Influence of mesenchymal stromal cells and 2-methoxyestradiol in a murine model of spontaneous mammary carcinoma

by

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Declaration

I, Kimberly Thando Peta, affirm that this thesis titled '**Influence of mesenchymal stromal cells 2-methoxyestradiol in a murine model of spontaneous mammary carcinoma**' is my original work. I am submitting it for the degree of Philosophiæ Doctor (PhD) in Medical Immunology. I confirm that this thesis has not been previously submitted for any other degree or examination at any other university. Additionally, I have appropriately referenced and acknowledged all the resources used in this thesis.

Kimberly Thando Peta

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Abstract

Breast cancer (BC) is the most prevalent cancer in females and the leading cause of cancer deaths. Treatment options include mastectomy, chemotherapy, and radiotherapy. While these treatments can improve 5-year survival rates and reduce recurrence risk, they also affect healthy cells and not are effective for metastatic BC. To address these limitations, alternative therapies targeting only cancerous cells such as mesenchymal stromal/stem cell (MSC) therapy and a novel chemotherapeutic agent, 2-methoxyestradiol (2-ME), have been explored.

MSCs have the ability to "home" to the tumour microenvironment (TME) and either promote or suppress tumour progression. Previous studies resulted in inconsistent results because of varied experimental designs including xenograft models that yielded conflicting results due to cross-species variations, limiting their interpretation. To overcome this, an isogenic mouse model of spontaneous BC was utilized to investigate the effect of MSCs on BC development. MSCs isolated from FVB/N mouse adipose tissue (mASC) were administered to heterozygous FVB/N-Tg(MMTV-PyVT)634Mul/J female mice that develop palpable mammary tumours. While no significant change in mammary tumour mass and volume was observed with mASC treatment, necrosis in lung lesions increased. Also, there was reduced number of CD163+ M2 macrophages and increased CD3+ T cells in the lungs but not mammary tumours in treatment group. *Vegfr1*, *cd105* and *mtdh* were downregulated in the lungs suggesting an anti-tumour effect, potentially due to the presence of trapped mASCs. Overall, 13 of the measured cytokines were higher in the mASC treated group. These findings indicate that mASCs have an anti-tumour effect on pulmonary metastatic BC.

The effect of 2-ME, a compound known for its anti-proliferative and anti-angiogenic properties, on the different stages of BC tumour development, is still unknown and was therefore investigated. The effects of 2-ME treatment on early- and late-stage BC were compared. While 2-ME treatment of early-stage BC led to reduced tumour necrosis with increased mass and volume of mammary tumours, a greater number of necrotic lesions and CD163 macrophages were observed in pulmonary metastatic tumours. Conversely, 2-ME treatment of late-stage BC inhibited tumour growth, increased CD3+ T cells and induced tumour necrosis. However, survival rates were not improved. Cytokine measurements of

early-stage BC indicated that 2-ME may have a pro-rumour effect. These findings suggest that 2-ME treatment has an antitumour effect on late-stage BC but does not enhance survival while no significant benefits were observed with 2-ME treatment of early-stage BC.

Interestingly, 2-ME treatment before the appearance of palpable tumours resulted in a significant increase in tumour mass. This pro-tumour activity was accompanied by lower numbers of CD3+ T cells in the TME and elevated levels of the pro-inflammatory cytokine interleukin (IL)-1β. However, 2-ME treatment also led to fewer CD163+ macrophages in the TME, increased tumour necrosis, increased IL-10, and reduced IL-6 and IL-27 levels. This suggests that 2-ME may promote tumour development at the onset and early stages of BC development.

In summary, BC is a complex disease with various stages, including tumour initiation, growth, progression and metastasis, and treatment effectiveness varies according to cancer stage. While mASCs show promise in treating pulmonary metastatic BC, 2-ME demonstrates an anti-tumour effect in late-stage BC but lacks efficacy in early-stage BC. Understanding the diverse responses to these treatments is crucial for developing targeted therapies that can effectively combat BC at different stages of progression.

Keywords: Breast cancer, mesenchymal stromal/stem cells, 2-methoxyestradiol, tumour microenvironment, metastasis, tumour progression, macrophages, T cells, cytokines, targeted therapies.

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ATM	Ataxia-telangiectasia mutated
APC	Allophycocyanin
APCs	Antigen presenting cells
ASCs	Adipose-derived stem cells
ASC-CM	ASC conditioned media
BALB	Bagg and Albino
BC	Breast cancer
BCL-2	B-cell lymphoma 2
BM-MSCs	Bone marrow mesenchymal stem cells
Bp	Base pairs
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BSA	Bovine serum albumin
BSC	Biosafety cabinet
^{o}C	Degree celsius
C57BL/6	C57 black 6
CAFs	Cancer-associated fibroblasts
CCL	Chemokine ligand
CCM	Complete culture media
CD	Cluster of differentiation
CD105	Endoglin
CDH1	Cadherin 1
CDH2	Cadherin 2
cDNA	Complementary deoxyribonucleic acid
CHEK2	Checkpoint kinase 2
cm	Centimetre
CO_2	Carbon dioxide

