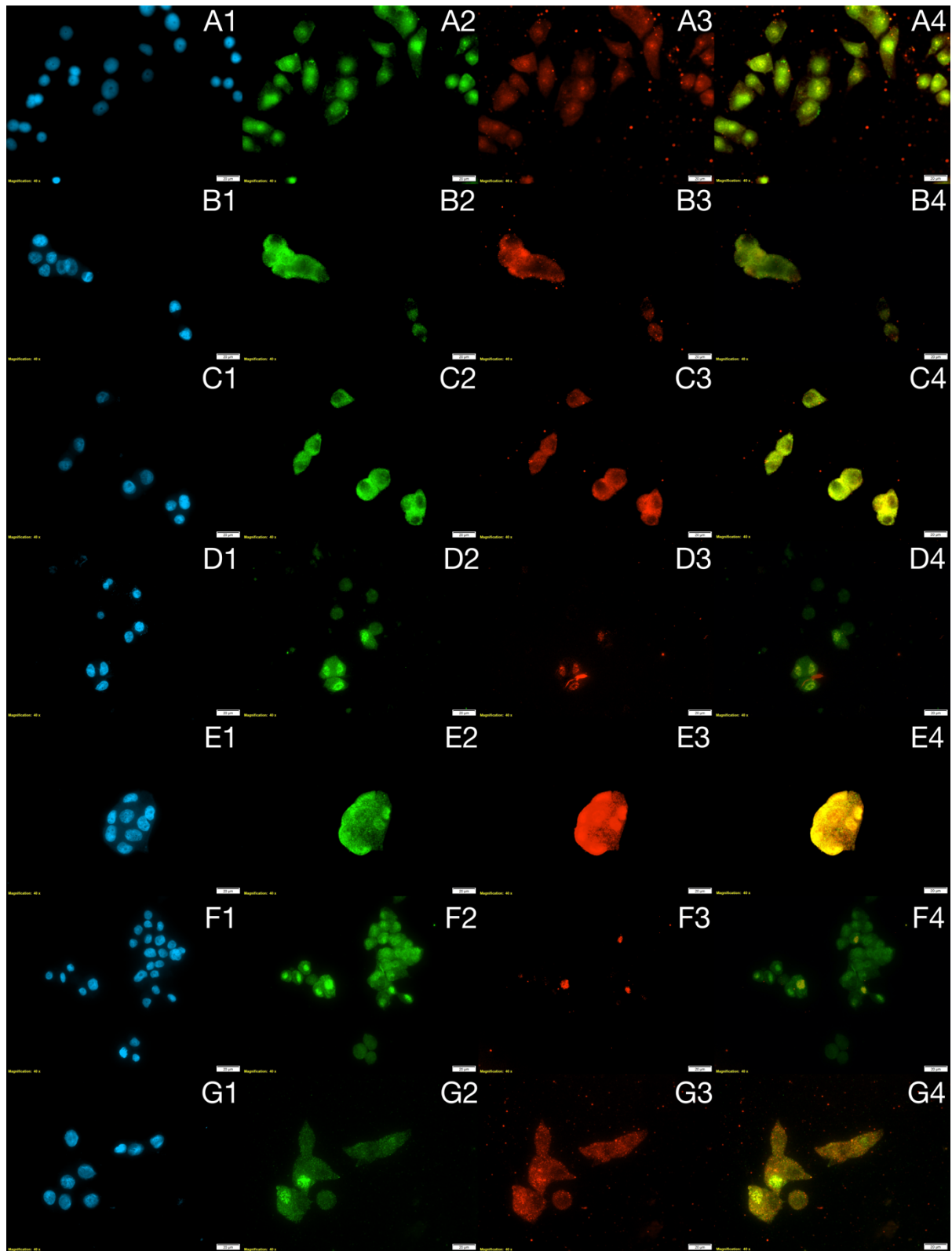


Figure 7.10: T47D cells. A: T47D cells treated Tamoxifen. B: T47D cells treated with Tamoxifen exposed to WB. C: T47D cells treated with Tamoxifen exposed to PRP. D: T47D cells treated with Tamoxifen exposed to PPP. E: WB treated with Tamoxifen exposed to T47D cells. F: PRP treated with Tamoxifen exposed to T47D cells. G: PPP treated with Tamoxifen exposed to T47D cells.

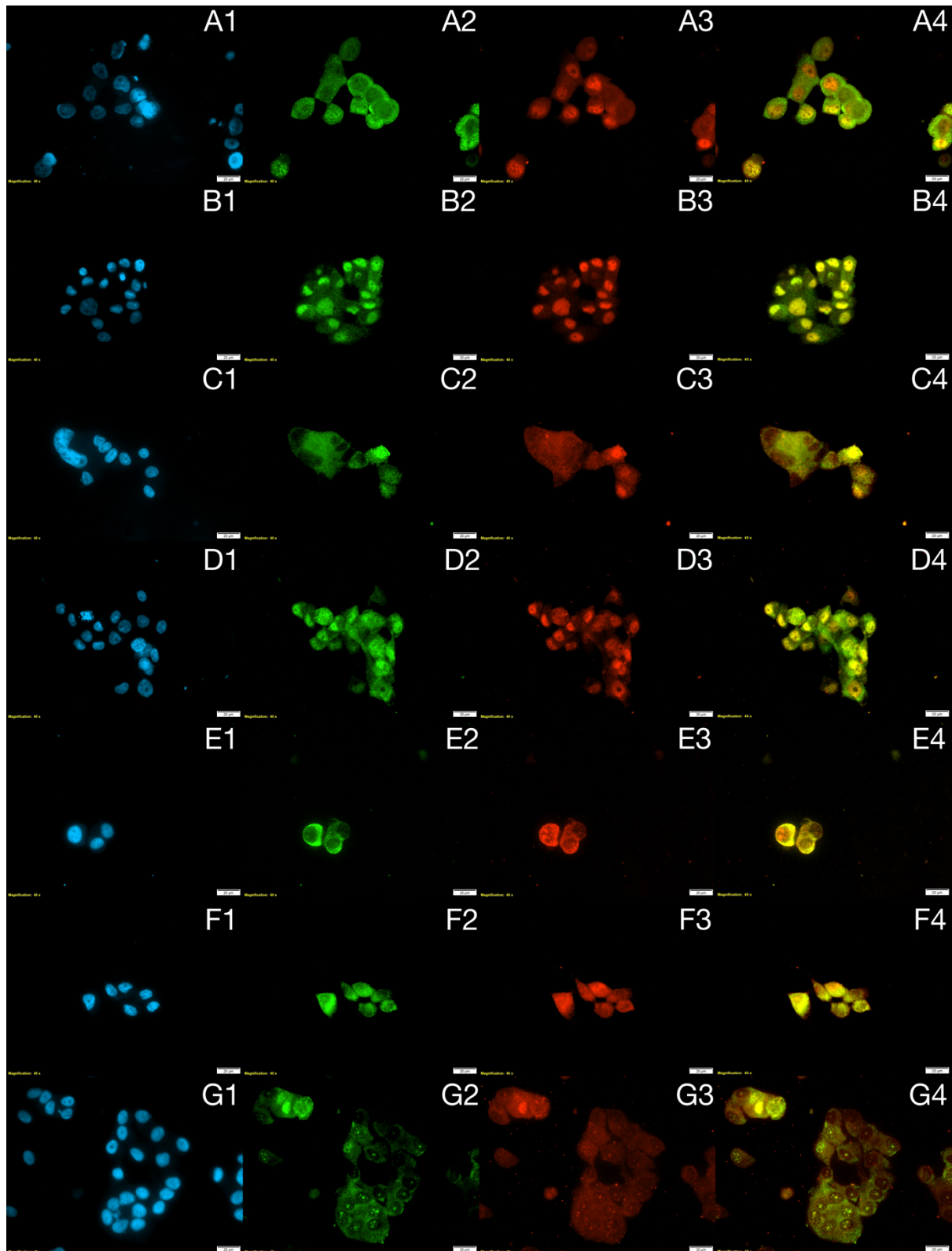


Figure 7.11: T47D cells. A: T47D cells treated Anastrozole. B: T47D cells treated with Anastrozole exposed to WB. C: T47D cells treated with Anastrozole exposed to PRP. D: T47D cells treated with Anastrozole exposed to PPP. E: WB treated with Anastrozole exposed to T47D cells. F: PRP treated with Anastrozole exposed to T47D cells. G: PPP treated with Anastrozole exposed to T47D cells.



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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 Ca²⁺ 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 cm² 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% CO₂ 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 (NH₄Cl) 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, Lasac, Johannesburg, South Africa) in 24-well plates and was incubated at 37 °C, 5% CO₂ 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 $200 \times g$ for 5 min and the resulting pellet resuspended in 150 μ l 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 $200 \times g$ for 5 min. The resulting pellet was resuspended in 500 μ l 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: $IPA = gMFI(CD62P) \times n(CD41a^+ CD62P^+ \text{ events})$ 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 $p < 0.05$.

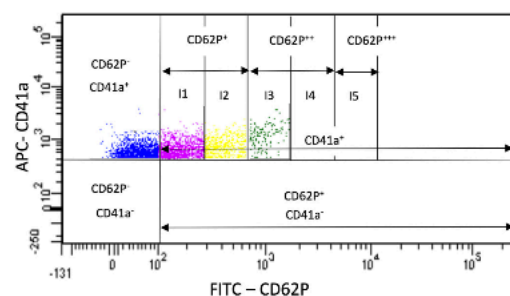


Fig. 1. Representative scatter plot showing the spread of platelet activation across the interval gates. Quadrant 1 is the inactive platelet population ($CD41a^+ CD62P^-$) population, while the activated population of interest ($CD41a^+ CD62P^+$) was within the 2nd quadrant. Interval gates (I1–I5) were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

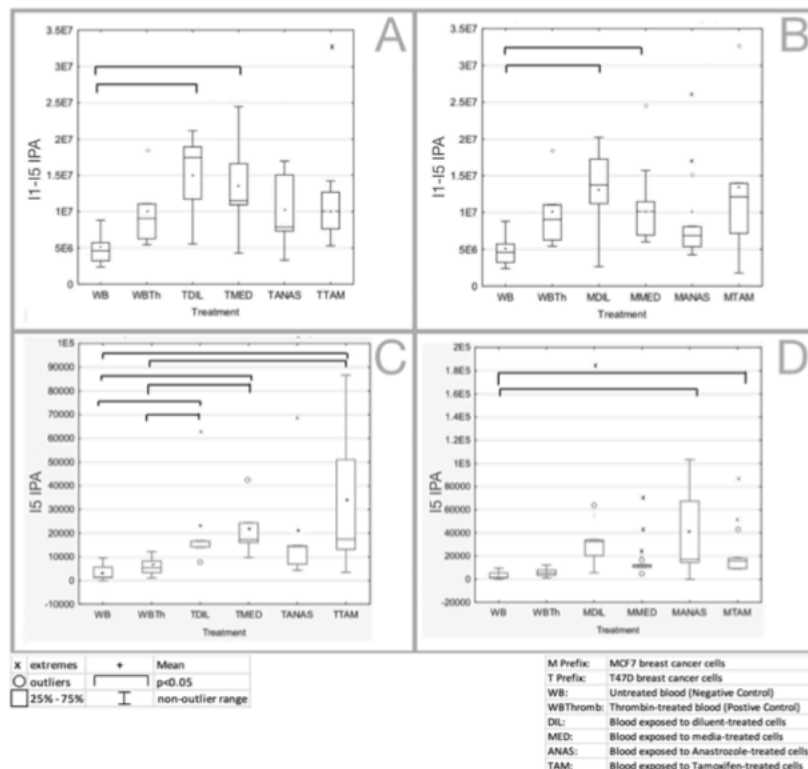


Fig. 2. Box and whisker plots indicating the Index of Platelet Activation (IPA) in lysed whole blood (WB) samples incubated with breast cancer cells and hormone-therapy treatment, as presented in intervals 11–15. Overall IPA presented in pre-treated MCF7 (A) and T47D (B) breast cancer cell lines. Overall 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),

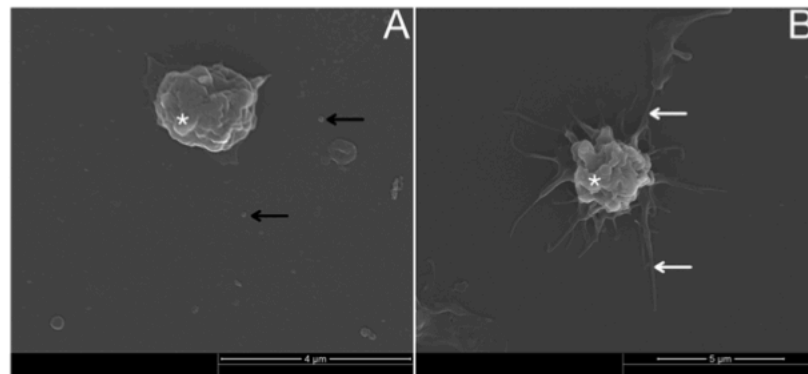


Fig. 3. Scanning electron microscopy images of platelets in lysed whole blood (WB) samples. A: Untreated, negative control, showing an inactive platelet with a smooth membrane and membrane folds (*), as well as the presence of microvesicles (black arrows). B: Platelet exposed to 0.1 U/ml thrombin displaying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

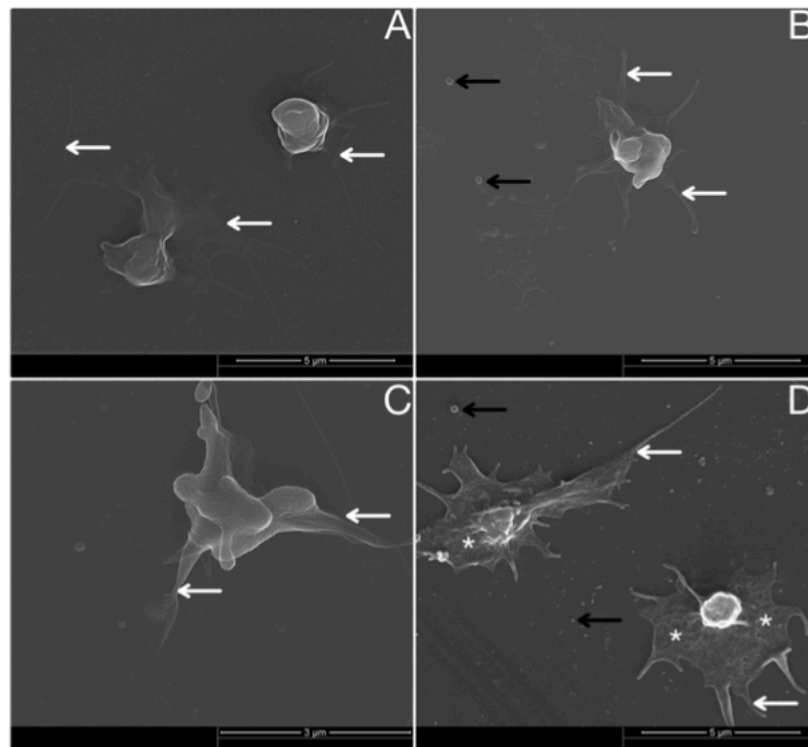


Fig. 4. Scanning electron microscopy images of platelets in lysed whole blood (WB) samples co-incubated with MCF7 cells and corresponding treatments. A: Platelets exposed to diluent-treated MCF7 cells displaying a smooth membrane with membrane folds and extending lamellipodia. B: Platelet exposed to 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).

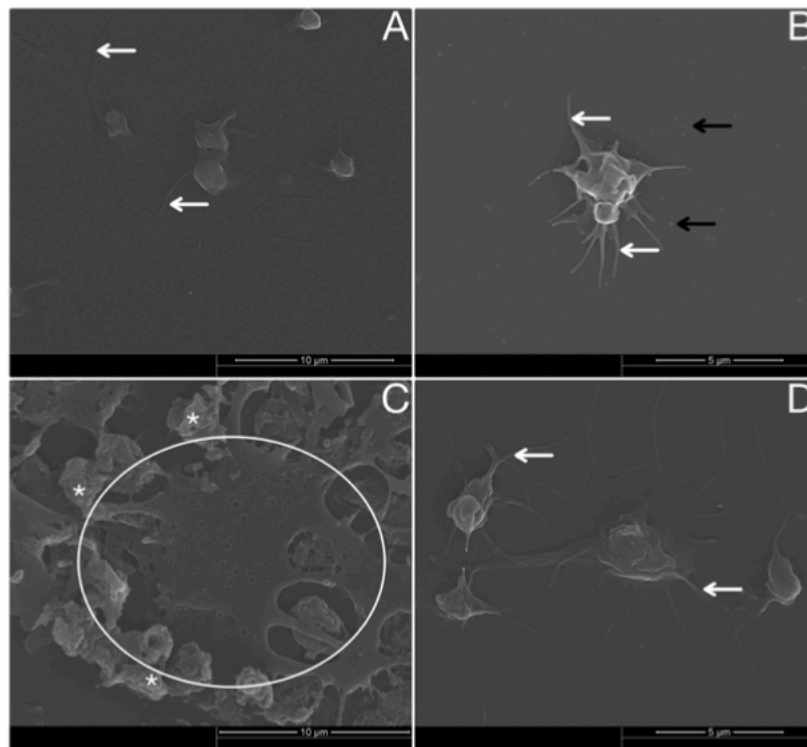


Fig. 5. Scanning electron microscopy images of platelets in lysed whole blood (WB) samples co-incubated with T47D cells and its corresponding treatments. A: Platelet exposed to diluent-treated T47D cells displaying a smooth membrane with extending filipodia. B: Platelet exposed to media-treated T47D 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 μ M Tamoxifen used in our study compared to a range of 3 μ M–20 μ M [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 hypercoagulable 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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Appendix I

Statistical Analyses

Early Platelet Activation – CD62P expression. Green in Dunn's Post Hoc indicates significant values.