COMT	Catechol-O-methyltransferase
CSCs	Cancer stem cells
CTCs	Circulating tumour cells
CTLs	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte-associated antigen
DAB	3,3-Diaminobenzidine
DAPI	6-Diamidino-2-phenylindole
DCs	Dendritic cells
dH_2O	Distilled water
DNA	Deoxyribose nucleic acid
DMEM	Dulbecco's modified eagle medium
DMEM/F12	Dulbecco's modified eagle medium/F12
DMSO	Dimethyl sulfoxide
DPX	Dibutylphthalate polystyrene xylene
E2	17β-Estradiol
E	Eosin
ECD	Extracellular domain
ECs	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ER+	Estrogen receptor positive
ER-	Estrogen receptor negative
EVs	Extracellular vesicles
FBS	Fetal bovine solution
FITC	Fluorescein isothiocyanate
FFPE	Formalin fixed paraffin embedded
FVB	Friend leukemia virus B

g	Grams
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H	Haematoxylin
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HLA-DR	Human Leukocyte Antigen – DR isotype
HRP	Horseradish peroxidase
IBMX	3-Isobutyl-methlyxanthine
ICMM	Institute of Cellular and Molecular Medicine
IDO	Indoleamine 2,3-dioxygenase
IGF1	Insulin-like growth factor 1
IFN-γ	Interferon gamma
IngWAT	Inguinal white adipose tissue
IL	Interleukin
IP	Intraperitoneal
kg	Kilogram
L	Length
LT	Lung tumour
2-ME	2-Methoxyestradiol
2-OHE2	2-hydroxyestradiol
µg	Microgram
µL	Microliters
µm	Micromolar
mASCS	Mouse adipose mesenchymal stem cells
M1	Proinflammatory macrophage
M2	Anti-inflammatory macrophage
MCP-1	M*onocyte chemoattractant protein-1*
mg	Milligrams
MHC I	Major histocompatibility complex class I

MHC II	Major histocompatibility complex class II
Min	Minute
mL	Millilitre
mm	Millimeters
mM	Millimolar
MMP9	Matrix metallopeptidase 9
MMTV-PyVT	Mouse mammary tumour virus- Polyoma Virus middle T antigen
mRNA	Messenger RNA
MSCs	Mesenchymal stromal/stem cells
MT	Mammary tumour
MTDH	Metadherin
N	Number
NBF	Neutral buffered formalin
NBSI	Nijmegen breakage syndrome 1 gene
NCCN	National Comprehensive Cancer Network
NCDs	Non-communicable diseases
n*g*	*Nanograms*
NK	*Natural killer*
ObASCs	Obesity-altered ASCs
OVARU	Onderstepoort Veterinary Animal Research Unit
PALB2	Partner and Localizer of BRCA2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PD-L1	Programmed death ligand-1
PE	Phycoerythrin
Pen/Strep	Penicillin Streptomycin
pg/mL	picograms per milliliter
PR	Progesterone receptor

PTEN	Phosphotase and TENsin homologue deleted on chromosome ten
PyVT	Polyomavirus middle T
RPM	Revolutions per minute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
S	Seconds
Sca-1	Stem cells antigen 1
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDF-1α	Stroma-derived factor 1α
SER	Standard error of regression
SNAIL1	Drosophila embryonic protein SNAI1
SNAIL2	Drosophila embryonic protein SNAI2
STK11	Serine/threonine kinase 11
TAMs	Tumour associated macrophages
TGFβ	Transforming growth factor beta
TGFβ1	Transforming growth factor beta 1
TGFβ3	Transforming growth factor beta 3
Th1	Helper T cell type 1
Th2	Helper T cell type 2
TNF-α	Tumour necrosis factor alpha
TME	Tumour microenvironment
TNBC	Triple negative breast cancer
Treg	Regulatory T cells
TWIST1	Twist-related protein 1
UC-MSCs	Umbilical cord mesenchymal stem cells
UGT	UDP-glucuronosyltransferase
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1

WAT	White adipose tissue
W	Width

Chapter 1

1. Introduction

Cancer is a non-communicable disease (NCD) that is a leading cause of death and reduces life expectancy in all nations worldwide [1]. According to the World Health Organisation's (WHO) estimates in 2019, cancer ranks among the top two causes of death under the age 70 years of age in 112 out of 183 countries [2]. Moreover, cancer causes 30% of all premature deaths of people aged between 30 and 69 years [3]. It was estimated that there were 19.3 million new cancer cases and 9.9 million cancer-related deaths in 2020 [1], which is an increase from the 2018 statistics of 18.1 million new cases and 9.6 million deaths [1-2]. The incidence and mortality rates are continuously increasing worldwide [2]. Female breast cancer (BC) ranks second as most frequently diagnosed cancer with 2.3 million new cases and is also the most prevalent cancer in females, and the leading cause of cancer death in women [1-2].

1.1 Breast Cancer Incidence and Risk Factors

1.1.1 Breast cancer incidence

Current BC prevalence is based on new cases documented in 159 of 185 countries and is the leading cause of death in more than 110 countries [1]. In 2020, BC has overtaken lung cancer to become the leading cause of global cancer incidence with 2.3 million new cases diagnosed worldwide, resulting in over six hundred thousand deaths. BC is responsible for 1 in every 4 cancer cases and 1 in every 6 cancer deaths in women [1]. BC incidence has increased over the past decades in numerous countries in Asia, Africa, and South America [2]. The mortality rate in sub-Saharan Africa is now the world's highest with late-stage presentation (stages III/IV) being the reason behind the low survival rates [1]. Africa has the second-lowest incidence rate (30 per 100 000) compared to the other continents like Europe and North America (90 -120 per 100 000), but with the highest BC mortality rate. The BC incidence varies across Africa, with southern Africa having the highest incidence rate in sub-Saharan Africa. However, taking Africa as a whole, southern Africa has the second-highest incidence rate after northern Africa and the second-lowest mortality rate [4-5].

The rise in BC incidence in Africa has significant socioeconomic impact, as the cost of treating BC exceeds what the average household can afford [4,6]. Moreover, the high BC mortality rate

may be attributed to a lack of appropriate treatment strategies, competing health priorities, and a late stage of diagnosis [4,6]. In South Africa, BC is the leading cancer in female and women have a lifetime risk of 1 in 29 of developing the disease, with an age-adjusted incidence rate of 31.4 per 100 000 women and a mortality rate of 16.4 [6-7]. South Africa is a middle- to low-income country, and 80% of women in low-resource communities are unaware of BC warning signs. This lack of knowledge is common in older women living in rural areas and this may contribute to a late diagnosis [5].

1.1.2 Risk factors

There are several BC risk factors, including family BC history, genetic mutations, increased age, and lifestyle choices [8]. These risk factors contribute to the increase in the development of BC in African women [5].

Lifestyle

The adoption of a westernized lifestyle includes changes in diet [5]. Traditionally, an African diet has consisted of mainly of vegetables, fibre and grain [8] which has been replaced with a high-fat diet and ultra-processed carbohydrates which may promote cancer [8-10]. Furthermore, a high-fat diet may lead to obesity [11], which is a risk factor since oestrogen production is increased in obesity and may in turn increase the risk of breast, ovarian and other cancers [12]. Besides diet, lifestyle changes include late childbearing age, heavy alcohol intake and reduced physical activity [5].

Genetic mutations

Two high-risk genes that are associated with BC are *BRCA1* and *BRCA2* [13]. Mutations in these genes have been implicated in hereditary BC [13]. Mutations in the BRCA genes have been detected in approximately 3-5% of BC patients [13]. Females carrying BRCA germline mutations are at a high risk of developing BC by the age of 70 years. Moreover, the frequency of mutation at that age is 85% and 84% for *BRCA1* and *BRCA2* respectively [14]. In South Africa, the most notable BC mutation is *BRCA2* 5999del4, which is predominantly found among Afrikaners, stemming from a founder effect. This mutation was also detected in mixed-race and Xhosa women in the Western Cape province [15-16]. Numerous other genes have been implicated in BC development including *p53, ATM, CHEK2, PTEN, NBS1, PALB2, CDH1, and STK11* [13-14].

Other risk factors

Additionalrisk factors for increased BC among African women. These include prolonged life expectancy that permits the disease to manifest, poor awareness and/or the availability of healthcare services, poor availability of diagnostic technology and skills, or a combination of these factors [4]. Moreover, late menopause, early menarche, environmental contaminants, drug exposures, and immunologic and hormonal factors are also BC risk factors [5].

1.2 Breast Cancer Types and Metastasis

Breast cancer tumours vary in size, morphology, and behaviour. There are different types of BC, including ductal carcinoma *in situ*, lobular carcinoma *in situ* and invasive lobular carcinoma among others. BC is divided into three types: triple-negative BC (TNBC), luminal, and human epidermal growth factor receptor 2 (HER-2) [17]. Furthermore, the grade, stage, and hormonal receptors of these types vary [18]. Moreover, these types have various risk factors, pathological features, and clinical presentations. TNBC lacks the expression of progesterone (PR), oestrogen (ER), and HER-2 [19-20]. These tumours are large (4.1 ± 2.7 cm), basal-like, and highly invasive, and the mass has a well-circumscribed margin and acoustic enhancement [19,21]. The pathological features of TNBC are the internal fluid component at the posterior enhancement [21]. This subtype is found mainly in women under the age of 40 years and is detected in 15-20% of BC patients [19-20,22]. In comparison with other subtypes, the survival time is shorter and the mortality rate in the first 5 years after initial diagnosis is 40% [19]. Distant metastasis occurs in about 46% of TNBC patients and the median survival time is 13.3 months after metastasis [19]. Metastasis frequently involves visceral organs and the brain [19].

The luminal A subtype lacks the expression of HER2, but is positive for ER and PR and accounts for about 60-70% of all BC cases [19,23-24]. Luminal A accounts for approximately 60% BC cases and is correlated with a good prognosis [22]. The luminal B subtype accounts for roughly 10-30% of all BC cases, is associated with a poor prognosis, and is differentiated by the presence of an ER+ and/or PR+/HER2+ status [22,25]. However, luminal A expresses low Ki-67 (<14% tumour cell positivity) and luminal B expresses high Ki-67 (≥14% tumour cell positivity), suggesting that luminal A has lower proliferation rate compared to luminal B [23]. Luminal A tumours are low grade, with infiltrating ductal carcinoma being the most common histologic subtype and absent distant metastasis in approximately 61.5% of BC patients diagnosed with

luminal A tumours [25-26]. Luminal B tumours is associated with absent lymphovascular invasion, with well-differentiated infiltrating ductal carcinoma being the most common histologic subtype [26]. The ten-year survival rate for patients with luminal A is higher (54.4%) than for patients with luminal B (46.1%) [27]. Luminal tumours metastasize to the bone, the liver and the lung [27]. The median survival time after metastasis is 2.2 years and 1.6 years for luminal A and luminal B respectively [27]. That notwithstanding, the median survival time for brain metastasis is 4 months and 7.3 months for luminal A and B respectively [28].