P7 - P11 IPA on Geo Mean			
	All		All
N	30	N	30
Shapiro-Wilk W	0,8802	Shapiro-Wilk W	0,945
p(normal)	0,00285	p(normal)	0,1242
Anderson- Darling A	1,032	Anderson- Darling A	0,5405
p(normal)	0,008746	p(normal)	0,152
p(Monte Carlo)	0,0093	p(Monte Carlo)	0,1586
Jarque-Bera JB	13,53	Jarque-Bera JB	2,121
p(normal)	0,001152	p(normal)	0,3463
p(Monte Carlo)	0,0054	p(Monte Carlo)	0,1534

<i>T47D</i>		<i>MCF7</i>	
Kruskal- Wallis test for equal medians		Kruskal- Wallis test for equal medians	
H (chi2):	9,196	H (chi2):	6,528
Hc (tie corrected):	9,196	Hc (tie corrected):	6,528
p (same):	0,01015	p (same):	0,02582

<i>Dunns Post Hoc</i>						
	TDIL	TMED	TANAS	TTAM	WBUnt	WBThromb
TDIL		0,5414	0,196	0,222	0,004057	0,222
TMED	0,5414		0,4949	0,5414	0,02363	0,5414
TANAS	0,196	0,4949		0,9427	0,114	0,9427
TTAM	0,222	0,5414	0,9427		0,09846	1
WBUnt	0,004057	0,02363	0,114	0,09846		0,09846
WBThromb	0,222	0,5414	0,9427	1	0,09846	

	WBUnt	WBThromb	MDIL	MMED	MANAS	MTAM
WBUnt		0,106	0,02363	0,04249	0,2655	0,05241
WBThromb	0,106		0,5179	0,8575	0,615	0,7465
MDIL	0,02363	0,5179		0,6405	0,2504	0,7465
MMED	0,04249	0,8575	0,6405		0,4949	0,8857
MANAS	0,2655	0,615	0,2504	0,4949		0,4087
MTAM	0,05241	0,7465	0,7465	0,8857	0,4087	

Two Sample Paired Tests							
TDIL	MDIL			TMED	MMED		
N:	5			N:	5		
Mean:	1,50E+07	Mean:	1,30E+07	Mean:	1,36E+07	Mean:	1,01E+07
Median:	1,75E+07	Median:	1,37E+07	Median:	1,15E+07	Median:	1,01E+07
t test				t test			
Mean difference:	1,94E+06	95% conf.:	(-3,924E06 7,8096E06)	Mean difference:	3,50E+06	95% conf.:	(-5,9309E06 1,2938E07)
t :	0,91943	p (same mean):	0,4099	t :	1,0311	p (same mean):	0,36077
Exact:	p (same mean):	0,375		Exact:	p (same mean):	0,375	
Sign test				Sign test			
r :	4	p (same median):	0,375	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	11			W :	12		
Normal appr. z :	0,94388	p (same median):	0,34523	Normal appr. z :	1,2136	p (same median):	0,22492
Monte Carlo (n=99999):	p (same median):	0,43562		Monte Carlo (n=99999):	p (same median):	0,31496	
Exact:	p (same median):	0,4375		Exact:	p (same median):	0,3125	

TANAS	MANAS			TTAM	MTAM		
N:	5			N:	5		
Mean:	1,01E+07	Mean:	1,01E+07	Mean:	9,98E+06	Mean:	1,36E+07
Median:	7,89E+06	Median:	6,90E+06	Median:	1,00E+07	Median:	1,21E+07
t test				t test			
Mean difference:	21624	95% conf.:	(-1,0522E07 1,0565E07)	Mean difference:	3,58E+06	95% conf.:	(-8,3624E06 1,5516E07)
t :	-0,005694	p (same mean):	0,99573	t :	-0,83175	p (same mean):	0,45232
Exact:	p (same mean):	1		Exact:	p (same mean):	0,5625	
Sign test				Sign test			
r :	3	p (same median):	1	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	8			W :	10		
Normal appr. z :	0,13484	p (same median):	0,89274	Normal appr. z :	0,6742	p (same median):	0,50018
Monte Carlo (n=99999):	p (same median):	1		Monte Carlo (n=99999):	p (same median):	0,62584	
Exact:	p (same median):	1		Exact:	p (same median):	0,625	

WBUnt	WBThromb		
N:	5		
Mean:	4,96E+06	Mean:	1,01E+07
Median:	4,61E+06	Median:	9,08E+06
t test			
Mean difference:	5,10E+06	95% conf.:	(-1,9734E06 1,218E07)
t :	-2,0022	p (same mean):	0,11582
Exact:	p (same mean):	0,1875	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	13		
Normal appr. z :	1,4832	p (same median):	0,13801
Monte Carlo (n=99999):	p (same median):	0,18641	
Exact:	p (same median):	0,1875	

P7 IPA on Geo Mean			
<i>Kruskal Wallis for T47D</i>		<i>Kruskal Wallis for MCF7</i>	
Kruskal-Wallis test for equal medians		Kruskal-Wallis test for equal medians	
H (chi2):	4,84	H (chi2):	4,36
Hc (tie corrected):	4,84	Hc (tie corrected):	4,36
p (same):	0,04357	p (same):	0,04988

<i>Dunns Post Hoc</i>						
	TDIL	TMED	TANAS	TTAM	WBUnt	WBThromb
TDIL		0,615	0,3886	0,3321	0,03721	0,5179
TMED	0,615		0,7194	0,6405	0,114	0,8857
TANAS	0,3886	0,7194		0,9142	0,222	0,8294
TTAM	0,3321	0,6405	0,9142		0,2655	0,7465
WBUnt	0,03721	0,114	0,222	0,2655		0,1508
WBThromb	0,5179	0,8857	0,8294	0,7465	0,1508	

	WBUnt	WBThromb	MDIL	MMED	MANAS	MTAM
WBUnt		0,1612	0,106	0,1508	0,3503	0,06696
WBThromb	0,1612		0,8294	0,9713	0,6405	0,6664
MDIL	0,106	0,8294		0,8575	0,4949	0,8294
MMED	0,1508	0,9713	0,8575		0,615	0,6927
MANAS	0,3503	0,6405	0,4949	0,615		0,3692
MTAM	0,06696	0,6664	0,8294	0,6927	0,3692	

<i>Two Sample Paired Test</i>			
TDIL	MDIL		
N:	5		
Mean:	1,50E+05	Mean:	1,25E+05
Median:	1,64E+05	Median:	1,14E+05
t test			
Mean difference:	25363	95% conf.:	(-55151 1,0588E05)
t :	0,87462	p (same mean):	0,43115
Exact:	p (same mean):	0,5	
Sign test			
r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :			
W :	10		
Normal appr. z :	0,6742	p (same median):	0,50018
Monte Carlo (n=99999):	p (same median):	0,62552	
Exact:	p (same median):	0,625	

TMED	MMED		
N:	5		
Mean:	1,39E+05	Mean:	1,29E+05
Median:	1,22E+05	Median:	1,47E+05
t test			
Mean difference:	9987,4	95% conf.:	(-85597 1,0557E05)
t :	0,29011	p (same mean):	0,78615
Exact:	p (same mean):	0,8125	
Sign test			
r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :			
W :	9		
Normal appr. z :	0,40452	p (same median):	0,68583
Monte Carlo (n=99999):	p (same median):	0,81287	
Exact:	p (same median):	0,8125	

TANAS	MANAS		
N:	5		
Mean:	1,30E+05	Mean:	1,32E+05
Median:	1,01E+05	Median:	79562
t test			
Mean difference:	2570,3	95% conf.:	(-1,3413E05 1,3927E05)
t :	-0,052204	p (same mean):	0,96087
Exact:	p (same mean):	0,9375	
Sign test			
r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :			
W :	8		
Normal appr. z :	0,13484	p (same median):	0,89274
Monte Carlo (n=99999):	p (same median):	1	
Exact:	p (same median):	1	

TTAM	MTAM		
N:	5		
Mean:	1,25E+05	Mean:	1,47E+05
Median:	1,02E+05	Median:	1,02E+05
t test			
Mean difference:	21978	95% conf.:	(-1,1156E05 1,5551E05)
t :	-0,45696	p (same mean):	0,67141
Exact:	p (same mean):	0,5625	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	11		
Normal appr. z :	0,94388	p (same median):	0,34523
Monte Carlo (n=99999):	p (same median):	0,43441	
Exact:	p (same median):	0,4375	

WBUnt	WBThromb		
N:	5		
Mean:	72206	Mean:	1,26E+05
Median:	82499	Median:	1,06E+05
t test			
Mean difference:	53351	95% conf.:	(-19969 1,2667E05)
t :	-2,0203	p (same mean):	0,11346
Exact:	p (same mean):	0,125	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	14		
Normal appr. z :	1,7529	p (same median):	0,079616
Monte Carlo (n=99999):	p (same median):	0,12454	
Exact:	p (same median):	0,125	

P8 IPA on Geo Mean			
<i>Kruskal Wallis for T47D</i>		<i>Kruskal Wallis for MCF7</i>	
Kruskal-Wallis test for equal medians		Kruskal-Wallis test for equal medians	
H (chi2):	3,41	H (chi2):	2,244
Hc (tie corrected):	3,41	Hc (tie corrected):	2,244
p (same):	0,0637	p (same):	0,048145

<i>Dunns Post Hoc</i>						
	TDIL	TMED	TANAS	TTAM	WBUnt	WBThromb
TDIL		0,3321	0,196	0,4725	0,09136	0,5414
TMED	0,3321		0,7465	0,8015	0,4725	0,7194
TANAS	0,196	0,7465		0,5655	0,6927	0,4949
TTAM	0,4725	0,8015	0,5655		0,3321	0,9142
WBUnt	0,09136	0,4725	0,6927	0,3321		0,2812
WBThromb	0,5414	0,7194	0,4949	0,9142	0,2812	

	WBUnt	WBThromb	MDIL	MMED	MANAS	MTAM
WBUnt		0,3145	0,2359	0,615	0,6927	0,2655
WBThromb	0,3145		0,8575	0,615	0,5414	0,9142
MDIL	0,2359	0,8575		0,4949	0,4294	0,9427
MMED	0,615	0,615	0,4949		0,9142	0,5414
MANAS	0,6927	0,5414	0,4294	0,9142		0,4725
MTAM	0,2655	0,9142	0,9427	0,5414	0,4725	

<i>Two Sample Paired Test</i>							
TDIL	MDIL			TMED	MMED		
N:	5			N:	5		
Mean:	2,31E+05	Mean:	2,03E+05	Mean:	1,84E+05	Mean:	1,70E+05
Median:	2,05E+05	Median:	2,40E+05	Median:	1,65E+05	Median:	1,89E+05
t test				t test			
Mean difference:	28134	95% conf.:	(-60944 1,1721E05)	Mean difference:	13108	95% conf.:	(-1,125E05 1,3872E05)
t :	0,8769	p (same mean):	0,43005	t :	0,28973	p (same mean):	0,78642
Exact:	p (same mean):	0,4375		Exact:	p (same mean):	0,75	
Sign test				Sign test			
r :	3	p (same median):	1	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	12			W :	9		
Normal appr. z :	1,2136	p (same median):	0,22492	Normal appr. z :	0,40452	p (same median):	0,68583
Monte Carlo (n=99999):	p (same median):	0,31367		Monte Carlo (n=99999):	p (same median):	0,81324	
Exact:	p (same median):	0,3125		Exact:	p (same median):	0,8125	

TANAS	MANAS		
N:	5		
Mean:	1,63E+05	Mean:	2,26E+05
Median:	1,79E+05	Median:	1,32E+05
t test			
Mean difference:	62952	95% conf.:	(-2,2735E05 3,5325E05)
t :	-0,60208	p (same mean):	0,57958
Exact:	p (same mean):	0,6875	
Sign test			
r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :			
W :	9		
Normal appr. z :	0,40452	p (same median):	0,68583
Monte Carlo (n=99999):	p (same median):	0,80985	
Exact:	p (same median):	0,8125	

TTAM	MTAM		
N:	5		
Mean:	1,88E+05	Mean:	2,49E+05
Median:	1,87E+05	Median:	2,13E+05
t test			
Mean difference:	60811	95% conf.:	(-1,8553E05 3,0715E05)
t :	-0,68538	p (same mean):	0,53076
Exact:	p (same mean):	0,5625	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	11		
Normal appr. z :	0,94388	p (same median):	0,34523
Monte Carlo (n=99999):	p (same median):	0,43733	
Exact:	p (same median):	0,4375	

WBUnt	WBThromb		
N:	5		
Mean:	1,40E+05	Mean:	2,17E+05
Median:	1,43E+05	Median:	1,87E+05
t test			
Mean difference:	76658	95% conf.:	(-18687 1,72E05)
t :	-2,2323	p (same mean):	0,089384
Exact:	p (same mean):	0,1875	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	13		
Normal appr. z :	1,4832	p (same median):	0,13801
Monte Carlo (n=99999):	p (same median):	0,18652	
Exact:	p (same median):	0,1875	

P9 IPA on Geo Mean			
Kruskall Wallis for T47D		Kruskall Wallis for MCF7	
Kruskal-Wallis test for equal medians		Kruskal-Wallis test for equal medians	
H (chi2):	1,867	H (chi2):	1,831
Hc (tie corrected):	1,867	Hc (tie corrected):	1,831
p (same):	0,048672	p (same):	0,03872

<i>Dunns Post Hoc</i>						
	TDIL	TMED	TANAS	TTAM	WBUnt	WBThromb
TDIL		0,7194	0,4087	0,615	0,3503	0,8857
TMED	0,7194		0,6405	0,8857	0,5655	0,615
TANAS	0,4087	0,6405		0,7465	0,9142	0,3321
TTAM	0,615	0,8857	0,7465		0,6664	0,5179
WBUnt	0,3503	0,5655	0,9142	0,6664		0,2812
WBThromb	0,8857	0,615	0,3321	0,5179	0,2812	

	WBUnt	WBThromb	MDIL	MMED	MANAS	MTAM
WBUnt		0,2812	0,3321	0,6927	0,4725	0,2975
WBThromb	0,2812		0,9142	0,4949	0,7194	0,9713
MDIL	0,3321	0,9142		0,5655	0,8015	0,9427
MMED	0,6927	0,4949	0,5655		0,7465	0,5179
MANAS	0,4725	0,7194	0,8015	0,7465		0,7465
MTAM	0,2975	0,9713	0,9427	0,5179	0,7465	

<i>Two Sample Paired Test</i>							
TDIL	MDIL			TMED	MMED		
N:	5			N:	5		
Mean:	3,44E+05	Mean:	3,20E+05	Mean:	2,70E+05	Mean:	2,22E+05
Median:	2,67E+05	Median:	3,01E+05	Median:	2,58E+05	Median:	2,28E+05
t test				t test			
Mean difference:	23602	95% conf.:	(-3,6296E05 4,1017E05)	Mean difference:	48128	95% conf.:	(-1,8163E05 2,7789E05)
t :	0,16952	p (same mean):	0,87362	t :	0,58158	p (same mean):	0,59205
Exact:	p (same mean):	1		Exact:	p (same mean):	0,8125	
Sign test				Sign test			
r :	4	p (same median):	0,375	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	10			W :	8		
Normal appr. z :	0,6742	p (same median):	0,50018	Normal appr. z :	0,13484	p (same median):	0,89274
Monte Carlo (n=99999):	p (same median):	0,62643		Monte Carlo (n=99999):	p (same median):	1	
Exact:	p (same median):	0,625		Exact:	p (same median):	1	

TANAS	MANAS			TTAM	MTAM		
N:	5			N:	5		
Mean:	2,27E+05	Mean:	3,11E+05	Mean:	2,62E+05	Mean:	3,31E+05
Median:	2,02E+05	Median:	1,48E+05	Median:	1,79E+05	Median:	3,13E+05
t test				t test			
Mean difference:	84194	95% conf.:	(-4,1506E05 5,8345E05)	Mean difference:	69755	95% conf.:	(-1,591E05 2,9861E05)
t :	-0,46822	p (same mean):	0,664	t :	-0,84627	p (same mean):	0,44506
Exact:	p (same mean):	0,5625		Exact:	p (same mean):	0,5	
Sign test				Sign test			
r :	4	p (same median):	0,375	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	11			W :	10		
Normal appr. z :	0,94388	p (same median):	0,34523	Normal appr. z :	0,6742	p (same median):	0,50018
Monte Carlo (n=99999):	p (same median):	0,43862		Monte Carlo (n=99999):	p (same median):	0,62427	
Exact:	p (same median):	0,4375		Exact:	p (same median):	0,625	

WBUnt	WBThromb		
N:	5		
Mean:	1,85E+05	Mean:	3,43E+05
Median:	2,25E+05	Median:	2,74E+05
t test			
Mean difference:	1,58E+05	95% conf.:	(-47618 3,635E05)
t :	-2,1333	p (same mean):	0,09984
Exact:	p (same mean):	0,1875	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	13		
Normal appr. z :	1,4832	p (same median):	0,13801
Monte Carlo (n=99999):	p (same median):	0,18791	
Exact:	p (same median):	0,1875	

P10 IPA on Geo Mean

Kruskal Wallis for T47D

Kruskal-Wallis test for equal medians

H (chi2):

1,676

Hc (tie corrected):

1,676

p (same):

0,8919

Kruskal Wallis for MCF7

Kruskal-Wallis test for equal medians

H (chi2):

1,335

Hc (tie corrected):

1,335

p (same):

0,9312

<i>Dunns Post Hoc</i>						
	TDIL	TMED	TANAS	TTAM	WBUnt	WBThromb
TDIL		1	0,6405	0,9713	0,3321	0,9427
TMED	1		0,6405	0,9713	0,3321	0,9427
TANAS	0,6405	0,6405		0,6664	0,615	0,59
TTAM	0,9713	0,9713	0,6664		0,3503	0,9142
WBUnt	0,3321	0,3321	0,615	0,3503		0,2975
WBThromb	0,9427	0,9427	0,59	0,9142	0,2975	

	TDIL	TMED	TANAS	TTAM	WBUnt	WBThromb
TDIL		1	0,6405	0,9713	0,3321	0,9427
TMED	1		0,6405	0,9713	0,3321	0,9427
TANAS	0,6405	0,6405		0,6664	0,615	0,59
TTAM	0,9713	0,9713	0,6664		0,3503	0,9142
WBUnt	0,3321	0,3321	0,615	0,3503		0,2975
WBThromb	0,9427	0,9427	0,59	0,9142	0,2975	

<i>Two Sample Paired Test</i>							
TDIL	MDIL			TMED	MMED		
N:	5			N:	5		
Mean:	3,22E+05	Mean:	3,68E+05	Mean:	2,35E+05	Mean:	1,86E+05
Median:	2,44E+05	Median:	1,86E+05	Median:	1,92E+05	Median:	1,38E+05
t test				t test			
Mean difference:	46753	95% conf.:	(-7,176E05 8,1111E05)	Mean difference:	49554	95% conf.:	(-2,6028E05 3,5939E05)
t :	-0,16982	p (same mean):	0,87339	t :	0,44406	p (same mean):	0,67996
Exact:	p (same mean):	0,6875		Exact:	p (same mean):	0,9375	
Sign test				Sign test			
r :	4	p (same median):	0,375	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	10			W :	9		
Normal appr. z :	0,6742	p (same median):	0,50018	Normal appr. z :	0,40452	p (same median):	0,68583
Monte Carlo (n=99999):	p (same median):	0,62763		Monte Carlo (n=99999):	p (same median):	0,81047	
Exact:	p (same median):	0,625		Exact:	p (same median):	0,8125	

TANAS	MANAS			TTAM	MTAM		
N:	5			N:	5		
Mean:	2,15E+05	Mean:	2,91E+05	Mean:	2,63E+05	Mean:	2,21E+05
Median:	1,18E+05	Median:	67185	Median:	1,22E+05	Median:	2,15E+05
t test				t test			
Mean difference:	76121	95% conf.:	(-5,8292E05 7,3516E05)	Mean difference:	42014	95% conf.:	(-2,546E05 3,3863E05)
t :	-0,32069	p (same mean):	0,7645	t :	0,39327	p (same mean):	0,71418
Exact:	p (same mean):	0,875		Exact:	p (same mean):	0,9375	
Sign test				Sign test			
r :	3	p (same median):	1	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	8			W :	9		
Normal appr. z :	0,13484	p (same median):	0,89274	Normal appr. z :	0,40452	p (same median):	0,68583
Monte Carlo (n=99999):	p (same median):	1		Monte Carlo (n=99999):	p (same median):	0,8104	
Exact:	p (same median):	1		Exact:	p (same median):	0,8125	

WBUnt	WBThromb		
N:	5		
Mean:	1,01E+05	Mean:	2,18E+05
Median:	68102	Median:	1,58E+05
t test			
Mean difference:	1,17E+05	95% conf.:	(-57687 2,9099E05)
t :	-1,8577	p (same mean):	0,13676
Exact:	p (same mean):	0,1875	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	13		
Normal appr. z :	1,4832	p (same median):	0,13801
Monte Carlo (n=99999):	p (same median):	0,18771	
Exact:	p (same median):	0,1875	

P11 IPA on Geo Mean			
<i>Kruskall Wallis for T47D</i>		<i>Kruskall Wallis for MCF7</i>	
Kruskal-Wallis test for equal medians		Kruskal-Wallis test for equal medians	
H (chi2):	14,42	H (chi2):	12,15
Hc (tie corrected):	14,43	Hc (tie corrected):	12,16
p (same):	0,01311	p (same):	0,03262

<i>Dunns Post Hoc</i>						
	TDIL	TMED	TANAS	TTAM	WBUnt	WBThromb
TDIL		0,7464	0,5654	0,8857	0,01318	0,04059
TMED	0,7464		0,3691	0,8574	0,005076	0,01774
TANAS	0,5654	0,3691		0,4724	0,05691	0,1408
TTAM	0,8857	0,8574	0,4724		0,008728	0,02842
WBUnt	0,01318	0,005076	0,05691	0,008728		0,6664
WBThromb	0,04059	0,01774	0,1408	0,02842	0,6664	

	WBUnt	WBThromb	MDIL	MMED	MANAS	MTAM
WBUnt		0,706	0,00598	0,07529	0,02359	0,03555
WBThromb	0,706		0,01771	0,1611	0,05923	0,08457
MDIL	0,00598	0,01771		0,332	0,6276	0,5178
MMED	0,07529	0,1611	0,332		0,6276	0,7464
MANAS	0,02359	0,05923	0,6276	0,6276		0,8715
MTAM	0,03555	0,08457	0,5178	0,7464	0,8715	

<i>Two Sample Paired Test</i>							
TDIL	MDIL			TTAM	MTAM		
N:	5			N:	5		
Mean:	23602	Mean:	55391	Mean:	34385	Mean:	18858
Median:	16650	Median:	32612	Median:	17491	Median:	15899
t test				t test			
Mean difference:	31789	95% conf.:	(-32266 95844)	Mean difference:	15527	95% conf.:	(-24734 55787)
t :	-1,3779	p (same mean):	0,2403	t :	1,0707	p (same mean):	0,34459
Exact:	p (same mean):	0,125		Exact:	p (same mean):	0,4375	
Sign test				Sign test			
r :	4	p (same median):	0,375	r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	14			W :	11		
Normal appr. z :	1,7529	p (same median):	0,079616	Normal appr. z :	0,94388	p (same median):	0,34523
Monte Carlo (n=99999):	p (same median):	0,12473		Monte Carlo (n=99999):	p (same median):	0,43729	
Exact:	p (same median):	0,125		Exact:	p (same median):	0,4375	

TMED	MMED			WBUnt	WBThromb		
N:	5			N:	5		
Mean:	22012	Mean:	22029	Mean:	3578,8	Mean:	6073,6
Median:	17336	Median:	11773	Median:	1269,2	Median:	5386
t test				t test			
Mean difference:	16,916	95% conf.:	(-20885 20919)	Mean difference:	2494,8	95% conf.:	(-7240 12230)
t :	-0,002247	p (same mean):	0,99831	t :	-0,71154	p (same mean):	0,51605
Exact:	p (same mean):	1		Exact:	p (same mean):	0,5	
Sign test				Sign test			
r :	3	p (same median):	1	r :	3	p (same median):	1
Wilcoxon test (normal approximation inaccurate) :				Wilcoxon test (normal approximation inaccurate) :			
W :	9			W :	10		
Normal appr. z :	0,40452	p (same median):	0,68583	Normal appr. z :	0,6742	p (same median):	0,50018
Monte Carlo (n=99999):	p (same median):	0,81047		Monte Carlo (n=99999):	p (same median):	0,62509	
Exact:	p (same median):	0,8125		Exact:	p (same median):	0,625	

TANAS	MANAS		
N:	5		
Mean:	21857	Mean:	40519
Median:	14432	Median:	17227
t test			
Mean difference:	18662	95% conf.:	(-11976 49300)
t :	-1,6912	p (same mean):	0,16606
Exact:	p (same mean):	0,1875	
Sign test			
r :	4	p (same median):	0,375
Wilcoxon test (normal approximation inaccurate) :			
W :	13		
Normal appr. z :	1,4832	p (same median):	0,13801
Monte Carlo (n=99999):	p (same median):	0,18692	
Exact:	p (same median):	0,1875	

ACTB, ESR1 and ESR2 Analysis.

Wilcoxon matched pair test. Green indicating significance.

GENE		MANAS - MCELLS	MTAM - MCELLS	MWB - MCELLS	MWBANAS - MCELLS
ACTB	Z	-2,023b	-2,023b	-2,023b	-2,023b
	Asymp, Sig, (2-tailed)	0,043	0,043	0,043	0,043
ESR1	Z	-,674c	-1,483c	-,405c	-,674c
	Asymp, Sig, (2-tailed)	0,5	0,138	0,686	0,5
ESR2	Z	-1,095c	,000d	-,365c	-1,000c
	Asymp, Sig, (2-tailed)	0,0273	1	0,715	0,317

GENE		MWBTAM - MCELLS	MDIL - MCELLS	MWBANAS - MWB	MWBTAM - MWB
ACTB	Z	-2,023b	-2,023b	-,135b	-,674b
	Asymp, Sig, (2-tailed)	0,043	0,043	0,893	0,5
ESR1	Z	-,405c	-,944c	-,405c	-,674c
	Asymp, Sig, (2-tailed)	0,686	0,345	0,686	0,5
ESR2	Z	-,365c	-1,105d	-,365c	-1,604c
	Asymp, Sig, (2-tailed)	0,715	0,269	0,715	0,109

GENE		MANAS - MWB	MTAM - MWB	MDIL - MWB	MTAM - MANAS
ACTB	Z	-2,023b	-1,753b	-,135b	-,135b
	Asymp, Sig, (2-tailed)	0,043	0,08	0,893	0,893
ESR1	Z	-,135c	-,135c	-,674c	-,135c
	Asymp, Sig, (2-tailed)	0,893	0,893	0,5	0,893
ESR2	Z	-,730c	-,552d	,000c	-1,604c
	Asymp, Sig, (2-tailed)	0,465	0,581	1	0,109

GENE		MWBTAM - MWBANAS	MANAS - MWBANAS	MTAM - MWBTAM	TANAS - TCELLS
ACTB	Z	-,135b	-,674b	-1,483b	-1,214b
	Asymp, Sig, (2-tailed)	0,893	0,5	0,138	0,225
ESR1	Z	-,135c	-1,214c	-,135c	-,405c
	Asymp, Sig, (2-tailed)	0,893	0,225	0,893	0,686
ESR2	Z	-,184c	-1,095d	-,184c	-,365c
	Asymp, Sig, (2-tailed)	0,854	0,273	0,854	0,715

GENE		TTAM - TCELLS	TWB - TCELLS	TWBANAS - TCELLS	TWBTAM - TCELLS
ACTB	Z	-1,483b	-,135b	-2,023b	-1,483b
	Asymp, Sig, (2-tailed)	0,138	0,893	0,043	0,138
ESR1	Z	-1,214c	-,944c	-,944c	-1,219c
	Asymp, Sig, (2-tailed)	0,0225	0,345	0,345	0,223
ESR2	Z	-,730c	-,730d	-,730c	,000c
	Asymp, Sig, (2-tailed)	0,465	0,465	0,465	0,02

GENE		TDIL - TCELLS	TWBANAS - TWB	TWBTAM - TWB	TANAS - TWB
ACTB	Z	-2,023b	-1,483b	-,944b	-,405b
	Asymp, Sig, (2-tailed)	0,043	0,138	0,345	0,686
ESR1	Z	-,674c	-,365c	-1,214c	-1,625c
	Asymp, Sig, (2-tailed)	0,5	0,715	0,225	0,104
ESR2	Z	-,365c	-1,461d	,000c	-1,095c
	Asymp, Sig, (2-tailed)	0,715	0,144	1	0,273

GENE		TTAM - TWB	TDIL - TWB	TTAM - TANAS	TWBTAM - TWBANAS
ACTB	Z	-1,483b	-2,023b	-,405b	-,674b
	Asymp, Sig, (2-tailed)	0,138	0,043	0,686	0,5
ESR1	Z	-,944c	-,135c	-1,214c	-,135c
	Asymp, Sig, (2-tailed)	0,345	0,893	0,225	0,893
ESR2	Z	-1,289c	-,730d	-,730c	,000c
	Asymp, Sig, (2-tailed)	0,197	0,465	0,465	1

GENE		TWBANAS - TANAS	TWBTAM - TTAM
ACTB	Z	-,405b	-,135b
	Asymp, Sig, (2-tailed)	0,686	0,893
ESR1	Z	-1,214c	-,405c
	Asymp, Sig, (2-tailed)	0,225	0,686
ESR2	Z	-,365c	,000d
	Asymp, Sig, (2-tailed)	0,715	1

Platelet Activation – Green indicates significance

Q2

Friedman			
Test for equal medians			
chi2:	95,591	Degrees of freedom	17
chi2, tie corrected:	95,591		
chi2, continuity corrected:	95,574		
p (same), asymptotic:	5,84E-13		

CD62																	
Wilcoxon Pair																	
	PPP all stains	PRP all stains	PRP Thromb	PRP w Anas	PRP w Tam	PRP MCF7 Anas	PRP MCF7 Dil	PRP MCF7 Med	PRP MCF7 Tam	PRP w Anas M	PRP w Tam M	PRP T47 Anas	PRP T47 Dil	PRP T47 Med	PRP T47 Tam	PRP w Anas T4	PRP w Tam T4
PPP all stains		0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,4462	0,04615	0,4462	0,8462
PRP all stains	0,04615		0,04615	0,04615	0,04615	0,0615	0,04615	0,0615	0,04615	0,04615	0,04615	0,0615	0,0615	0,0615	0,0615	0,0615	0,0615
PRP Thromb	0,04615	0,04615		0,04615	0,04615	0,07692	0,07692	0,04692	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615
PRP w Anas	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,0615	0,04615	0,04615	0,1077	0,04615	0,04615	0,07692	0,04615	0,07692	0,07692
PRP w Tam	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,5692	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615
PRP MCF7 Anas	0,04615	0,0615	0,07692	0,04615	0,04615		0,07692	0,1692	0,04615	0,0615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Dil	0,04615	0,04615	0,07692	0,04615	0,04615	0,07692		0,04615	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Med	0,0615	0,0615	0,04692	0,0615	0,04615	0,1692	0,04615		0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Tam	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,0615	0,1077	0,04615	0,04615	0,5692	0,04615	0,04615
PRP w Anas MCF7	0,04615	0,04615	0,04615	0,04615	0,5692	0,0615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615
PRP w Tam MCF7	0,04615	0,04615	0,04615	0,1077	0,04615	0,04615	0,0615	0,04615	0,0615	0,04615		0,04615	0,04615	0,4462	0,04615	0,07692	0,07692
PRP T47 Anas	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,1077	0,04615	0,04615		0,04615	0,0615	0,04615	0,04615	0,04615
PRP T47 Dil	0,04615	0,0615	0,04615	0,04615	0,04615	0,07692	0,07692	0,04615	0,04615	0,04615	0,04615	0,04615		0,0615	0,04615	0,04615	0,04615
PRP T47 Med	0,4462	0,0615	0,04615	0,07692	0,07692	0,04615	0,04615	0,04615	0,07692	0,4462	0,0615	0,0615	0,0615		0,04615	0,0462	0,4462
PRP T47 Tam	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,5692	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615
PRP w Anas T47	0,4462	0,0615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,462	0,04615		0,07692
PRP w Tam T47	0,8462	0,0615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,4462	0,04615	0,07692	

CD63															
Wilcoxon Pair															
	PRP all stain	PRP Thromb	PRP w Anas	PRP w Tam	PRP MCF7 D	PRP MCF7 Med	PRP MCF7 Tam	PRP w Anas	PRP w Tam	PRP T47 Ana	PRP T47 Dil	PRP T47 Med	PRP T47 Tam	PRP w Anas	PRP w Tam T
PRP all stains		0,04692	0,04615	0,04615	0,0615	0,04615	0,04615	0,07692	0,0615	0,04615	0,04615	0,0615	0,0615	0,04462	0,04615
PRP Thromb	0,04692		0,04615	0,04615	0,07692	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615	0,04615	0,4462	0,04615	0,04615
PRP w Anas	0,04615	0,04615		0,4462	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,8462	0,04615
PRP w Tam	0,04615	0,04615	0,4462		0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,8462	0,04615
PRP MCF7 Anas	0,0615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,0615	0,04615	0,04615	0,1692	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Dil	0,0615	0,07692	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,04615	0,1692	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Med	0,04615	0,04615	0,0615	0,04615	0,04615		0,04615	0,04615	0,04615	0,4462	0,07692	0,8462	0,04615	0,04615	0,04615
PRP MCF7 Tam	0,07692	0,07692	0,04615	0,04615	0,04615	0,04615		0,04615	0,0615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615
PRP w Anas MCF7	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615
PRP w Tam MCF7	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,04615	0,04615
PRP T47 Anas	0,04615	0,04615	0,04615	0,04615	0,04615	0,4462	0,04615	0,04615	0,04615		0,07692	0,04692	0,04615	0,04615	0,04615
PRP T47 Dil	0,0615	0,04615	0,04615	0,04615	0,1692	0,07692	0,04615	0,04615	0,04615	0,07692		0,07692	0,04615	0,04615	0,04615
PRP T47 Med	0,0615	0,04615	0,04615	0,04615	0,04615	0,8462	0,04615	0,04615	0,04615	0,07692	0,07692		0,04615	0,04615	0,04615
PRP T47 Tam	0,04462	0,4462	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,0615
PRP w Anas T47	0,04615	0,04615	0,8462	0,8462	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615
PRP w Tam T47	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,0615	

Friedman			
Test for equal medians			
chi2:	69,007	Degrees of f	16
chi2, tie co	69,007		
chi2, conti	68,991		
p (same), as	1,50E-08		