Human epidermal growth factor receptor 2 (HER2+) BC is ER and PR negative and HER2 positive [29]. It is one of the most aggressive BC subtypes and is incurable when diagnosed at stage IV [30]. HER-2 is overexpressed in 15-30% of invasive BC [31]. HER2+ BC metastasizes to the brain, liver, bones, lung and lymph nodes [32-33]. Median overall survival for HER2+ metastatic BC patients is 63 months [34]. Patients with stage I, II, and III BC who received drug treatment had a 5-year recurrence risk of 7, 11, and 13% respectively [35]. All these factors make BC a heterogeneous disease that also differs in clinical behaviour resulting in differences in response to therapies and clinical outcome [36].

Metastatic tumours are the cause of most deaths from BC and not the primary tumour. Metastasis occurs when circulating tumour cells (CTCs) migrate from the primary tumour site to a secondary location via the bloodstream. The process of metastasis begins when the cell-to-cell adhesion of tumour cells and cell adhesion to the extracellular matrix (ECM) are altered. Cell-cell adhesion is maintained by E-cadherin, when this protein is switched off, N-cadherin is switched on and induces an epithelial-to-mesenchymal transition (EMT). At the EMT state, dissociated tumour cells migrate and invade the local surrounding tissues and intravasate into lymphatic vessels or the blood stream (Figure 1). After tumour cells intravasate, they spread to distant organs and proliferate. Within the target organ, these cells attach to capillary beds before extravasating into the parenchyma of the organ, proliferating and stimulating angiogenesis within the organ [37].

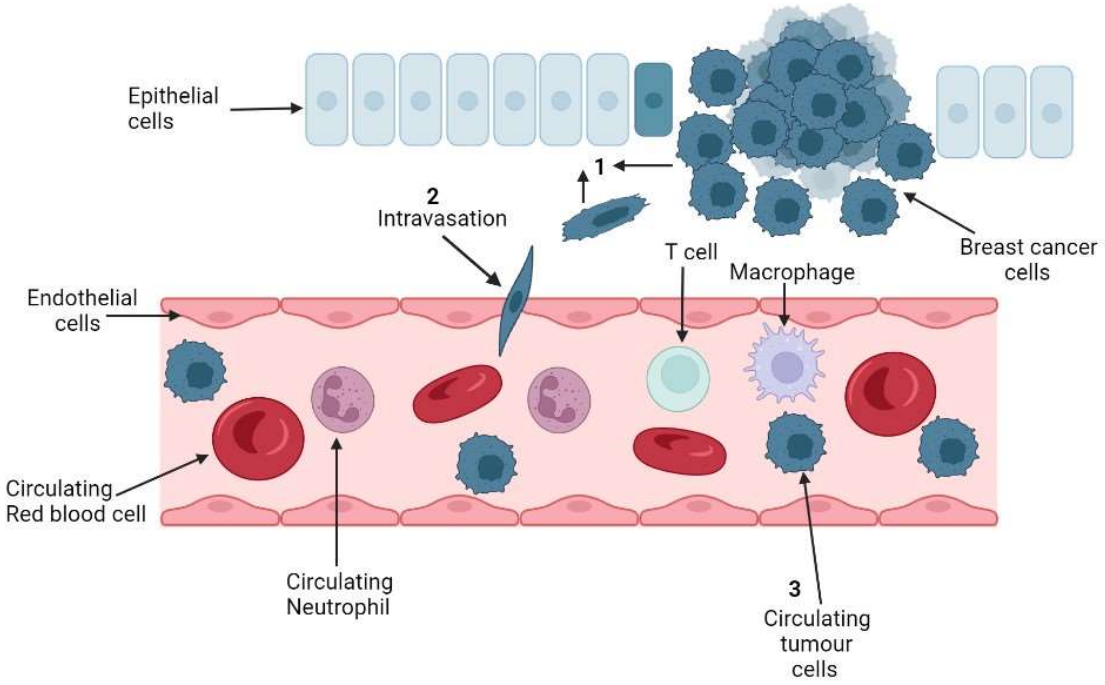

Figure 1: Dissociated breast cancer cells intravasate into the bloodstream and circulate. 1. Dissociated BC cells, 2. Intravasation, and 3. Circulating tumour cells (created in BioRender.com).

Tumour cells undergo all these steps while simultaneously evading apoptotic signals and the host immune response. The successful infiltration of tumour cells into secondary sites is dependent on the completion of these steps [37]. Additionally, the process is repeated to induce further metastasis. Evidence suggests that EMT plays a crucial role in tumour progression when it comes to invasion and intravasation into the bloodstream and extracellular matrix (ECM) degradation. CTCs are rare in healthy individuals and are therefore used as markers to detect cancer metastasis and are important for predicting clinical outcome [37].

1.3 Tumour microenvironment

The tumour microenvironment (TME) consists of various cell types such as endothelial progenitor cells, hematopoietic cells, and carcinoma-associated fibroblasts to name some, which are present in the same environment as the tumour cells. Also, in the TME are inflammatory cells that arrive early in the TME during tumour development, and their crosstalk determines tumour progression or antitumor immunity [38-39].

1.3.1 Immune response to tumours

Antigens bound on the major histocompatibility complex class I (MHC I) of tumour cells are recognized by antigen-presenting cells (APCs) such as dendritic cells (DCs). DCs migrate to the lymph node and present the antigen to cytotoxic CD8+ T cells, resulting in activation, differentiation and clonal expansion of these cells. The cytotoxic (CD8+) T lymphocytes, leave the lymph node, enter circulation, migrate to sites if inflammation, including TME, and infiltrate the TME to secrete granzymes and perforin that damages the tumour cell membranes resulting in cell lysis [17,40-41].

Helper T cells (CD4+ T cells) recognize antigen peptides bound to major histocompatibility complex class II (MHC II) expressed by APCs. Subsequently, CD4+ T cells release cytokines that assist DCs to activate CTLs which will stimulate a cytotoxic response. Furthermore, effector CD4+ T cells differentiate into helper T cell type 1 (Th1) that stimulates a cytotoxic response or an antibody immune response. Th1 cells secrete cytokines including interferon-gamma (IFNγ), transforming growth factor β (TGFβ), tumour necrosis factor-alpha (TNFα) and interleukin 2 (IL-2). Except for TGFβ, all these cytokines are pro-inflammatory cytokines that play a role in CTL activation, enhance tumour antigen presentation, stimulate proinflammatory macrophage (M1) and natural killer (NK) cell antitumour activity, and regulate T cells and antibody production by B cell. Subsequently, cytokines such as TNFα trigger the production of interleukin 1 (IL-1), interleukin 6 (IL-6) and interleukin 8 (IL-8). Each of these interleukins engages in various roles including enhancing lymphocyte and monocyte binding to endothelial cells (ECs), increases B cell activation and T cell proliferation, activates neutrophils to produce prostaglandins and induce differentiation of B cells into plasma cells [40-41]. Taken together, these cytokines oppose tumourigenesis [41].

Effector CD4+ T cells also differentiate into helper T cell type 2 (Th2) which stimulates an anti-inflammatory response. Th2 cells produce cytokines such as interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13) and TGFβ that promote tumour progression. These cytokines suppress M1 polarization while inducing anti-inflammatory macrophage (M2) polarization, stimulate M1 to M2 transition of tumour-associated macrophages (TAMs) and promote the metastasis of mammary cancer cells into the lungs [39,41]. Furthermore, by producing immunoglobulins and cytokines, chronically activated B cells promote the accumulation of innate cells such as DCs, NK cells and macrophages in the neoplastic stroma. When these

innate cells become polarized, as during chronic inflammation, a rich proangiogenic and protumoral microenvironment is provided [41]. Additionally, IL-6, IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) decreases macrophage cytotoxicity, promoting tumour progression by preventing antigen presentation by macrophages and monocyte differentiation into DC [41]. Myeloid cells, cancer cells and T cells secrete TGFβ which is an important regulator of EMT and metastasis [39]. Regulatory T cells (Treg) are other anti-inflammatory cells that function to prevent autoimmune diseases by repressing self-reactive cells. Treg inhibits the cell-to-cell contact ability of CTLs, DCs, NK cells and B cells, therefore, inhibiting immunosuppression and promoting cancer progression [17,41]. Anti-inflammatory cytokines stimulate pro-tumour B cell responses and accelerate tumour progression by suppressing anti-tumour activity [41].

1.3.2 The complexity of the TME

Cancer is described as a "wound that never heals" because tumours are in a chronic state of inflammation, similar to chronic wounds [42]. However, the immune response is similar to that of wound healing pathways except that it is abnormal [43]. Abnormal refers to tumour stroma forming as a result of activation of abnormal wound healing pathways [43]. The immune response to cancer is highly complex, with some immune factors promoting pro-inflammation while other factors promoting an anti-inflammatory response. These opposing responses are crucial for adequate wound-healing mechanisms that stem from mechanical injuries. Proinflammatory cells are necessary for tumour cell destruction; however, high levels of these cells cause chronic wounds [44].

The immune response abnormality may in part be due to the tumour cells producing immunosuppressants that suppress the immune response, causing an immune tolerance that permits tumour cells to spread to adjacent tissues [40]. Some of the immunosuppressive factors that tumour cells produce include cytokines such as TGFβ, IL-10, and cell types, such as TAMs, Tregs and tumour-associated fibroblasts. In the TME, TAMs are the most abundant infiltrating cells and can transform between M1 and M2 phenotypes [45]. The secretion of IL-4, IL-10 and TGF-β by tumour cells, polarizes TAMs into an M2 phenotype in the TME [45]. TAMS can also secrete molecules such as TNF-α, TGF-β, IL-10 and IL-13 that promote tumour cell invasion, EMT and metastasis [46-47].

Cancer stem cells (CSCs) which can self-renew and differentiate, are present in most solid cancers including breast, brain, ovarian, liver, pancreas and prostate cancers [48-50]. CSCs differentiation increases tumour aggressiveness, accelerating cancer progression [48-49]. CSCs are required for intratumoral heterogeneity and thus are involved in tumour onset, expansion, relapse, metastatic spread, and therapeutic resistance [48-49]. In the TME, various cell types such as cancer-associated fibroblasts (CAFS), mesenchymal stromal/stem cells (MSCs) and exomes secrete factors that maintain and promote CSCs phenotypic transition [48-49]. However, the transition mechanism between non-CSCs and various CSC subsets is still poorly understood [49].