CD62																
Wilcoxon Pair																
	WB all stain	WB Thromb	WB w Anas	WB w Anas	WB MCF7 An	WB MCF7 Dil	WB MCF7 Med	WB MCF7 Ta	WB T47 Ana	WB T47 Dil	WB T47 Med	WB T47 Tam	WB w Anas	WB w Tam	WB w Tam	WB w Tam T
WB all stains		0,4615	0,4615	0,04615	0,4615	0,4615	0,04615	0,4615	0,4615	0,4462	0,1077	0,04615	0,04692	0,4615	0,4615	0,04615
WB Thromb	0,4615		0,1077	0,4462	0,07692	0,04615	0,1077	0,04615	0,1077	0,1077	0,07692	0,4462	0,04615	0,04615	0,04615	0,04615
WB w Anas	0,4615	0,1077		0,07692	0,6923	0,04615	0,07692	0,04615	0,4462	0,1077	0,07692	0,07692	0,04615	0,04615	0,04615	0,1692
WB w Anas MCF7	0,04615	0,4462	0,07692		0,04615	0,04615	0,07692	0,04615	0,1077	0,1077	0,04615	0,1692	0,04615	0,07692	0,04615	0,07692
WB MCF7 Anas	0,4615	0,07692	0,6923	0,04615		0,04615	0,07692	0,04615	0,1077	0,1077	0,07692	0,04615	0,04615	0,04615	0,04615	0,07692
WB MCF7 Dil	0,4615	0,04615	0,04615	0,04615	0,04615		0,4615	0,5692	0,04615	0,1077	0,1077	0,04615	0,07692	0,04615	0,04615	0,04615
WB MCF7 Med	0,04615	0,1077	0,07692	0,07692	0,07692	0,4615		0,4615	0,1077	0,1077	0,04615	0,2308	0,04615	0,07692	0,04615	0,4462
WB MCF7 Tam	0,4615	0,04615	0,04615	0,04615	0,04615	0,5692	0,4615		0,07692	0,1077	0,07692	0,04615	0,04615	0,04615	0,04615	0,07692
WB T47 Ana	0,4615	0,1077	0,4462	0,1077	0,1077	0,04615	0,01077	0,07692		0,1077	0,04615	0,07692	0,04615	0,07692	0,07692	0,1077
WB T47 Dil	0,4462	0,1077	0,1077	0,1077	0,1077	0,1077	0,1077	0,1077	0,01077		0,04615		0,1077	0,07692	0,1077	0,1077
WB T47 Med	0,1077	0,07692	0,07692	0,04615	0,07692	0,1077	0,04615	0,07692	0,04615	0,04615		0,04615	0,1077	0,4615	0,04615	0,04692
WB T47 Tam	0,04615	0,4462	0,07692	0,1692	0,04615	0,04615	0,2308	0,04615	0,07692	0,1077	0,04615		0,04615	0,07692	0,04615	0,01692
WB w Anas T47	0,04692	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,1077	0,01077	0,04615		0,04615	0,04615	0,04615
WB w Tam	0,4615	0,04615	0,04615	0,07692	0,04615	0,04615	0,07692	0,4615	0,07692	0,07692	0,4615	0,07692	0,04615		0,2308	0,04615
WB w Tam MCF7	0,4615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,4615	0,07692	0,1077	0,04615	0,04615	0,04615	0,2308		0,04615
WB w Tam T47	0,04615	0,04615	0,1692	0,07692	0,07692	0,04615	0,4462	0,07692	0,1077	0,1077	0,04692	0,1692	0,04615	0,04615	0,04615	

CD63																
Wilcoxon Pair																
	WB all stain	WB Thromb	WB w Anas	WB w Anas	WB MCF7 An	WB MCF7 Dil	WB MCF7 Med	WB MCF7 Ta	WB T47 Ana	WB T47 Dil	WB T47 Med	WB T47 Tam	WB w Anas	WB w Tam	WB w Tam	WB w Tam T
WB all stains		0,04615	0,4615	0,04615	0,4615	0,4615	0,4615	0,4615	0,04615	0,4462	0,01077	0,04615	0,07692	0,4615	0,4615	0,04615
WB Thromb	0,04615		0,1077	0,4462	0,07692	0,04615	0,1077	0,04615	0,1077	0,1077	0,07692	0,4462	0,04615	0,04615	0,04615	0,04615
WB w Anas	0,4615	0,1077		0,07692	0,6923	0,04615	0,07692	0,04615	0,4462	0,1077	0,07692	0,07692	0,04615	0,04615	0,04615	0,1692
WB w Anas MCF7	0,04615	0,4462	0,07692		0,04615	0,04615	0,04692	0,04615	0,1077	0,1077	0,04615	0,1692	0,04615	0,07692	0,04615	0,07692
WB MCF7 Anas	0,4615	0,07692	0,6923	0,04615		0,04615	0,07692	0,04615	0,1077	0,1077	0,07692	0,04615	0,04615	0,04615	0,04615	0,07692
WB MCF7 Dil	0,4615	0,04615	0,04615	0,04615	0,04615		0,4615	0,5692	0,04615	0,1077	0,1077	0,04615	0,07692	0,04615	0,04615	0,04615
WB MCF7 Med	0,4615	0,1077	0,07692	0,04692	0,07692	0,4615		0,4615	0,1077	0,1077	0,04615	0,2308	0,04615	0,07692	0,04615	0,4462
WB MCF7 Tam	0,4615	0,04615	0,04615	0,04615	0,04615	0,5692	0,4615		0,07692	0,1077	0,07692	0,04615	0,04615	0,04615	0,04615	0,07692
WB T47 Ana	0,04615	0,1077	0,4462	0,1077	0,1077	0,04615	0,1077	0,07692		0,1077	0,04615	0,07692	0,04615	0,07692	0,07692	0,1077
WB T47 Dil	0,4462	0,1077	0,1077	0,1077	0,1077	0,1077	0,1077	0,1077	0,1077		0,04615	0,1077	0,1077	0,07692	0,1077	0,1077
WB T47 Med	0,01077	0,07692	0,07692	0,04615	0,07692	0,1077	0,04615	0,07692	0,04615	0,04615		0,04615	0,1077	0,04615	0,04615	0,07692
WB T47 Tam	0,04615	0,4462	0,07692	0,1692	0,04615	0,04615	0,2308	0,04615	0,07692	0,1077	0,04615		0,04615	0,07692	0,04615	0,1692
WB w Anas T47	0,07692	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,1077	0,1077	0,04615		0,04615	0,04615	0,04615
WB w Tam	0,4615	0,04615	0,04615	0,07692	0,04615	0,04615	0,07692	0,04615	0,07692	0,07692	0,04615	0,07692	0,04615		0,2308	0,04615
WB w Tam MCF7	0,4615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,07692	0,1077	0,04615	0,04615	0,04615	0,2308		0,04615
WB w Tam T47	0,04615	0,04615	0,1692	0,07692	0,07692	0,04615	0,4462	0,07692	0,1077	0,1077	0,07692	0,01692	0,04615	0,04615	0,04615	

11

Friedman			
Test for equal medians			
chi2:	94,144	Degrees of	16
chi2, tie co	94,144		
chi2, conti	94,124		
p (same), as	4,32E-13		

Wilcoxon Pair	62														
	PRP all stain	PRP Thromb	PRP w Anas	PRP w Tam	PRP MCF7 Dil	PRP MCF7 Med	PRP MCF7 Tam	PRP w Anas	PRP w Tam	PRP T47 Ana	PRP T47 Dil	PRP T47 Med	PRP T47 Tam	PRP w Anas	PRP w Tam T
PRP all stains		0,4615	0,04615	0,04615	0,4615	0,07692	0,04615	0,07692	0,4615	0,04615	0,4615	0,4615	0,4615	0,4615	0,04615
PRP Thromb	0,4615		0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615
PRP w Anas	0,04615	0,04615		0,6923	0,04615	0,4615	0,04615	0,04615	0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615
PRP w Tam	0,04615	0,04615	0,6923		0,04615	0,4615	0,04615	0,04615	0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615
PRP MCF7 Anas	0,4615	0,04615	0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615	0,04615	0,6923	1	0,4615	0,04615	0,04615
PRP MCF7 Dil	0,07692	0,04615	0,04615	0,04615		0,4615	0,04615	0,04615	0,04615	0,1692	0,2308	0,4615	0,04615	0,04615	0,04615
PRP MCF7 Med	0,07692	0,4615	0,4615	0,4615	0,4615		0,4615	0,4615	0,04615	0,4615	0,4615	0,6923	0,4615	0,04615	0,04615
PRP MCF7 Tam	0,07692	0,04615	0,04615	0,04615	0,04615	0,4615		0,04615	0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615
PRP w Anas MCF7	0,4615	0,04615	0,04615	0,04615	0,04615	0,4615	0,04615		0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615
PRP w Tam MCF7	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,4615	0,04615	0,1692	0,04615
PRP T47 Anas	0,4615	0,04615	0,04615	0,04615	0,1692	0,4615	0,04615	0,04615	0,04615		0,5692	0,4615	0,04615	0,04615	0,04615
PRP T47 Dil	0,4615	0,04615	0,04615	0,04615	0,2308	0,4615	0,04615	0,04615	0,04615	0,5692		0,4615	0,04615	0,04615	0,04615
PRP T47 Med	0,4615	0,4615	0,4615	0,4615	0,4615	0,4615	0,6923	0,4615	0,4615	0,4615	0,4615		0,4615	0,04615	0,04615
PRP T47 Tam	0,4615	0,04615	0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615	0,04615	0,04615	0,4615		0,04615	0,04615
PRP w Anas T47	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,1692	0,04615	0,04615	0,04615	0,04615		0,04615
PRP w Tam T47	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	

63																	
Wilcoxon Pair																	
	PRP all stain	PRP Thromb	PRP w Anas	PRP w Tam	PRP MCF10	PRP MCF7 Dil	PRP MCF7 Med	PRP MCF7 T	PRP w Anas	PRP w Tam	PRP T47 Ana	PRP T47 Dil	PRP T47 Med	PRP T47 Tam	PRP w Anas	PRP w Tam T	
PRP all stains		0,4615	0,04615	0,04615	0,4615	0,04692	0,4615	0,04692	0,04615	0,04615	0,04615	0,0615	0,0615	0,0615	0,04615	0,04615	
PRP Thromb	0,4615		0,04615	0,04615	0,04615	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,0615	0,04615	0,04615	0,04615	
PRP w Anas	0,04615	0,04615		0,6923	0,04615	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	
PRP w Tam	0,04615	0,04615	0,6923		0,04615	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	
PRP MCF7 Anas	0,4615	0,04615	0,04615	0,04615	0,1692	0,04615	0,0615	0,04615	0,04615	0,04615	0,6923	1	0,0615	0,04615	0,04615	0,04615	
PRP MCF7 Dil	0,04692	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,1692	0,2308	0,0615	0,04615	0,04615	0,04615	
PRP MCF7 Med	0,4615	0,0615	0,0615	0,0615	0,0615	0,04615		0,04615	0,04615	0,04615	0,0615	0,04615	0,6923	0,4615	0,04615	0,04615	
PRP MCF7 Tam	0,04692	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,0615	0,04615	0,04615	0,04615	
PRP w Anas MCF7	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	
PRP w Tam MCF7	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,0615	0,04615	0,1692	0,04615	
PRP T47 Anas	0,04615	0,04615	0,04615	0,04615	0,04615	0,1692	0,0615	0,04615	0,04615	0,04615		0,5692	0,0615	0,04615	0,04615	0,04615	
PRP T47 Dil	0,0615	0,04615	0,04615	0,04615	0,04615	0,2308	0,04615	0,04615	0,04615	0,04615	0,5692		0,0615	0,04615	0,04615	0,04615	
PRP T47 Med	0,0615	0,0615	0,04615	0,04615	0,0615	0,0615	0,6923	0,0615	0,04615	0,0615	0,0615	0,0615		0,0615	0,04615	0,04615	
PRP T47 Tam	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,4615	0,04615	0,04615	0,04615	0,04615	0,04615	0,0615		0,04615	0,04615	
PRP w Anas T47	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,1692	0,04615	0,04615	0,04615	0,04615		0,04615	
PRP w Tam T47	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		

Friedman			
Test for equal medians			
chi2:	71,778	Degrees of f	16
chi2, tie co	71,778		
chi2, conti	71,761		
p (same), as	4,89E-09		

Wilcoxon Pair															
	WB all stains	WB Thromb	WB w Anas	WB w Anas	WB MCF7 An	WB MCF7 Dil	WB MCF7 Med	WB MCF7 T4	WB T47 Ana	WB T47 Dil	WB T47 Med	WB T47 Tam	WB w Anas	WB w Tam	WB w Tam T
WB all stains		0,07692	0,04615	0,04615	0,07692	0,0615	0,0615	0,0615	0,0615	0,0615	0,0615	0,07692	0,04615	0,0615	0,04615
WB Thromb	0,07692		0,04615	0,04615	0,07692	0,04615	0,0615	0,04615	0,04615	0,04615	0,0615	0,07692	0,04615	0,04615	0,04615
WB w Anas	0,04615	0,04615		0,07692	0,07692	0,07692	0,07692	0,04615	0,07692	0,4462	0,0615	0,07692	0,07692	0,07692	0,04615
WB w Anas MCF7	0,04615	0,04615	0,07692		0,4462	0,4462	0,0615	0,07692	0,04615	0,04615	0,07692	0,04615	0,2308	0,07692	0,04615
WB MCF7 Anas	0,07692	0,07692	0,07692	0,4462		0,07692	0,8462	0,4462	0,07692	0,04615	0,4462	0,07692	0,4462	0,07692	0,4462
WB MCF7 Dil	0,0615	0,04615	0,07692	0,4462	0,07692		0,07692	0,07692	0,04615	0,04615	0,07692	0,04615	0,1077	0,07692	0,4462
WB MCF7 Med	0,0615	0,0615	0,07692	0,0615	0,8462	0,07692		0,07692	0,0615	0,0615	0,4462	0,0615	0,4462	0,07692	0,0615
WB MCF7 Tam	0,0615	0,04615	0,04615	0,07692	0,4462	0,07692	0,07692		0,4462	0,04615	0,6923	0,07692	0,07692	0,04615	0,4462
WB T47 Ana	0,0615	0,04615	0,07692	0,04615	0,07692	0,04615	0,0615	0,4462		0,04615	0,8462	0,04615	0,07692	0,07692	0,8462
WB T47 Dil	0,0615	0,04615	0,4462	0,04615	0,04615	0,04615	0,0615	0,04615	0,04615		0,0615	0,04615	0,04615	0,07692	0,04615
WB T47 Med	0,0615	0,0615	0,0615	0,07692	0,4462	0,07692	0,4462	0,6923	0,8462	0,0615		0,04692	0,3231	0,0615	0,4462
WB T47 Tam	0,07692	0,07692	0,07692	0,04615	0,07692	0,04615	0,0615	0,07692	0,04615	0,04615	0,04692		0,04615	0,07692	0,07692
WB w Anas T47	0,04615	0,04615	0,07692	0,2308	0,4462	0,1077	0,4462	0,07692	0,07692	0,04615	0,3231	0,04615		0,07692	0,04615
WB w Tam	0,0615	0,04615	0,07692	0,07692	0,07692	0,07692	0,07692	0,04615	0,07692	0,07692	0,0615	0,07692	0,07692		0,04615
WB w Tam MCF7	0,04615	0,04615	0,07692	0,04615	0,07692	0,07692	0,0615	0,4462	0,8462	0,04615	0,4462	0,04615	0,04615	0,07692	0,04615
WB w Tam T47	0,04615	0,04615	0,04615	0,07692	0,4462	0,4462	0,07692	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	

Wilcoxon Pair															
	WB all stains	WB Thromb	WB w Anas	WB w Anas	WB MCF7 An	WB MCF7 Dil	WB MCF7 Med	WB MCF7 T4	WB T47 Ana	WB T47 Dil	WB T47 Med	WB T47 Tam	WB w Anas	WB w Tam	WB w Tam T
WB all stains		0,04692	0,04615	0,04615	0,04692	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04692	0,04615	0,04615	0,04615
WB Thromb	0,04692		0,04615	0,04615	0,07692	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615
WB w Anas	0,04615	0,04615		0,07692	0,07692	0,07692	0,0692	0,04615	0,07692	0,4462	0,04615	0,07692	0,07692	0,07692	0,04615
WB w Anas MCF7	0,04615	0,04615	0,07692		0,4462	0,4462	0,0615	0,07692	0,04615	0,04615	0,07692	0,04615	0,2308	0,07692	0,07692
WB MCF7 Anas	0,04692	0,07692	0,07692	0,4462		0,07692	0,8462	0,4462	0,07692	0,04615	0,4462	0,07692	0,4462	0,07692	0,4462
WB MCF7 Dil	0,0615	0,04615	0,07692	0,4462	0,07692		0,07692	0,07692	0,04615	0,04615	0,07692	0,04615	0,1077	0,07692	0,4462
WB MCF7 Med	0,04615	0,0615	0,0692	0,0615	0,8462	0,07692		0,07692	0,0615	0,0615	0,4462	0,04615	0,4462	0,07692	0,0615
WB MCF7 Tam	0,04615	0,04615	0,04615	0,07692	0,4462	0,07692	0,07692		0,4462	0,04615	0,6923	0,07692	0,07692	0,04615	0,4462
WB T47 Ana	0,04615	0,04615	0,07692	0,04615	0,07692	0,04615	0,0615	0,4462		0,04615	0,8462	0,04615	0,07692	0,07692	0,8462
WB T47 Dil	0,04615	0,04615	0,4462	0,04615	0,04615	0,04615	0,0615	0,04615	0,04615		0,04615	0,04615	0,04615	0,07692	0,04615
WB T47 Med	0,04615	0,04615	0,04615	0,07692	0,4462	0,07692	0,4462	0,6923	0,8462	0,04615		0,07692	0,3231	0,04615	0,4462
WB T47 Tam	0,04692	0,07692	0,07692	0,04615	0,07692	0,04615	0,04615	0,07692	0,04615	0,04615	0,07692		0,04615	0,07692	0,07692
WB w Anas T47	0,04615	0,04615	0,07692	0,2308	0,4462	0,1077	0,4462	0,07692	0,07692	0,04615	0,3231	0,04615		0,07692	0,04615
WB w Tam	0,04615	0,04615	0,07692	0,07692	0,07692	0,07692	0,07692	0,04615	0,07692	0,07692	0,04615	0,07692	0,07692		0,04615
WB w Tam MCF7	0,04615	0,04615	0,07692	0,04615	0,07692	0,07692	0,0615	0,4462	0,8462	0,04615	0,4462	0,04615	0,04615	0,07692	0,04615
WB w Tam T47	0,04615	0,04615	0,04615	0,07692	0,4462	0,4462	0,04615	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	

12

Friedman			
Test for equal medians			
chi2:	93,438	Degrees of f	16
chi2, tie co	93,438		
chi2, conti	93,419		
p (same), as	5,84E-13		

62																
Wilcoxon Pair																
	PRP all stain	PRP Thromb	PRP w Anas	PRP w Tam	PRP MCF7 D	PRP MCF7 Med	PRP MCF7 Tam	PRP w Anas	PRP w Tam	PRP T47 Ana	PRP T47 Dil	PRP T47 Me	PRP T47 Tar	PRP w Anas	PRP w Tam	
PRP all stains		0,04615	0,0615	0,0615	0,0615	0,04615	0,04692	0,0615	0,04615	0,04615	0,04615	0,04615	0,04692	0,0615	0,04615	
PRP Thromb	0,04615		0,04615	0,04615	0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615
PRP w Anas	0,0615	0,04615		0,04615	0,04615	0,0615	0,04615	0,04615	0,1692	0,04615	0,04615	0,04615	0,04615	0,8462	0,04615	0,04615
PRP w Tam	0,0615	0,04615	0,04615		0,04615	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Anas	0,04615	0,04615	0,04615	0,04615	0,4462	0,04615	0,04615	0,04615	0,04615	0,04615	0,2308	0,04615	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Dil	0,0615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,04615	0,2308	0,04615	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Med	0,04615	0,0615	0,0615	0,0615	0,04615		0,0615	0,04615	0,04615	0,8462	0,07692	0,8462	0,04615	0,04615	0,04615	0,04615
PRP MCF7 Tam	0,04692	0,04615	0,04615	0,04615	0,04615	0,0615		0,04615	0,04615	0,04615	0,04615	0,04615	0,5692	0,04615	0,04615	0,04615
PRP w Anas MCF7	0,0615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615
PRP w Tam MCF7	0,04615	0,04615	0,1692	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615	0,04615	0,2308	0,04615	0,04615
PRP T47 Anas	0,04615	0,04615	0,04615	0,04615	0,04615	0,8462	0,04615	0,04615	0,04615		0,07692	0,8462	0,04615	0,04615	0,04615	0,04615
PRP T47 Dil	0,04615	0,04615	0,04615	0,04615	0,2308	0,07692	0,04615	0,04615	0,04615	0,07692		0,07692	0,04615	0,04615	0,04615	0,04615
PRP T47 Med	0,04615	0,04615	0,04615	0,04615	0,04615	0,8462	0,04615	0,04615	0,04615	0,8462	0,07692		0,04615	0,04615	0,04615	0,04615
PRP T47 Tam	0,04692	0,04615	0,04615	0,04615	0,04615	0,04615	0,5692	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615	0,04615	0,04615
PRP w Anas T47	0,0615	0,04615	0,8462	0,04615	0,04615	0,04615	0,04615	0,04615	0,2308	0,04615	0,04615	0,04615	0,04615	0,04615		0,04615
PRP w Tam T47	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	

	WB all stair	WB Thromb	WB w Anas	WB w Anas	WB MCF7 A	WB MCF7 C	WB MCF7 D	WB MCF7 T	WB T47 An	WB T47 Dil	WB T47 Me	WB T47 Tar	WB w Anas	WB w Tam	WB w Tam	WB w Tam
WB all stains		0,07692	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615
WB Thromb	0,07692		0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615	0,04615
WB w Anas	0,04615	0,04615		0,07692	0,07692	0,07692	0,07692	0,04615	0,4462	0,4462	0,04615	0,07692	0,07692	0,04615	0,5692	0,07692
WB w Anas	0,04615	0,04615	0,07692		0,1077	0,4462	0,4462	0,07692	0,04615	0,04615	0,8462	0,04615	0,4462	0,07692	0,04615	0,4462
WB MCF7 A	0,04615	0,04615	0,07692	0,4462	0,1077		0,1077	0,4462	0,07692	0,04615	0,04615	0,8462	0,04615	0,8462	0,07692	0,8462
WB MCF7 C	0,07692	0,04615	0,07692	0,1077		0,1077		0,1077	0,07692	0,04615	0,04615	0,4462	0,04615	0,4462	0,07692	0,4462
WB MCF7 D	0,04615	0,04615	0,07692	0,4462	0,1077		0,1077	0,07692	0,04615	0,04615	0,4462	0,04615	0,4462	0,07692	0,04615	0,8462
WB MCF7 T	0,04615	0,04615	0,07692	0,4462	0,1077	0,1077		0,07692	0,04615	0,04615	0,8462	0,04615	0,4462	0,07692	0,04615	0,4462
WB MCF7 A	0,04615	0,04615	0,04615	0,07692	0,07692	0,07692	0,07692		0,07692	0,04615	0,04615	0,07692	0,07692	0,04615	0,07692	0,07692
WB T47 An	0,04615	0,04615	0,4462	0,04615	0,04615	0,04615	0,04615	0,07692		0,4462	0,04615	0,04615	0,04615	0,8462	0,4462	0,04615
WB T47 Dil	0,04615	0,04615	0,4462	0,04615	0,04615	0,04615	0,04615	0,4462	0,4462		0,04615	0,04615	0,04615	0,4462	0,04615	0,04615
WB T47 Me	0,04615	0,04615	0,8462	0,8462	0,4462	0,8462	0,04615	0,04615		0,07692		0,07692	0,4462	0,04615	0,04615	0,4462
WB T47 Tar	0,07692	0,04615	0,07692	0,04615	0,04615	0,04615	0,04615	0,07692	0,04615	0,04615	0,07692		0,04615	0,04615	0,04615	0,07692
WB w Anas	0,04615	0,04615	0,07692	0,4462	0,4462	0,4462	0,4462	0,07692	0,04615	0,04615	0,4462	0,04615		0,07692	0,04615	0,4462
WB w Tam	0,04615	0,04615	0,04615	0,07692	0,07692	0,07692	0,07692	0,04615	0,8462	0,4462	0,04615	0,04615	0,07692		0,07692	0,04615
WB w Tam	0,04615	0,04615	0,5692	0,04615	0,04615	0,04615	0,04615	0,07692	0,4462	0,04615	0,04615	0,04615	0,04615	0,07692		0,04615
WB w Tam	0,04615	0,04615	0,07692	0,4462	0,4462	0,8462	0,4462	0,07692	0,04615	0,04615	0,4462	0,07692	0,4462	0,04615	0,04615	

62																	
Wilcoxon Pair																	
	PRP all stains	PRP Thromb	PRP w Anas	PRP w Tam	PRP MCF7 10	PRP MCF7 Dil	PRP MCF7 Med	PRP MCF7 T	PRP w Anas	PRP w Tam	PRP T47 Ana	PRP T47 Dil	PRP T47 Med	PRP T47 Tam	PRP w Anas	PRP w Tam	T
PRP all stains		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PRP Thromb	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PRP w Anas	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1
PRP w Tam	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1
PRP MCF7 Anas	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1
PRP MCF7 Dil	1	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1
PRP MCF7 Med	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1	1
PRP MCF7 Tam	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1
PRP w Anas MCF7	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
PRP w Tam MCF7	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1
PRP T47 Anas	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1
PRP T47 Dil	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1
PRP T47 Med	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1
PRP T47 Tam	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1
PRP w Anas T47	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1
PRP w Tam T47	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1

62																	
Wilcoxon Pair																	
	WB all stains	WB Thromb	WB w Anas	WB w Anas M	WB MCF7 Ana	WB MCF7 Dil	WB MCF7 Med	WB MCF7 Tan	WB T47 Ana	WB T47 Dil	WB T47 Mec	WB T47 Tam	WB w Anas	WB w Tam	WB w Tam M	WB w Tam T	T
WB all stains		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
WB Thromb	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
WB w Anas	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1
WB w Anas MCF7	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1
WB MCF7 Ana	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1
WB MCF7 Dil	1	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1
WB MCF7 Med	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1	1
WB MCF7 Tam	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1
WB T47 Ana	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
WB T47 Dil	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1
WB T47 Med	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1
WB T47 Tam	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1
WB w Anas T47	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1
WB w Tam	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1
WB w Tam MCF7	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1
WB w Tam T47	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1

Thrombin Activity Assay. Friedman's test for equal medians with Posthoc Wilcoxon. Green indicates significance

Fried			
Test for equal medians			
chi2:	671,67	Degrees of freedom	22
chi2, tie corr	671,67		
chi2, continuity	671,67		
p (same), asymptotic	7,32E-128		

	WB Only	WB thrombin	WB Anas	PRP Only	*PRP Throm	PPP Only	PRP Anas	PPP Anas	MCF7 Med	MCF7 Dil	MCF7 WB	MCF7 Dil WB	MCF7 Anas	MCF7 Anas	WB Anas M	MCF7 PRP	MCF7 PPP	MCF7 Dil PR	MCF7 Dil PP	MCF7 Anas	MCF7 Anas	PRP Anas M	PPP Anas M
WB Only		0,3833	0,6755	4,77E-30	4,47E-33	1,31E-06	0,0002306	0,001098	7,08E-17	3,35E-14	0,513	0,6813	8,71E-10	0,1847	0,1653	4,85E-23	5,89E-21	2,24E-10	2,83E-13	1,53E-13	8,03E-12	1,24E-13	1,30E-18
WB thrombin	0,3833		0,6504	7,24E-26	1,13E-28	7,29E-05	0,004938	0,01674	7,79E-14	1,92E-11	0,8277	0,6446	1,44E-07	0,6495	0,6062	2,01E-19	1,59E-17	4,44E-08	1,27E-10	7,36E-11	2,43E-09	6,12E-11	2,13E-15
WB Anas	0,6755	0,6504		5,34E-28	6,39E-31	9,88E-06	0,001098	0,004433	2,25E-15	7,75E-13	0,8138	0,9936	1,11E-08	0,3641	0,3327	2,91E-21	2,88E-19	3,11E-09	5,83E-12	3,25E-12	1,37E-10	2,67E-12	4,99E-17
PRP Only	4,77E-30	7,24E-26	5,34E-28		0,035	5,75E-11	1,30E-14	4,50E-16	0,2343	0,1421	7,02E-27	4,88E-28	1,46E-01	8,11E-24	1,51E-23	0,01326	0,04588	4,55E-07	4,39E-05	6,24E-05	5,35E-06	7,01E-05	0,009797
*PRP Throm	4,47E-33	1,13E-28	6,39E-31	0,035		9,16E-13	1,06E-16	2,86E-18	0,0002775	1,10E-05	9,66E-30	5,82E-31	4,93E-09	1,66E-26	3,19E-26	0,03604	0,009623	1,73E-08	2,89E-06	4,31E-06	2,70E-07	4,92E-06	0,001496
PPP Only	1,31E-06	7,29E-05	9,88E-06	5,75E-11	9,16E-13		0,2479	0,1154	0,4528	0,6038	2,86E-05	9,52E-06	0,196	0,0004445	0,0005583	4,51E-07	5,27E-06	0,1321	0,1374	0,01088	0,4554	0,01004	7,27E-05
PRP Anas	0,0002306	0,004938	0,001098	1,30E-14	1,06E-16	0,2479		0,6755	3,12E-06	9,57E-05	0,002456	0,001067	0,01435	0,01844	0,0217	5,59E-10	1,14E-08	0,007786	0,0002953	0,0002139	0,001604	0,0001916	3,01E-07
PPP Anas	0,001098	0,01674	0,004433	4,50E-16	2,86E-18	0,1154	0,6755		3,75E-07	1,56E-05	0,009053	0,004323	0,004142	0,05263	0,06053	3,58E-11	8,91E-10	0,002071	5,39E-05	3,78E-05	0,0003518	3,35E-05	3,00E-08
MCF7 Med	7,08E-17	7,79E-14	2,25E-15	0,2343	0,0002775	0,4528	3,12E-06	3,75E-07		0,4464	1,46E-14	2,11E-15	0,02682	2,23E-12	3,44E-12	0,1238	0,2954	0,04534	0,2968	0,3367	0,1317	0,3509	0,6455
MCF7 Dil	3,35E-14	1,92E-11	7,75E-13	0,1421	1,10E-05	0,6038	9,57E-05	1,56E-05	0,4464		4,21E-12	7,31E-13	0,1463	3,90E-10	5,76E-10	0,02142	0,07063	0,215	0,7781	0,8421	0,4556	0,8639	0,2219
MCF7 WB	0,513	0,8277	0,8138	7,02E-27	9,66E-30	2,86E-05	0,002456	0,009053	1,46E-14	4,21E-12		0,8076	4,32E-08	0,5015	0,4635	2,70E-20	2,38E-18	1,27E-08	2,97E-11	1,69E-11	6,26E-10	1,39E-11	3,60E-16
MCF7 Dil WB	0,6813	0,6446	0,9936	4,88E-28	5,82E-31	9,52E-06	0,001067	0,004323	2,11E-15	7,31E-13	0,8076		1,06E-08	0,3599	0,3287	2,69E-21	2,68E-19	2,97E-09	5,51E-12	3,07E-12	1,30E-10	2,52E-12	4,66E-17
MCF7 Anas	8,71E-10	1,44E-07	1,11E-08	1,46E-01	4,93E-09	0,196	0,01435	0,004142	0,02682	0,1463	4,32E-08	1,06E-08		1,55E-06	2,09E-06	0,0001746	0,001112	0,8315	0,2416	0,21	0,4798	0,2001	0,007491
MCF7 Anas	0,1847	0,6495	0,3641	8,11E-24	1,66E-26	0,0004445	0,01844	0,05263	2,23E-12	3,90E-10	0,5015	0,3599	1,55E-06		0,9513	1,15E-17	7,28E-16	5,23E-07	2,29E-09	1,37E-09	3,55E-08	1,16E-09	7,47E-14
WB Anas M	0,1653	0,6062	0,3327	1,51E-23	3,19E-26	0,0005583	0,0217	0,06053	3,44E-12	5,76E-10	0,4635	0,3287	2,09E-06	0,9513		1,94E-17	1,20E-15	7,17E-07	3,32E-09	2,00E-09	5,02E-08	1,69E-09	1,19E-13
MCF7 PRP	4,85E-23	2,01E-19	2,91E-21	0,01326	0,03604	4,51E-07	5,59E-10	3,58E-11	0,1238	0,02142	2,70E-20	2,69E-21	0,0001746	1,15E-17	1,94E-17		0,6223	0,0003994	0,009814	0,01243	0,002314	0,01344	0,2806
MCF7 PPP	5,89E-21	1,59E-17	2,88E-19	0,04588	0,009623	5,27E-06	1,14E-08	8,91E-10	0,2954	0,07063	2,38E-18	2,68E-19	0,001112	7,28E-16	1,20E-15	0,6223		0,002305	0,03665	0,04475	0,01065	0,04779	0,5576
MCF7 Dil PR	2,24E-10	4,44E-08	3,11E-09	4,55E-07	1,73E-08	0,1321	0,007786	0,002071	0,04534	0,215	1,27E-08	2,97E-09	0,8315	5,23E-07	7,17E-07	0,0003994	0,002305		0,338	0,298	0,6214	0,2853	0,01384
MCF7 Dil PP	2,83E-13	1,27E-10	5,83E-12	4,39E-05	2,89E-06	0,1374	0,0002953	5,39E-05	0,2968	0,7781	2,97E-11	5,51E-12	0,2416	2,29E-09	3,32E-09	0,009814	0,03665	0,338		0,9342	0,6424	0,9121	0,1328
MCF7 Anas	1,53E-13	7,36E-11	3,25E-12	6,24E-05	4,31E-06	0,01088	0,0002139	3,78E-05	0,3367	0,8421	1,69E-11	3,07E-12	0,21	1,37E-09	2,00E-09	0,01243	0,04475	0,298	0,9342		0,5844	0,009779	0,1554
MCF7 Anas	8,03E-12	2,43E-09	1,37E-10	5,35E-06	2,70E-07	0,4554	0,001604	0,0003518	0,1317	0,4556	6,26E-10	1,30E-10	0,4798	3,55E-08	5,02E-08	0,002314	0,01065	0,6214	0,6424	0,5844		0,5655	0,04912
PRP Anas M	1,24E-13	6,12E-11	2,67E-12	7,01E-05	4,92E-06	0,01004	0,0001916	3,35E-05	0,3509	0,8639	1,39E-11	2,52E-12	0,2001	1,16E-09	1,69E-09	0,01344	0,04779	0,2853	0,9121	0,009779	0,5655		0,1637
PPP Anas M	1,30E-18	2,13E-15	4,99E-17	0,009797	0,001496	7,27E-05	3,01E-07	3,00E-08	0,6455	0,2219	3,60E-16	4,66E-17	0,007491	7,47E-14	1,19E-13	0,2806	0,5576	0,01384	0,1328	0,1554	0,04912	0,1637	

Fried			
Test for equal medians			
chi2:	668,84	Degrees of f	22
chi2, tie corr	668,84		
chi2, continu	668,84		
p (same), as	2,88E-127		