MSCs are found throughout the body and can differentiate into multiple stromal cell lineages [50]. In response to the production of IL-10, vascular endothelial growth factor (VEGF) and GM-CSF by tumour cells, MSCs infiltrate the tumour and secrete fatty acids and exosomes into the TME thus reducing anti-tumour T-cell activity in BC by increasing chemoresistance [50]. Moreover, MSCs support tumour growth through various mechanisms such as suppressing the immune response, CSCs enrichment, differentiate into pro-tumorigenic stromal components, and promotes tumour cell survival, EMT, angiogenesis and metastasis [51]. Moreover, driven by cytokines, MSCs can transdifferentiate into a M2-like phenotype or M2 macrophages [52]. Additionally, exosomes are used by tumours to connect to MSCs and re-program their functional profile from trophic to pro-tumorigenic. This is achieved when exosomes interact with MSCs surface receptors and are absorbed by MSCs. This interaction leads to molecular, transcriptional and translational changes converting MSCs into producers of factors required for tumour growth [52]. All these anti-inflammatory factors skew TME towards an immunosuppressive and anti-inflammatory state [53]. However, contradictory reports have shown that MSCs also have anti-tumorigenic functions such as encouraging immune response, cellular signalling regulation, angiogenesis inhibition and promoting tumour cell apoptosis [51]. However, the majority of studies support a pro-tumorigenic effect on MSCs [51].

Some anti-inflammatory factors appear to support both the progress and inhibition of tumour cells, such as TGFβ which is an anti-inflammatory growth factor that encourages tumourigenesis but also inhibits early tumour development and epithelial cell differentiation [39,54]. TGF-β play an essential role in other crucial pathways involved in tumorigeneses such as

the PI3K/AKT, Ras, Wnt, IFN-γ, TNF-α, Hedgehog, Hippo and Notch pathways [55]. The GM-CSF activates phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) which results in cell lysis through complement activation [40].

Tumour cells that secrete cytokines suppress the immune system, and the dual functionality of some of these cytokines contributes to the TME's complexity. Several cancer types have cures, while other cancers such as BC have no current cure due to its complex nature. However, some treatments can eradicate, ease the severity, or slow down tumour progression.

1.4 Treatments

There are several treatments for BC such as mastectomy or breast-conserving surgery, tumourectomy, chemotherapy, hormone therapy and radiotherapy. These therapies have increased the 5-year relative survival rate of American women from 74.8% to 90.3% from years 1975 to 1977 and 2003 to 2009 respectively [56]. However, metastatic BC remains incurable [57]. Treatment is dependent on the type or subtype of BC a patient has been diagnosed with. Treatment options for TNBC are limited because of the lack of expression of ER, HER2 and PR [19,58]. Nonetheless, the main treatment for TNBC is chemotherapy, combined with the angiogenesis inhibitor bevacizumab, or neoadjuvant chemotherapeutic drugs [19,58]. The United States National Comprehensive Cancer Network (NCCN) guidelines suggest chemotherapeutic combinations based on anthracycline, cyclophosphamide, taxane, fluorouracil and cisplatin. For example the following combinations are currently used to treat BC; a combination of docetaxel + cyclophosphamide (TC), adriamycin + cyclophosphamide (TAC), fluorouracil + methotrexate + cyclophosphamide, paclitaxel + fluorouracil + epirubicin + cyclophosphamide, and taxel/docetaxel + adriamycin + cyclophosphamide (TAC) [19]. All these are the favoured TNBC adjuvant regimens [19]. Taxanes inhibit microtubule depolymerization by causing spindle fibres not to form during mitosis thus inhibiting cell division and inducing apoptosis [19,58]. Anthracyclines are antibiotics derived from *Streptomyces peucetius*, a class of chemotherapeutic drugs that decrease the risk of relapse and mortality rate in 25-30% of BC patients [19,58]. Cyclophosphamide is converted in the liver to aldophosphamide and is activated in tumour cells by cytochrome P450 to produce acrolein and nitrogen mustard with alkylating activity [19]. Nitrogen mustard is cytotoxic to tumour cells [19]. Thymidylate phosphorylase in the

tumour catalyses capecitabine which transforms into cytotoxic 5-Fluorouracil (5-Fu) [19]. Cisplatin is a platinum agent that prevents replication fork formation and produces double-strand breaks by generating inter- and intra-strand double strand DNA crosslinks [58]. Capecitabine is used as a treatment for metastatic or advanced BC in combination with paclitaxel chemotherapy [19]. Immunotherapy drug atezolizumab has been used in combination with microtubule stabilizers paclitaxel to block PD-L1 in BC [19]. Surgery and radiotherapy are also used as standard treatment strategies [58] TNBC has high heterogeneity thus treating this subtype is challenging and an increasing number of BC patients have developed resistance to chemotherapy drugs such as taxanes and anthracyclines, which are used to for high risk patients and are standard treatments for early-stage TNBC patients [19,58]. Hence, new treatment strategies are needed to improve patient outcomes and eliminate residual TNBC.