	WB Only	WB thrombi	WB Tam	PRP Only	*PRP Throm	PPP Only	PRP Tam	PPP Tam	MCF7 Med	MCF7 Dil	MCF7 WB	MCF7 Dil WB	MCF7 Tam	MCF7 Tam V	WB Tam MC	PPP Tam MC	MCF7 PRP	MCF7 PPP	MCF7 Dil PR	MCF7 Dil PP	MCF7 Tam P	MCF7 Tam PP	PRP Tam MC
WB Only		0,3713	0,9946	1,34E-29	1,33E-32	1,06E-05	4,75E-07	7,73E-07	1,27E-16	9,05E-14	0,5011	0,6719	2,26E-05	0,309	0,1587	1,66E-16	7,79E-23	2,46E-20	3,49E-10	3,59E-13	4,01E-10	1,66E-11	9,37E-15
WB thrombi	0,3713		0,3749	2,38E-25	3,98E-28	0,0004456	3,44E-05	5,17E-05	1,56E-13	5,38E-11	0,8248	0,638	0,0008282	0,9019	0,6062	1,98E-13	3,78E-19	7,05E-17	7,41E-08	1,82E-10	8,35E-08	5,25E-09	7,21E-12
WB Tam	0,9946	0,3749		1,45E-29	1,44E-32	1,09E-05	4,92E-07	8,01E-07	1,35E-16	9,53E-14	0,5055	0,6768	2,33E-05	0,3122	0,1607	1,76E-16	8,33E-23	2,62E-20	3,65E-10	3,77E-13	4,19E-10	1,74E-11	9,88E-15
PRP Only	1,34E-29	2,38E-25	1,45E-29		0,035	5,49E-12	3,81E-10	2,07E-10	0,2512	0,1211	2,28E-26	1,53E-27	1,66E-12	8,63E-25	4,68E-23	0,002263	0,01441	0,03963	5,09E-07	5,64E-05	4,55E-07	5,00E-06	0,0003847
*PRP Throm	1,33E-32	3,98E-28	1,44E-32	0,035		7,16E-14	7,18E-12	3,69E-12	0,0003009	9,15E-06	3,34E-29	1,94E-30	1,96E-14	1,55E-27	1,06E-25	0,0002664	0,04005	0,008046	1,96E-08	3,84E-06	1,73E-08	2,51E-07	3,43E-05
PPP Only	1,06E-05	0,0004456	1,09E-05	5,49E-12	7,16E-14		0,5282	0,5917	0,1087	0,23	0,0001893	6,83E-05	0,8663	0,0007035	0,002735	0,0001236	5,59E-08	1,33E-06	0,06155	0,4174	0,06461	0,01993	0,0008319
PRP Tam	4,75E-07	3,44E-05	4,92E-07	3,81E-10	7,18E-12	0,5282		0,9248	0,001197	0,01562	1,28E-05	3,97E-06	0,4242	5,85E-05	0,0002869	0,001336	1,58E-06	2,62E-05	0,2154	0,02549	0,2235	0,08972	0,006705
PPP Tam	7,73E-07	5,17E-05	8,01E-07	2,07E-10	3,69E-12	0,5917	0,9248		0,0008563	0,012	1,96E-05	6,23E-06	0,481	8,69E-05	0,0004117	0,0009583	9,82E-07	1,72E-05	0,1825	0,0199	0,1897	0,07326	0,005024
MCF7 Med	1,27E-16	1,56E-13	1,35E-16	0,2512	0,3009	0,1087	0,001197	0,0008563		0,4111	2,89E-14	4,08E-15	5,38E-05	3,91E-13	6,58E-12	0,9749	0,1185	0,3349	0,04541	0,3146	0,04313	0,1229	0,5972
MCF7 Dil	9,05E-14	5,38E-11	9,53E-14	0,1211	9,15E-06	0,0023	0,01562	0,012	0,4111		1,19E-11	2,06E-12	0,001296	1,22E-10	1,50E-09	0,4292	0,01717	0,07405	0,2384	0,8542	0,2299	0,471	0,7691
MCF7 WB	0,5011	0,8248	0,5055	2,28E-26	3,34E-29	0,0001893	1,28E-05	1,96E-05	2,89E-14	1,19E-11		0,8033	0,0003645	0,7303	0,4612	3,68E-14	4,98E-20	1,06E-17	2,11E-08	4,18E-11	2,39E-08	1,36E-09	1,50E-12
MCF7 Dil WB	0,6719	0,638	0,6768	1,53E-27	1,94E-30	6,83E-05	3,97E-06	6,23E-06	4,08E-15	2,06E-12	0,8033		0,0001369	0,5527	0,3242	5,24E-15	4,80E-21	1,18E-18	4,87E-09	7,56E-12	5,55E-09	2,80E-10	2,41E-13
MCF7 Tam	2,26E-05	0,0008282	2,33E-05	1,66E-12	1,96E-14	0,8663	0,4242	0,481	5,38E-05	0,001296	0,0003645	0,0001369		0,001283	0,004688	6,14E-05	2,14E-08	5,64E-07	0,04156	0,002421	0,04377	0,01256	0,0004476
MCF7 Tam V	0,309	0,9019	0,3122	8,63E-25	1,55E-27	0,0007035	5,85E-05	8,69E-05	3,91E-13	1,22E-10	0,7303	0,5527	0,001283		0,6949	4,93E-13	1,15E-18	1,99E-16	1,46E-07	4,03E-10	1,64E-07	1,09E-08	1,70E-11
WB Tam MC	0,1587	0,6062	0,1607	4,68E-23	1,06E-25	0,002735	0,0002869	0,0004117	6,58E-12	1,50E-09	0,4612	0,3242	0,004688	0,6949		8,20E-12	3,53E-17	4,85E-15	1,14E-06	4,61E-09	1,27E-06	1,02E-07	2,33E-10
PPP Tam MC	1,66E-16	1,98E-13	1,76E-16	0,002263	0,0002664	0,0001236	0,001336	0,0009583	0,9749	0,4292	3,68E-14	5,24E-15	6,14E-05	4,93E-13	8,20E-12		0,1112	0,3193	0,04891	0,3299	0,04648	0,1307	0,6192
MCF7 PRP	7,79E-23	3,78E-19	8,33E-23	0,01441	0,04005	5,59E-08	1,58E-06	9,82E-07	0,1185	0,01717	4,98E-20	4,80E-21	2,14E-08	1,15E-18	3,53E-17	0,1112		0,5506	0,0003679	0,01026	0,0003388	0,00191	0,03665
MCF7 PPP	2,46E-20	7,05E-17	2,62E-20	0,03963	0,008046	1,33E-06	2,62E-05	1,72E-05	0,3349	0,07405	1,06E-17	1,18E-18	5,64E-07	1,99E-16	4,85E-15	0,3193	0,5506		0,003025	0,04884	0,002819	0,01217	0,1355
MCF7 Dil PR	3,49E-10	7,41E-08	3,65E-10	5,09E-07	1,96E-08	0,06155	0,2154	0,1825	0,04541	0,2384	2,11E-08	4,87E-09	0,04156	1,46E-07	1,14E-06	0,04891	0,0003679	0,003025		0,3196	0,9828	0,6469	0,1409
MCF7 Dil PP	3,59E-13	1,82E-10	3,77E-13	5,64E-05	3,84E-06	0,4174	0,02549	0,0199	0,3146	0,8542	4,18E-11	7,56E-12	0,002421	4,03E-10	4,61E-09	0,3299	0,01026	0,04884	0,3196		0,3093	0,5912	0,6332
MCF7 Tam P	4,01E-10	8,35E-08	4,19E-10	4,55E-07	1,73E-08	0,06461	0,2235	0,1897	0,04313	0,2299	2,39E-08	5,55E-09	0,04377	1,64E-07	1,27E-06	0,04648	0,0003388	0,002819	0,9828	0,3093		0,6314	0,1352
MCF7 Tam P	1,66E-11	5,25E-09	1,74E-11	5,00E-06	2,51E-07	0,01993	0,08972	0,07326	0,1229	0,471	1,36E-09	2,80E-10	0,01256	1,09E-08	1,02E-07	0,1307	0,00191	0,01217	0,6469	0,5912	0,6314		0,3104
PRP Tam MC	9,37E-15	7,21E-12	9,88E-15	0,0003847	3,43E-05	0,0008319	0,006705	0,005024	0,5972	0,7691	1,50E-12	2,41E-13	0,0004476	1,70E-11	2,33E-10	0,6192	0,03665	0,1355	0,1409	0,6332	0,1352	0,3104	

Fried			
Test for equal medians			
chi2:	665,15	Degrees of freedom	22
chi2, tie correction	665,15		
chi2, continuity correction	665,15		
p (same), asymptotic	1,73E-126		

	WB Only	WB thrombolytic	WB Anas	PRP Only	*PRP Thrombolytic	PPP Only	PRP Anas	PPP Anas	T47 Med	T47 Dil	T47 Med WB	T47 Dil WB	T47 Anas	T47Anas WB	WB Anas T4	T47 PRP	T47 PPP	T47 Dil PRP	T47 Dil PPP	T47 Anas PR	T47 Anas PP	PRP Anas T4	PPP Anas T4
WB Only		0,576	0,7805	5,97E-33	4,00E-36	3,89E-10	1,34E-05	0,000382	3,04E-13	4,63E-06	0,1227	0,02851	1,36E-16	9,03E-05	0,08078	2,63E-20	2,01E-18	4,25E-15	1,68E-15	2,86E-14	1,03E-16	2,99E-18	2,02E-29
WB thrombolytic	0,576		0,7791	4,29E-30	3,99E-33	1,20E-08	0,0001479	0,002763	1,66E-11	5,78E-05	0,3251	0,1029	1,27E-14	0,0007904	0,2352	4,18E-18	2,45E-16	3,14E-13	1,33E-13	1,85E-12	9,74E-15	3,56E-16	9,87E-27
WB Anas	0,7805	0,7791		1,65E-31	1,30E-34	2,24E-09	4,60E-05	0,001062	2,32E-12	1,69E-05	0,206	0,05595	1,36E-15	0,0002762	0,1422	3,42E-19	2,29E-17	3,77E-14	1,54E-14	2,38E-13	1,03E-15	3,36E-17	4,59E-28
PRP Only	5,97E-33	4,29E-30	1,65E-31		0,035	1,21E-08	2,89E-14	4,29E-17	3,09E-06	1,63E-13	2,15E-25	1,56E-22	0,0002251	8,86E-16	1,77E-24	0,006447	0,001374	3,96E-05	6,49E-05	1,35E-05	0,000257	0,001174	0,4871
*PRP Thrombolytic	4,00E-36	3,99E-33	1,30E-34	0,035		3,15E-10	2,49E-16	2,31E-19	1,46E-07	1,60E-15	3,57E-28	3,79E-25	1,86E-05	5,91E-18	3,32E-27	0,000911	0,000149	2,57E-06	4,50E-06	7,61E-07	2,16E-05	0,0001242	0,1979
PPP Only	3,89E-10	1,20E-08	2,24E-09	1,21E-08	3,15E-10		0,05682	0,006805	0,3011	0,09339	2,42E-06	4,74E-05	0,04449	0,01913	6,41E-06	0,002935	0,01247	0,1121	0,08833	0,1783	0,04101	0,01415	5,63E-07
PRP Anas	1,34E-05	0,0001479	4,60E-05	2,89E-14	2,49E-16	0,05682		0,4228	0,003296	0,8205	0,004947	0,03049	9,07E-05	0,6611	0,009118	1,07E-06	1,07E-05	0,0004765	0,0003075	0,001151	7,88E-05	1,31E-05	4,90E-12
PPP Anas	0,000382	0,002763	0,001062	4,29E-17	2,31E-19	0,006805	0,4228		0,0001838	0,3037	0,04455	0,1732	2,41E-06	0,7165	0,07092	1,34E-08	1,94E-07	1,75E-05	1,03E-05	5,07E-05	2,04E-06	2,47E-07	1,26E-14
T47 Med	3,04E-13	1,66E-11	2,32E-12	3,09E-06	1,46E-07	0,3011	0,003296	0,0001838		0,006692	8,97E-09	3,36E-07	0,3293	0,0007325	2,92E-08	0,05233	0,1431	0,5789	0,5027	0,755	0,3128	0,1558	7,20E-05
T47 Dil	4,63E-06	5,78E-05	1,69E-05	1,63E-13	1,60E-15	0,09339	0,8205	0,3037	0,006692		0,002387	0,01682	0,0002267	0,5059	0,00459	3,28E-06	2,96E-05	0,001088	0,0007195	0,002496	0,0001983	3,61E-05	2,37E-11
T47 Med WB	0,1227	0,3251	0,206	2,15E-25	3,57E-28	2,42E-06	0,004947	0,04455	8,97E-09	0,002387		0,5178	1,76E-11	0,01769	0,8392	1,48E-14	5,45E-13	2,90E-10	1,37E-10	1,35E-09	1,39E-11	7,58E-13	2,51E-22
T47 Dil WB	0,02851	0,1029	0,05595	1,56E-22	3,79E-25	4,74E-05	0,03049	0,1732	3,36E-07	0,01682	0,5178		1,22E-09	0,08448	0,6571	1,89E-12	5,14E-11	1,54E-08	7,81E-09	6,15E-08	9,86E-10	6,95E-11	1,17E-19
T47 Anas	1,36E-16	1,27E-14	1,36E-15	0,0002251	1,86E-05	0,04449	9,07E-05	2,41E-06	0,3293	0,0002267	1,76E-11	1,22E-09		1,35E-05	6,95E-11	0,3346	0,6249	0,6741	0,7602	0,507	0,9729	0,6571	0,002752
T47Anas WB	9,03E-05	0,0007904	0,0002762	8,86E-16	5,91E-18	0,01913	0,6611	0,7165	0,0007325	0,5059	0,01769	0,08448	1,35E-05		0,03007	1,05E-07	1,29E-06	8,42E-05	5,18E-05	0,0002251	1,15E-05	1,62E-06	2,03E-13
WB Anas T4	0,08078	0,2352	0,1422	1,77E-24	3,32E-27	6,41E-06	0,009118	0,07092	2,92E-08	0,00459	0,8392	0,6571	6,95E-11	0,03007		7,06E-14	2,37E-12	1,05E-09	5,08E-10	4,68E-09	5,54E-11	3,27E-12	1,80E-21
T47 PRP	2,63E-20	4,18E-18	3,42E-19	0,006447	0,000911	0,002935	1,07E-06	1,34E-08	0,05233	3,28E-06	1,48E-14	1,89E-12	0,3346	1,05E-07	7,06E-14		0,6341	0,1659	0,204	0,1034	0,3518	0,6024	0,04244
T47 PPP	2,01E-18	2,45E-16	2,29E-17	0,001374	0,000149	0,01247	1,07E-05	1,94E-07	0,1431	2,96E-05	5,45E-13	5,14E-11	0,6249	1,29E-06	2,37E-12	0,6341		0,3631	0,4271	0,2491	0,6491	0,9641	0,01224
T47 Dil PRP	4,25E-15	3,14E-13	3,77E-14	3,96E-05	2,57E-06	0,1121	0,0004765	1,75E-05	0,5789	0,001088	2,90E-10	1,54E-08	0,6741	8,42E-05	1,05E-09	0,1659	0,3631		0,9082	0,9081	0,6495	0,3873	0,0006385
T47 Dil PPP	1,68E-15	1,33E-13	1,54E-14	6,49E-05	4,50E-06	0,08833	0,0003075	1,03E-05	0,5027	0,0007195	1,37E-10	7,81E-09	0,7602	5,18E-05	5,08E-10	0,204	0,4271	0,9082		0,7202	0,7345	0,4538	0,0009689
T47 Anas PR	2,86E-14	1,85E-12	2,38E-13	1,35E-05	7,61E-07	0,1783	0,001151	5,07E-05	0,755	0,002496	1,35E-09	6,15E-08	0,507	0,0002251	4,68E-09	0,1034	0,2491	0,8081	0,7202		0,4856	0,02681	0,0002545
T47 Anas PP	1,03E-16	9,74E-15	1,03E-15	0,000257	2,16E-05	0,04101	7,88E-05	2,04E-06	0,3128	0,0001983	1,39E-11	9,86E-10	0,9729	1,15E-05	5,54E-11	0,3518	0,6491	0,6495	0,7345	0,04856		0,6818	0,003074
PRP Anas T4	2,99E-18	3,56E-16	3,36E-17	0,001174	0,0001242	0,01415	1,31E-05	2,47E-07	0,1558	3,61E-05	7,58E-13	6,95E-11	0,6571	1,62E-06	3,27E-12	0,6024	0,9641	0,3873	0,4538	0,2681	0,6818		0,01076
PPP Anas T4	2,02E-29	9,87E-27	4,59E-28	0,4871	0,1979	5,63E-07	4,90E-12	1,26E-14	7,20E-05	2,37E-11	2,51E-22	1,17E-19	0,002752	2,03E-13	1,80E-21	0,04244	0,01224	0,0006385	0,0009689	0,0002545	0,003074	0,01076	

Fried			
Test for equal medians			
chi2:	664,51	Degrees of fr	22
chi2, tie corr	664,51		
chi2, continu	664,51		
p (same), as	2,35E-126		

	WB Only	WB thrombi	WB Tam	PRP Only	*PRP Throm	PPP Only	PRP Tam	PPP Tam	T47 Med	T47 Dil	T47 Med WB	T47 Dil WB	T47 Tam	T47 Tam WB	WB Tam T47	PPP Tam T47	T47 PRP	T47 PPP	T47 Dil PRP	T47 Dil PPP	T47 Tam PR	T47 Tam PP	PRP Tam T47
WB Only		0,5768	0,9946	2,60E-31	7,98E-36	4,52E-07	8,74E-09	1,72E-08	2,46E-09	0,0004214	0,1536	0,05377	4,63E-29	0,07486	1,48E-25	2,48E-25	1,07E-14	5,23E-13	8,02E-11	4,98E-11	1,79E-05	1,48E-19	8,91E-17
WB thrombi	0,5768		0,5722	1,54E-28	7,62E-33	7,20E-06	2,04E-07	3,77E-07	6,43E-08	0,002995	0,385	0,1705	2,14E-26	0,2212	4,56E-23	7,41E-23	7,38E-13	2,72E-11	2,81E-09	1,81E-09	0,0001902	2,10E-17	8,46E-15
WB Tam	0,9946	0,5722		2,40E-31	7,33E-36	4,36E-07	8,40E-09	1,65E-08	2,36E-09	0,0004107	0,1517	0,05293	4,29E-29	0,07376	1,38E-25	2,31E-25	1,02E-14	4,98E-13	7,67E-11	4,75E-11	1,74E-05	1,39E-19	8,41E-17
PRP Only	2,60E-31	1,54E-28	2,40E-31		0,03924	4,29E-11	3,96E-09	1,96E-09	1,39E-08	4,93E-16	1,74E-24	2,71E-22	0,6522	6,32E-23	0,2338	0,2152	9,26E-05	9,85E-06	2,76E-07	4,02E-07	1,99E-13	0,009503	0,000897
*PRP Throm	7,98E-36	7,62E-33	7,33E-36	0,03924		9,40E-14	1,57E-11	7,05E-12	6,56E-11	3,01E-19	1,80E-28	4,28E-26	0,1916	8,81E-27	0,04077	0,03621	1,89E-06	1,32E-07	2,04E-09	3,16E-09	2,31E-16	0,0005635	2,96E-05
PPP Only	4,52E-07	7,20E-06	4,36E-07	4,29E-11	9,40E-14		0,479	0,5535	0,3582	0,1287	0,000296	0,001828	8,09E-10	0,001098	6,55E-08	8,58E-08	0,00726	0,02974	0,1458	0,127	0,4497	6,32E-05	0,001065
PRP Tam	8,74E-09	2,04E-07	8,40E-09	3,96E-09	1,57E-11	0,479		0,9082	0,833	0,02594	1,51E-05	0,0001309	5,47E-08	7,12E-05	2,66E-06	3,37E-06	0,04806	0,1427	0,4552	0,4132	0,1432	0,0009926	0,01032
PPP Tam	1,72E-08	3,77E-07	1,65E-08	1,96E-09	7,05E-12	0,5535	0,9082		0,7443	0,0347	2,54E-05	0,0002077	2,85E-08	0,0001149	1,50E-06	1,92E-06	0,03643	0,1139	0,3887	0,3505	0,1775	0,0006546	0,007355
T47 Med	2,46E-09	6,43E-08	2,36E-09	1,39E-08	6,56E-11	0,3582	0,833	0,7443		0,01477	5,69E-06	5,45E-05	1,75E-07	2,88E-05	7,31E-06	9,17E-06	0,07741	0,2096	0,5921	0,5436	0,094	0,002058	0,01856
T47 Dil	0,0004214	0,002995	0,0004107	4,93E-16	3,01E-19	0,1287	0,02594	0,0347	0,01477		0,03577	0,1101	1,82E-14	0,08099	4,44E-12	6,25E-12	2,62E-05	0,0002218	0,002941	0,002324	0,4453	3,39E-08	1,65E-06
T47 Med WB	0,1536	0,385	0,1517	1,74E-24	1,80E-28	0,000296	1,51E-05	2,54E-05	5,69E-06	0,03577		0,6157	1,64E-22	0,007229	1,85E-19	2,88E-19	2,91E-10	6,94E-09	3,91E-07	2,68E-07	0,004198	2,55E-14	5,51E-12
T47 Dil WB	0,05377	0,1705	0,05293	2,71E-22	4,28E-26	0,001828	0,0001309	0,0002077	5,45E-05	0,1101	0,6157		2,04E-20	0,8828	1,59E-17	2,42E-17	6,57E-09	1,22E-07	4,84E-06	3,43E-06	0,01823	1,10E-12	1,66E-10
T47 Tam	4,63E-29	2,14E-26	4,29E-29	0,6522	0,1916	8,09E-10	5,47E-08	2,85E-08	1,75E-07	1,82E-14	1,64E-22	2,04E-20		5,09E-21	0,4594	0,4303	0,0005432	7,20E-05	2,75E-06	3,89E-06	5,23E-12	0,03214	0,004101
T47 Tam WB	0,07486	0,2212	0,07376	6,32E-23	8,81E-27	0,001098	7,12E-05	0,0001149	2,88E-05	0,08099	0,7229	0,8828	5,09E-21		4,42E-18	6,77E-18	2,70E-09	5,40E-08	2,37E-06	1,67E-06	0,01213	3,74E-13	6,27E-11
WB Tam T47	1,48E-25	4,56E-23	1,38E-25	0,2338	0,04077	6,55E-08	2,66E-06	1,50E-06	7,31E-06	4,44E-12	1,85E-19	1,59E-17	0,4594	4,42E-18		0,9611	0,006556	0,001239	7,86E-05	0,0001057	7,31E-10	0,1607	0,03314
PPP Tam T47	2,48E-25	7,41E-23	2,31E-25	0,2152	0,03621	8,58E-08	3,37E-06	1,92E-06	9,17E-06	6,25E-12	2,88E-19	2,42E-17	0,4303	6,77E-18	0,9611		0,007588	0,001468	9,62E-05	0,000129	9,93E-10	0,1757	0,03738
T47 PRP	1,07E-14	7,38E-13	1,02E-14	9,26E-05	1,89E-06	0,00726	0,04806	0,03643	0,07741	2,62E-05	2,91E-10	6,57E-09	0,0005432	2,70E-09	0,006556	0,007588		0,6092	0,2187	0,2466	0,0005804	0,1882	0,5564
T47 PPP	5,23E-13	2,72E-11	4,98E-13	9,85E-06	1,32E-07	0,02974	0,1427	0,1139	0,2096	0,0002218	6,94E-09	1,22E-07	7,20E-05	5,40E-08	0,001239	0,001468	0,6092		0,4722	0,5174	0,003396	0,0677	0,2716
T47 Dil PRP	8,02E-11	2,81E-09	7,67E-11	2,76E-07	2,04E-09	0,1458	0,4552	0,3887	0,5921	0,002941	3,91E-07	4,84E-06	2,75E-06	2,37E-06	7,86E-05	9,62E-05	0,2187	0,4722		0,943	0,02707	0,0109	0,06901
T47 Dil PPP	4,98E-11	1,81E-09	4,75E-11	4,02E-07	3,16E-09	0,127	0,4132	0,3505	0,5436	0,002324	2,68E-07	3,43E-06	3,89E-06	1,67E-06	0,0001057	0,000129	0,2466	0,5174	0,943		0,02249	0,01335	0,08067
T47 Tam PR	1,79E-05	0,0001902	1,74E-05	1,99E-13	2,31E-16	0,4497	0,1432	0,1775	0,094	0,4453	0,004198	0,01823	5,23E-12	0,01213	7,31E-10	9,93E-10	0,0005804	0,003396	0,02707	0,02249		1,97E-06	5,61E-05
T47 Tam PP	1,48E-19	2,10E-17	1,39E-19	0,009503	0,0005635	6,32E-05	0,0009926	0,0006546	0,002058	3,39E-08	2,55E-14	1,10E-12	0,03214	3,74E-13	0,1607	0,1757	0,1882	0,0677	0,0109	0,01335	1,97E-06		0,04669
PRP Tam T47	8,91E-17	8,46E-15	8,41E-17	0,000897	2,96E-05	0,001065	0,01032	0,007355	0,01856	1,65E-06	5,51E-12	1,66E-10	0,004101	6,27E-11	0,03314	0,03738	0,5564	0,2716	0,06901	0,08067	5,61E-05	0,04669	

ER protein expression – Green indicates significance

	M10Med A	M10Med B	M10WB A	M10WB B	M10PRP A	M10PRP B	M10PPP A	M10PPP B
M10Med A		7,05E-63	6,61E-02	1,84E-62	3,12E-01	7,05E-63	1,21E-01	7,05E-63
M10Med B	7,05E-63		7,05E-63	7,05E-63	7,05E-63	3,19E-01	7,05E-63	7,05E-01
M10WB A	6,61E-02	7,05E-63		1,16E-62	2,15E-41	7,05E-63	1,03E-62	7,05E-63
M10WB B	1,84E-62	7,05E-63	1,16E-62		4,02E-42	7,05E-63	5,56E-13	7,05E-63
M10PRP A	3,12E-01	7,05E-63	2,15E-41	4,02E-42		7,05E-63	7,13E-58	7,05E-63
M10PRP B	7,05E-63	3,19E-01	7,05E-63	7,05E-63	7,05E-63		7,05E-63	1,55E-09
M10PPP A	1,21E-01	7,05E-63	1,03E-62	5,56E-13	7,13E-58	7,05E-63		7,05E-63
M10PPP B	7,05E-63	7,05E-01	7,05E-63	7,05E-63	7,05E-63	1,55E-09	7,05E-63	

	TMED ALPHA	TMED BETA	TMEDWB AL	TMEDWB BE	TMEDPRP AL	TMEDPRP BE	TMEDPPP AL	TMEDPPP BE
TMED ALPHA		2,56E-215	3,07E+00	5,67E-33	2,57E-20	6,10E-219	5,22E-02	1,61E-154
TMED BETA	2,56E-215		9,86E-76	3,41E-65	3,21E-68	0,7995	1,43E-104	9,85E-07
TMEDWB AL	3,07E+00	9,86E-76		0,2017	0,6279	1,41E-94	7,31E-06	3,55E-56
TMEDWB BE	5,67E-33	3,41E-65	0,2017		0,09482	3,13E-84	1,41E-08	2,64E-48
TMEDPRP AL	2,57E-20	3,21E-68	0,6279	0,09482		2,89E-87	0,0001773	1,40E-52
TMEDPRP BE	6,10E-219	0,7995	1,41E-94	3,13E-84	2,89E-87		6,59E-123	4,94E-06
TMEDPPP AL	5,22E-02	1,43E-104	7,31E-06	1,41E-08	0,0001773	6,59E-123		8,70E-81
TMEDPPP BE	1,61E-154	9,85E-07	3,55E-56	2,64E-48	1,40E-52	4,94E-06	8,70E-81	

	MMED ALPH	MMED BEM	MMEDWB A	MMEDWB B	MMEDPRP A	MMEDPRP B	MMEDPPP A	MMEDPPP B
MMED ALPHA		5,00E-63	3,16E-50	1,94E-62	0,6572	1,88E-61	3,14E-17	1,01E-58
MMED BEM	5,00E-63		7,35E-63	5,51E-20	5,08E-63	1,29E-01	5,33E-63	4,09E-16
MMEDWB A	3,16E-50	7,35E-63		8,60E-58	7,35E-22	3,65E-56	8,06E-49	3,43E-36
MMEDWB B	1,94E-62	5,51E-20	8,60E-58		1,89E-59	4,84E-24	1,15E-62	0,03727
MMEDPRP A	0,6572	5,08E-63	7,35E-22	1,89E-59		1,43E-56	2,04E-15	1,25E-56
MMEDPRP B	1,88E-61	1,29E-01	3,65E-56	4,84E-24	1,43E-56		5,16E-63	7,23E-14
MMEDPPP A	3,14E-17	5,33E-63	8,06E-49	1,15E-62	2,04E-15	5,16E-63		8,65E-49
MMEDPPP B	1,01E-58	4,09E-16	3,43E-36	0,03727	1,25E-56	7,23E-14	8,65E-49	

	MDIL ALPHA	MDIL BETA	MDILWB AL	MDILWB BE	MDILPRP AL	MDILPRP BE	MDILPPP AL	MDILPPP BE
MDIL ALPHA		2,41E-56	1,78E-62	1,67E-62	1,24E-01	4,84E-63	2,44E-01	4,84E-63
MDIL BETA	2,41E-56		1,28E-52	4,43E-24	3,26E-53	1,31E-19	7,04E-55	3,76E-09
MDILWB AL	1,78E-62	1,28E-52		9,42E-37	1,32E-09	6,95E-48	5,75E-48	6,99E-61
MDILWB BE	1,67E-62	4,43E-24	9,42E-37		3,28E-47	7,67E-08	3,46E-58	3,95E-40
MDILPRP AL	1,24E-01	3,26E-53	1,32E-09	3,28E-47		4,69E-52	1,10E-31	2,20E-62
MDILPRP BE	4,84E-63	1,31E-19	6,95E-48	7,67E-08	4,69E-52		6,73E-63	4,11E-07
MDILPPP AL	2,44E-01	7,04E-55	5,75E-48	3,46E-58	1,10E-31	6,73E-63		2,52E-60
MDILPPP BE	4,84E-63	3,76E-09	6,99E-61	3,95E-40	2,20E-62	4,11E-07	2,52E-60	

	TDIL ALPHA	TDIL BETA	TDILWB AL	TDILWB BE	TDILPRP AL	TDILPRP BE	TDILPPP AL	TDILPPP BE
TDIL ALPHA		4,84E-63	2,74E-23	5,16E-63	5,96E-48	4,84E-63	1,50E-01	4,84E-63
TDIL BETA	4,84E-63		5,10E-62	2,38E-01	6,27E-62	5,36E-02	4,84E-63	0,06328
TDILWB AL	2,74E-23	5,10E-62		2,36E-61	4,42E-50	4,84E-63	0,8169	6,79E-63
TDILWB BE	5,16E-01	2,38E-01	2,36E-61		7,88E-55	2,11E-01	5,83E-63	2,49E-01
TDILPRP AL	5,96E-48	6,27E-62	4,42E-50	7,88E-55		1,54E-58	9,25E-53	5,66E-59
TDILPRP BE	4,84E-02	5,36E-02	4,84E-63	2,11E-01	1,54E-58		5,00E-63	9,97E-02
TDILPPP AL	1,50E-01	4,84E-63	0,8169	5,83E-63	9,25E-53	5,00E-63		4,84E-63
TDILPPP BE	4,84E-63	0,06328	6,79E-63	2,49E-01	5,66E-59	9,97E-02	4,84E-63	

	TANAS A	TANAS B	TANASWB A	TANASWB B	TANASPRP A	TANASPRP B	TANASPPP A	TANASPPP B	WBANAST A	WBANAST B	PRPANAST A	PRPANAST B	PPPANAST A	PPPANAST B
TANAS A		4,84E-63	2,71E-52	5,42E-63	2,10E-40	8,23E-63	1,78E-01	4,84E-63	0,03072	6,22E-15	4,62E-01	1,28E-60	0,8964	4,84E-63
TANAS B	4,84E-63		4,84E-63	1,16E-01	4,84E-63	1,88E-01	4,84E-63	0,09559	4,84E-63	4,88E-63	4,84E-63	4,84E-63	4,84E-63	3,97E-01
TANASWB A	2,71E-52	4,84E-63		4,84E-63	2,03E-16	4,84E-63	4,37E-33	4,84E-63	5,25E-47	1,58E-44	8,72E-41	0,01295	3,40E-49	4,84E-63
TANASWB B	5,42E-63	1,16E-01	4,84E-63		4,88E-63	3,92E-09	5,04E-63	2,33E-11	5,60E-63	5,12E-63	5,25E-63	4,84E-63	5,42E-63	0,834
TANASPRP A	2,10E-40	4,84E-63	2,03E-16	4,88E-63		6,11E-63	3,85E-13	4,84E-63	1,38E-01	5,08E-23	8,06E-18	1,17E-26	8,10E-30	5,04E-63
TANASPRP B	8,23E-63	1,88E-01	4,84E-63	3,92E-09	6,11E-63		2,63E-62	4,57E-09	1,22E-62	6,57E-63	5,69E-63	4,84E-63	5,83E-61	1,71E-14
TANASPPP A	1,78E-01	4,84E-63	4,37E-33	5,04E-63	3,85E-13	2,63E-62		4,88E-63	4,88E-63	6,65E-08	0,003988	0,1148	7,24E-45	5,95E-13
TANASPPP B	4,84E-63	0,09559	4,84E-63	2,33E-11	4,84E-63	4,57E-09	4,88E-63		4,84E-63	4,92E-63	4,84E-63	4,84E-63	1,16E-62	6,76E-02
WBANAST A	0,03072	4,84E-63	5,25E-47	5,60E-63	1,38E-29	1,22E-62	6,65E-08	4,84E-63		0,0003425	5,40E-06	1,20E-56	0,07543	1,36E-62
WBANAST B	6,22E-15	4,88E-63	1,58E-44	5,12E-63	5,08E-01	6,57E-63	0,003988	4,92E-63	0,0003425		0,3228	2,32E-55	4,68E-08	8,63E-63
PRPANAST A	4,62E-01	4,84E-63	8,72E-41	5,25E-63	8,06E-18	5,69E-63	0,1148	4,84E-63	5,40E-06	0,3228		3,50E-52	1,81E-09	4,84E-63
PRPANAST B	1,28E-60	4,84E-63	0,01295	4,84E-63	1,17E-26	4,84E-63	7,24E-45	4,84E-63	1,20E-56	2,32E-55	3,50E-52		9,29E-57	4,84E-63
PPPANAST A	0,8964	4,84E-63	3,40E-49	5,42E-63	8,10E-30	5,83E-61	5,95E-13	1,16E-62	0,07543	4,68E-08	1,81E-09	9,29E-57		2,02E-58
PPPANAST B	4,84E-63	3,97E-01	4,84E-63	0,834	5,04E-63	1,71E-14	3,62E-62	6,76E-02	1,36E-62	8,63E-63	4,84E-63	4,84E-63	2,02E-58	

	MANAS A	MANAS B	MANASWB A	MANASWB B	MANASPRP A	MANASPRP B	MANASPPP A	MANASPPP B	WBANASM A	WBANASM B	PRPANASM A	PRPANASM B	PPPANASM A	PPPANASM B
MANAS A		1,68E-60	1,55E-01	1,37E-59	4,36E-01	5,04E-60	2,00E-01	1,93E-36	1,76E-22	0,03736	2,61E-01	0,0006607	7,08E-02	1,08E-51
MANAS B	1,68E-60		5,73E-63	7,67E-02	4,84E-63	6,22E-02	9,51E-63	9,81E-02	5,21E-63	2,29E-22	5,46E-63	2,59E-42	6,52E-63	0,2323
MANASWB A	1,55E-01	5,73E-63		9,97E-63	0,465	1,55E-62	9,31E-13	4,22E-54	0,4057	7,43E-08	0,04884	0,02562	0,03304	3,49E-61
MANASWB B	1,37E-59	7,67E-02	9,97E-63		5,55E-63	0,01465	8,29E-63	5,52E-05	7,12E-63	3,22E-18	6,79E-63	5,59E-35	8,23E-63	9,66E-09
MANASPRP A	4,36E-01	4,84E-63	0,465	5,55E-63		6,68E-63	3,80E-17	2,93E-55	0,6345	2,18E-06	0,951	0,9039	8,8903	1,67E-61
MANASPRP B	5,04E-60	6,22E-02	1,55E-62	0,01465	6,68E-63		8,83E-62	5,54E-05	1,05E-60	1,92E-16	2,32E-60	1,18E-32	5,24E-60	5,06E-08
MANASPPP A	2,00E-01	9,51E-63	9,31E-13	8,29E-63	3,80E-17	8,83E-62		2,42E-53	2,47E-16	0,01348	4,23E-16	0,01149	7,04E-02	1,93E-59
MANASPPP B	1,93E-36	9,81E-02	4,22E-54	5,52E-05	2,93E-55	5,54E-05	2,42E-53		8,48E-57	5,66E-18	2,04E-55	1,15E-27	2,69E-56	1,16E-01
WBANASM A	1,76E-22	5,21E-63	0,4057	7,12E-63	0,6345	1,05E-60	2,47E-16	8,48E-57		1,95E-08	0,596	0,3282	0,6813	6,67E-58
WBANASM B	0,03736	2,29E-22	7,43E-08	3,22E-18	2,18E-06	1,92E-16	0,01348	5,66E-18	1,95E-08		5,01E-06	0,009033	6,60E-07	6,32E-21
PRPANASM A	2,61E-01	5,46E-63	0,04884	6,79E-63	0,951	2,32E-60	4,23E-16	2,04E-55	0,596	5,01E-06		0,7606	0,841	1,06E-57
PRPANASM B	0,0006607	2,59E-42	0,02562	5,59E-35	0,9039	1,18E-32	0,01149	1,15E-27	0,3282	0,009033	0,7606		0,5164	1,84E-33
PPPANASM A	7,08E-02	6,52E-63	0,03304	8,23E-63	0,8903	5,24E-60	7,04E-02	2,69E-56	0,6813	6,60E-07	0,841	0,5164		3,89E-58
PPPANASM B	1,08E-51	0,2323	3,49E-61	9,66E-09	1,67E-61	5,06E-08	1,93E-59	1,16E-01	6,67E-58	6,32E-21	1,06E-57	1,84E-33	3,89E-58	