Treatment for HER2 positive BC includes trastuzumab and lapatinib, which are used for early-stage BC and have been shown to significantly reduce the risk of relapse [59-60]. However, there is a potential risk for heart and lung damage [59]. Trastuzumab is a monoclonal antibody that binds to the extracellular domain (ECD) of the HER2 receptor. The anti-tumour mechanisms include inhibiting cleavage of HER2 ECD, inhibiting dimerization of ligand-dependent HER receptor, inhibiting downstream signal transduction pathways, triggering cell cycle arrest, angiogenesis inhibition, DNA repair interference, and triggering apoptosis [59-60]. Lapatinib is the other treatment approved for HER2-positive patients with advanced BC [59]. Lapatinib inhibits HER2 and HER1 intracellular tyrosine kinase activity, suppressing tyrosine autophosphorylation and thus downstream pathways such as PI3K/Akt Akt (phosphoinositide 3-kinase/Ak strain transforming) and MAPK/Erk1/2 (mitogen-activated protein kinase/ extracellular signal-regulated kinase) [59-60]. The neoadjuvant drug pertuzumab in combination with trastuzumab resulted in a higher event-free survival rate in BC patients than either drug alone [61]. Cyclophosphamide (TC) is another commonly used neoadjuvant chemotherapy [19]. These drugs have improved the overall survival of patients but numerous patients develop resistance and thus do not benefit from these agents [60].

Luminal BC treatments include radiation, surgery, and chemotherapy [22]. Luminal A is treated with endocrine therapy that targets oestrogen receptors or oestrogen synthesis through inhibiting activation of Pi3K/ signalling pathways [22,62]. For luminal B advanced or metastatic BC, Lapatinib B is used in combination with an aromatase inhibitors [22]. Furthermore,

combining tamoxifen with luteinizing hormone-releasing hormone improved disease-free survival in patients with luminal B subtype but not the luminal A subtype [63]. Additional treatment for luminal B includes cixutumumab, dalotuzumub, and MK-0646 which targets the insulin growth factor pathway, MK-2206, XL-147 and XL-765 which inhibit the PI3k/AKT pathway, and TKI-258 and ADZ-4547 that target the fibroblastic growth factor pathway [64].

Traditional treatments are successful with early diagnosis, a good prognosis and a decreased risk of recurrence [65]. However, these treatments are non-discriminatory and also target healthy cells, not just cancerous cells. Additionally, these treatments are not effective for advanced and metastatic BC [66]. These limitations warrant a different therapeutic approach that targets only cancerous cells such as immunotherapy and cell therapy [66-67]. There are several immunotherapies such as antibody-based immunotherapy, cancer vaccine immunotherapy, adoptive T cell transfer immunotherapy, and T cell transfer immunotherapy. These therapies elicited an immune response that targeted only cancer cells [66]. Immunotherapies such as checkpoint antagonists have been approved for numerous cancers including programmed death ligand-1 (PD-L1) and cytotoxic T lymphocyte-associated antigen (CTLA-4) which may be beneficial for metastatic BC [17,68].

2-Methoxyestradiol (2-ME) and MSCs are two treatments that have been shown to have an effect on BC. MSCs are a cellular therapy and 2-ME is an anti-cancer agent.

1.4.1 2-Methoxyestradiol

2-ME is an anti-cancer agent that has been demonstrated both *in vitro* and *in vivo* to have anti-angiogenic and anti-proliferative properties [69]. 2-ME is a natural endogenous steroid that is a metabolite of 17β-estradiol (E2) [70-71]. E2 is generated by O-methylation of estradiol at the 2-position and sequential hepatic hydroxylation [72-73]. 2-ME is produced by catechol-O-methyltransferase and cytochrome P450 enzymes. Catechol-O-methyltransferase (COMT) transfers a methyl group to 2-OH or 4-OH groups from cofactor S-adenosyl methionine catalysing O-methylation of catecholestrogens to form 2-ME and 4-methoxyestradiol [73]. Cytochrome P450 hydroxylation yields either 2-, 4- or 16 α-hydroxyestradiol oestrogens provided that C-2, C-4 or C-16 are hydroxylated [73]. COMT is expressed in multiple tissues such as the brain, kidneys, liver and erythrocytes and it catalyses while cytochrome P450 is found in the liver [73-74]. Generally, oestrogens are crucial regulators for cell survival, cell proliferation

and differentiation in various tissues and organs, however, 2-ME is an anti-angiogenic and anti-proliferative molecule that induces apoptosis of actively dividing cells *in vitro and in vivo* [74]. This drug targets dividing cells and spares quiescent cells [75]. By targeting actively dividing cells, 2-ME displays a unique mechanism of action that distinguishes it from conventional oestrogens.