	MTAM A	MTAM B	MTAMWB A	MTAMWB B	MTAMPRP A	MTAMPRP B	MTAMPPP A	MTAMPPP B	WBTAMM A	WBTAMM B	PRPTAMM A	PRPTAMM B	PPPTAMM A	PPPTAMM B
MTAM A		8,95E-59	0,2289	5,88E-62	1,60E-01	3,63E-48	0,2062	7,35E-63	8,30E-21	4,04E-16	1,16E-01	1,40E-40	7,59E-02	5,87E-61
MTAM B	8,95E-59		7,03E-59	0,2119	2,64E-55	0,2955	2,86E-60	0,2564	5,21E-63	2,12E-38	5,55E-63	2,70E-10	5,55E-63	0,4523
MTAMWB A	0,2289	7,03E-59		2,95E-60	3,24E-22	4,35E-51	0,9876	3,30E-61	1,53E-01	3,39E-17	4,55E-25	8,20E-39	1,12E-25	5,43E-62
MTAMWB B	5,88E-62	0,2119	2,95E-60		9,65E-58	0,2942	1,78E-62	0,002433	7,12E-63	2,68E-39	8,03E-63	3,85E-07	7,90E-63	0,3507
MTAMPRP A	1,60E-01	2,64E-55	3,24E-22	9,65E-58		1,23E-42	5,44E-26	2,66E-55	5,77E-54	1,00E-23	6,25E-02	1,37E-35	2,41E-55	4,32E-51
MTAMPRP B	3,63E-48	0,2955	4,35E-51	0,2942	1,23E-42		1,26E-46	0,0001511	2,26E-60	2,50E-41	4,31E-60	1,19E-07	5,00E-60	0,9853
MTAMPPP A	0,2062	2,86E-60	0,9876	1,78E-62	5,44E-26	1,26E-46		1,12E-62	5,03E-36	2,49E-18	1,64E-36	2,93E-40	1,98E-01	4,07E-58
MTAMPPP B	7,35E-63	0,2564	3,30E-61	0,002433	2,66E-55	0,0001511	1,12E-62		1,24E-62	1,24E-33	1,16E-62	4,34E-08	1,16E-62	0,01563
WBTAMM A	8,30E-21	5,21E-63	1,53E-01	7,12E-63	5,77E-54	2,26E-60	5,03E-36	1,24E-62		3,53E-08	0,4167	1,49E-48	0,1727	1,90E-59
WBTAMM B	4,04E-16	2,12E-38	3,39E-17	2,68E-39	1,00E-23	2,50E-41	2,49E-18	1,24E-33	3,53E-08		9,40E-08	8,41E-40	8,76E-08	2,14E-37
PRPTAMM A	1,16E-01	5,55E-63	4,55E-25	8,03E-63	6,25E-02	4,31E-60	1,64E-36	1,16E-62	0,4167	9,40E-08		1,41E-47	0,5109	6,47E-60
PRPTAMM B	1,40E-40	2,70E-10	8,20E-39	3,85E-07	1,37E-35	1,19E-07	2,93E-40	4,34E-08	1,49E-48	8,41E-40	1,41E-47		7,30E-49	1,12E-07
PPPTAMM A	7,59E-02	5,55E-63	1,12E-25	7,90E-63	2,41E-55	5,00E-60	1,98E-01	1,16E-62	0,1727	8,76E-08	0,5109	7,30E-49		7,75E-60
PPPTAMM B	5,87E-61	0,4523	5,43E-62	0,3507	4,32E-51	0,9853	4,07E-58	0,01563	1,90E-59	2,14E-37	6,47E-60	1,12E-07	7,75E-60	

	TTAM A	TTAM B	TTAMWB A	TTAMWB B	TTAMPRP A	TTAMPRP B	TTAMPPP A	TTAMPPP B	WBTAMT A	WBTAMT B	PRPTAMT A	PRPTAMT B	PPPTAMT A	PPPTAMT B
TTAM A		9,09E-50	1,48E-25	0,0001407	1,29E-55	4,84E-63	2,93E-16	4,17E-60	1,99E-58	7,24E-63	1,68E-61	4,84E-63	2,64E-59	6,98E-09
TTAM B	9,09E-50		2,05E-62	2,86E-42	6,56E-08	1,36E-57	8,93E-30	2,77E-01	1,38E-06	4,84E-63	1,22E-11	4,84E-63	7,42E-06	9,55E-02
TTAMWB A	1,48E-25	2,05E-62		6,81E-49	5,87E-63	4,84E-63	3,04E-58	5,04E-63	4,11E-62	5,58E-55	6,31E-63	4,84E-63	5,00E-63	2,96E-52
TTAMWB B	0,0001407	2,86E-42	6,81E-49		7,97E-51	1,89E-62	1,00E-05	8,67E-59	4,23E-48	4,84E-63	1,39E-50	4,84E-63	4,44E-48	0,003087
TTAMPRP A	1,29E-55	6,56E-08	5,87E-63	7,97E-51		4,35E-01	1,03E-39	2,12E-05	0,5002	4,84E-63	0,07653	4,84E-63	0,4282	2,93E-39
TTAMPRP B	4,84E-63	1,36E-57	4,84E-63	1,89E-62	4,35E-01		4,34E-62	2,70E-31	3,50E-57	4,84E-63	1,47E-56	7,35E-63	5,24E-59	1,40E-59
TTAMPPP A	2,93E-01	8,93E-30	3,04E-58	1,00E-05	1,03E-39	4,34E-62		4,31E-60	3,22E-40	4,84E-63	3,51E-46	4,84E-63	6,25E-02	0,2742
TTAMPPP B	4,17E-60	2,77E-01	5,04E-63	8,67E-59	2,12E-05	2,70E-31	4,31E-60		2,32E-10	4,84E-63	4,82E-07	1,15E-62	4,51E-11	1,36E-54
WBTAMT A	1,99E-01	1,38E-06	4,11E-62	4,23E-48	0,5002	3,50E-57	3,22E-40	2,32E-10		4,84E-63	0,1736	4,84E-63	0,3808	1,72E-35
WBTAMT B	7,24E-63	4,84E-63	5,58E-55	4,84E-63	4,84E-63	4,84E-63	4,84E-63	4,84E-63	4,84E-63		4,84E-63	4,84E-63	4,84E-63	4,84E-63
PRPTAMT A	1,68E-61	1,22E-11	6,31E-63	1,39E-50	0,07653	1,47E-56	3,51E-46	4,82E-07	0,1736	4,84E-63		4,84E-63	0,01521	2,52E-39
PRPTAMT B	4,84E-63	4,84E-63	4,84E-63	4,84E-63	4,84E-63	7,35E-63	4,84E-63	1,15E-62	4,84E-63	4,84E-63	4,84E-63		4,84E-63	4,84E-63
PPPTAMT A	2,64E-01	7,42E-06	5,00E-63	4,44E-48	0,4282	5,24E-59	6,25E-02	4,51E-11	0,3808	4,84E-63	0,01521	4,84E-63		3,67E-36
PPPTAMT B	6,98E-09	9,55E-02	2,96E-52	0,003087	2,93E-39	1,40E-59	0,2742	1,36E-54	1,72E-35	4,84E-63	2,52E-39	4,84E-63	3,67E-36	

Spearman's Rank Order Correlations. Red indicating significance

All Groups Spearman Rank Order Correlations (Correlations Spearman) MD pairwise deleted Marked correlations are significant at p <,05000				
	ACTB	ESR1	ESR2	P7-P11 IPA
ACTB		1,000000	0,420561	0,113695
ESR1	0,420561		0,564082	0,186450
ESR2	0,623803	0,564082		0,022227
P7-P11 IPA	0,113695	0,186450	0,022227	

A=MMEDWB Spearman Rank Order Correlations (Correlations Spearman) MD pairwise deleted Marked correlations are significant at $p < ,05000$				
	ACTB	ESR1	ESR2	P7-P11 IPA
ACTB	1,000000	0,900000	0,800000	-0,200000
ESR1	0,900000	1,000000	1,000000	-0,500000
ESR2	0,800000	1,000000	1,000000	-0,800000
P7-P11 IPA	-0,200000	-0,500000	-0,800000	1,000000

A=MANASWB Spearman Rank Order Correlations (Correlations Spearman) MD pairwise deleted Marked correlations are significant at $p < ,05000$				
	ACTB	ESR1	ESR2	P7-P11 IPA
ACTB	1,000000	1,000000	0,948683	0,700000
ESR1	1,000000	1,000000	0,948683	0,700000
ESR2	0,948683	0,948683	1,000000	0,316228
P7-P11 IPA	0,700000	0,700000	0,316228	1,000000

A=MTAMWB Spearman Rank Order Correlations (Correlations Spearman) MD pairwise deleted Marked correlations are significant at $p < ,05000$				
	ACTB	ESR1	ESR2	P7-P11 IPA
ACTB	1,000000	0,900000	0,400000	0,300000
ESR1	0,900000	1,000000	0,200000	0,100000
ESR2	0,400000	0,200000	1,000000	-0,200000
P7-P11 IPA	0,300000	0,100000	-0,200000	1,000000

A=TMEDWB Spearman Rank Order Correlations (Correlations Spearman) MD pairwise deleted Marked correlations are significant at $p < ,05000$				
	ACTB	ESR1	ESR2	P7-P11 IPA
ACTB	1,000000	0,200000	0,400000	0,100000
ESR1	0,200000	1,000000	0,800000	0,900000
ESR2	0,400000	0,800000	1,000000	0,400000
P7-P11 IPA	0,100000	0,900000	0,400000	1,000000

A=TANASWB Spearman Rank Order Correlations (Correlations Spearman) MD pairwise deleted Marked correlations are significant at p <,05000				
	ACTB	ESR1	ESR2	P7-P11 IPA
ACTB	1,000000	-0,500000	0,000000	0,000000
ESR1	-0,500000	1,000000	1,000000	0,500000
ESR2	0,000000	1,000000	1,000000	0,200000
P7-P11 IPA	0,000000	0,500000	0,200000	1,000000

A=TTAMWB Spearman Rank Order Correlations (Correlations Spearman) MD pairwise deleted Marked correlations are significant at p <,05000				
	ACTB	ESR1	ESR2	P7-P11 IPA
ACTB	1,000000	0,000000	-0,200000	0,600000
ESR1	0,000000	1,000000	0,800000	0,200000
ESR2	-0,200000	0,800000	1,000000	-0,400000
P7-P11 IPA	0,600000	0,200000	-0,400000	1,000000

	All Groups				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,07563	-0,04732	0,016055	-0,18911
CD62P IPA	-0,07563	1	0,947854	0,089532	0,262309
CD63 IPA	-0,04732	0,947854	1	0,074512	0,264991
ERAlpha	0,016055	0,089532	0,074512	1	0,2858
ERBeta	-0,18911	0,262309	0,264991	0,2858	1

	Treatment=MCF10A WB				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,42857	-0,54286	0,428571	0,085714
CD62P IPA	-0,42857	1	0,885714	-0,14286	-0,2
CD63 IPA	-0,54286	0,885714	1	-0,08571	-0,08571
ERAlpha	0,428571	-0,14286	-0,08571	1	-0,48571
ERBeta	0,085714	-0,2	-0,08571	-0,48571	1

	Treatment=MCF10A PRP				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,54286	-0,6	0,6	-0,71429
CD62P IPA	-0,54286	1	0,942857	-0,08571	0,371429
CD63 IPA	-0,6	0,942857	1	-0,2	0,2
ERAlpha	0,6	-0,08571	-0,2	1	-0,2
ERBeta	-0,71429	0,371429	0,2	-0,2	1

	Treatment=MCF7 Dil PRP				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,2	0,2	0,257143	-0,42857
CD62P IPA	0,2	1	1	0,428571	-0,65714
CD63 IPA	0,2	1	1	0,428571	-0,65714
ERAlpha	0,257143	0,428571	0,428571	1	-0,31429
ERBeta	-0,42857	-0,65714	-0,65714	-0,31429	1

	Treatment=MCF7 Dil WB				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,142857	0,257143	-0,2	0,485714
CD62P IPA	0,142857	1	0,942857	0,314286	-0,37143
CD63 IPA	0,257143	0,942857	1	0,085714	-0,25714
ERAlpha	-0,2	0,314286	0,085714	1	0,142857
ERBeta	0,485714	-0,37143	-0,25714	0,142857	1

	Treatment=MCF7 PRP				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,085714	0,028571	0,029424	-0,26482
CD62P IPA	0,085714	1	0,942857	-0,79446	-0,61791
CD63 IPA	0,028571	0,942857	1	-0,70619	-0,76504
ERAlpha	0,029424	-0,79446	-0,70619	1	0,333333
ERBeta	-0,26482	-0,61791	-0,76504	0,333333	1

	Treatment=MCF7 Tam PRP				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,771429	0,771429	-0,48571	-0,14286
CD62P IPA	0,771429	1	1	-0,6	0,428571
CD63 IPA	0,771429	1	1	-0,6	0,428571
ERAlpha	-0,48571	-0,6	-0,6	1	-0,2
ERBeta	-0,14286	0,428571	0,428571	-0,2	1

	Treatment=MCF7 Tam WB				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,2	-0,42857	0,485714	0,485714
CD62P IPA	-0,2	1	0,942857	0,485714	-0,14286
CD63 IPA	-0,42857	0,942857	1	0,257143	-0,42857
ERAlpha	0,485714	0,485714	0,257143	1	0,314286
ERBeta	0,485714	-0,14286	-0,42857	0,314286	1

	Treatment=MCF7 WB				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,31429	-0,31429	-0,71429	-0,2
CD62P IPA	-0,31429	1	1	-0,2	-0,37143
CD63 IPA	-0,31429	1	1	-0,2	-0,37143
ERAlpha	-0,71429	-0,2	-0,2	1	0,542857
ERBeta	-0,2	-0,37143	-0,37143	0,542857	1

	Treatment=PRP Tam MCF7				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,48571	-0,48571	0,771429	-0,6
CD62P IPA	-0,48571	1	1	-0,82857	0,942857
CD63 IPA	-0,48571	1	1	-0,82857	0,942857
ERAlpha	0,771429	-0,82857	-0,82857	1	-0,94286
ERBeta	-0,6	0,942857	0,942857	-0,94286	1

	Treatment=PRP Tam T47				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,4	0,1	-0,1	-0,6
CD62P IPA	0,4	1	0,7	-0,7	0
CD63 IPA	0,1	0,7	1	-1	-0,3
ERAlpha	-0,1	-0,7	-1	1	0,3
ERBeta	-0,6	0	-0,3	0,3	1

	Treatment=T47 Dil PRP				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,14286	-0,25714	-0,65714	-0,25714
CD62P IPA	-0,14286	1	0,942857	0,485714	0,771429
CD63 IPA	-0,25714	0,942857	1	0,6	0,828571
ERAlpha	-0,65714	0,485714	0,6	1	0,257143
ERBeta	-0,25714	0,771429	0,828571	0,257143	1

	Treatment=T47 Dil WB				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,485714	0,142857	0,485714	-0,65714
CD62P IPA	0,485714	1	0,714286	0,257143	-0,94286
CD63 IPA	0,142857	0,714286	1	-0,37143	-0,48571
ERAlpha	0,485714	0,257143	-0,37143	1	-0,48571
ERBeta	-0,65714	-0,94286	-0,48571	-0,48571	1

	Treatment=T47 Med WB				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,23191	-0,11595	0,25714	-0,25714
CD62P IPA	-0,23191	1	0,941176	-0,63775	0,63775
CD63 IPA	-0,11595	0,941176	1	-0,57977	0,57977
ERAlpha	0,257143	-0,63775	-0,57977	1	-1
ERBeta	-0,25714	0,637748	0,579771	-1	1

	Treatment=T47 PRP				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,257143	0,428571	0,257143	0,142857
CD62P IPA	0,257143	1	0,942857	0,542857	0,771429
CD63 IPA	0,428571	0,942857	1	0,371429	0,885714
ERAlpha	0,257143	0,542857	0,371429	1	0,028571
ERBeta	0,142857	0,771429	0,885714	0,028571	1

	Treatment=T47 Tam PRP				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,314286	-0,08571	0,142857	-0,31429
CD62P IPA	0,314286	1	0,257143	0,142857	0,371429
CD63 IPA	-0,08571	0,257143	1	0,085714	0,771429
ERAlpha	0,142857	0,142857	0,085714	1	0,542857
ERBeta	-0,31429	0,371429	0,771429	0,542857	1

	Treatment=T47 Tam WB				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	0,3	-0,7	0,3	0,4
CD62P IPA	0,3	1	-0,3	-0,5	-0,6
CD63 IPA	-0,7	-0,3	1	0,2	-0,1
ERAlpha	0,3	-0,5	0,2	1	0,4
ERBeta	0,4	-0,6	-0,1	0,4	1

	Treatment=WB Tam MCF7				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,65714	0,898645	0,314286	0,428571
CD62P IPA	-0,65714	1	-0,81168	-0,65714	-0,08571
CD63 IPA	0,898645	-0,81168	1	0,463817	0,347863
ERAlpha	0,314286	-0,65714	0,463817	1	-0,14286
ERBeta	0,428571	-0,08571	0,347863	-0,14286	1

	Treatment=WB Tam T47				
Variable	Spearman Rank Order Correlations (Correlation 4 (1))				
	MD pairwise deleted				
	Marked correlations are significant at p <,05000				
	Thromb	CD62P IPA	CD63 IPA	ERAlpha	ERBeta
Thromb	1	-0,57977	0,6	-0,65714	-0,48571
CD62P IPA	-0,57977	1	-0,14494	0,115954	0,637748
CD63 IPA	0,6	-0,14494	1	-0,08571	0,085714
ERAlpha	-0,65714	0,115954	-0,08571	1	0,657143
ERBeta	-0,48571	0,637748	0,085714	0,657143	1

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