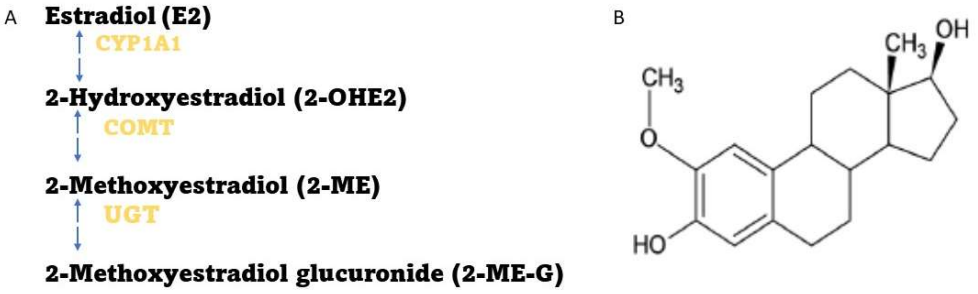

Figure 2: 2-ME metabolic pathway and chemical structure: (A) E2 undergoes oxidation by cytochrome P450 1A1 (CYP1A1) to form 2-hydroxyestradiol (2-OHE2). Subsequently, 2-OHE2 is methylated by COMT, leading to the generation of 2-ME. Further, metabolism of 2-ME can occur via UDP-glucuronosyltransferase (UGT), resulting in the production of 2-methoxyestradiol glucuronide (2-ME-G). The predominant metabolite of 2-ME, 2-ME-G, is subsequently excreted in the urine. (B) Chemical structure of 2-ME (Copied *from Mothibeli et al)* [76].

1.4.2 Mesenchymal stem/stromal cells

Cellular therapy is another alternative treatment for BC. MSCs are heterogenous cells that resemble fibroblastic cells [77-78]. These cells are commonly isolated from adipose tissue, bone marrow, and the umbilical cord [77]. MSCs can also be isolated from other tissues such as blood, dental pulp, skin, muscle, cartilage and amniotic fluid [77]. MSCs can differentiate into adipocytes, osteoblasts, chondroblasts and fibroblasts *in vitro* [78]. To be considered MSCs, they must exhibit plastic adherence, have the ability to self-renew but also to differentiate into adipocytes, osteoblasts, and chondrocytes, and express the following surface markers: CD73, CD90, and CD105, while lacking the expression of CD11b, CD14, CD19, CD79, and the human leukocyte antigen - DR isotype (HLA-DR) [77-78]. Mouse MSCs express Sca-1, CD29, CD44 and CD105 and lack the expression of CD11b, CD34, CD45 and TER-119 [79].

MSCs “home” to sites of injury and elicit an immune response to assist in the healing process of chronic wounds [80]. For this reason, MSCs have been used to treat bone, spinal cord, and neural injuries successfully in preclinical studies and clinical trials involving patients [81-83]. Furthermore, *in vivo* studies demonstrated that ASCs may be used to treat multiple sclerosis,

glioblastoma, acute kidney injuries, chronic kidney failure, osteoarthritis, obstructive pulmonary disease, myocardial infarction, inflammatory bowel syndrome and ischemic stroke [84]. MSCs have been identified as a potential delivery vehicle for anti-cancer agents due to their ability to migrate to sites of injury and TME [85]. In the TME, MSCs elicit an immune response, as well as anti- and pro-tumorigenic activities [86]. As previously mentioned, there are contradictory reports on the effect of MSCs in the TME and these reasons were reported by Oloyo *et al.* [87]. *In vivo* studies were conducted using xenogeneic models in which human MSCs are combined with BC cells and injected into an immunocompromised mouse strain. Cross-species experiments can increase genetic variability, resulting in contradictory results. Furthermore, the differences between BC cells with varying mutations and the different strains of mice used that are genetically different, add to the molecular complexity, contributing to the varying results.

Given the above, in this study aimed to investigate the influence of 2-ME and MSCs on tumorigenesis and metastasis *in vivo* using a mouse model of spontaneous mammary carcinoma. The objectives are as follows:

- To assess the impact of mASCs and 2-ME derivatives on the progression rate, volume, and mass of primary mammary tumours in transgenic mice.
- To quantitatively assess the pulmonary lesion burden in transgenic mice.
- To perform comprehensive histopathological analysis of mammary and metastatic tumours.
- To investigate the extent of infiltration of CD163 M2 macrophages and CD3 lymphocytes within the TME in both mammary and lung tissues.
- To explore the plasma cytokine profile of mice with and without tumour metastasis.

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Chapter 2

This chapter has been presented in the final manuscript version submitted for publication (appendix E)

Effect of adipose-derived mesenchymal stromal/stem cells on mouse mammary tumour growth and formation of lung metastases

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Abstract: Background: The role of mesenchymal stromal/stem cells (MSCs) in tumour development and progression remains a subject of debate. Previous studies have reported contradictory outcomes, possibly due to variations in experimental design and the use of xenograft models. Xenograft models limit interpretation and translation due to cross-species variability. To address these limitations, an isogenic mouse model of spontaneous breast cancer (BC) was employed to investigate the impact of murine MSCs on BC development and progression. Methods: MSCs isolated from FVB/N mouse adipose tissue (mASCs) were administered to female mice with palpable mammary tumours. Tumour volume and mass were assessed, and analysis of histopathological necrosis and gene expression was conducted on mammary (MT) and lung metastatic tumours (LT). Results: No change in MT mass and volume was observed between mASC-treated and control mice. However, mASC treatment led to increased necrosis in LT but not in MT. Immunohistochemistry revealed that mASC-treated mice had fewer CD163+ anti-inflammatory macrophages in the LT but not in the MT. Tgf-β3, vegfr1 and cd105 were observed and downregulated in both MT and LT in mASC-treated mice. The downregulation of cd36 and tgf-β3 contributes to pro-tumourigenic activities whereas the downregulation of vegfr1 and cd105 is associated with an anti-tumour effect. In the mASC treatment group, all cytokines tested for, except IL-27, were elevated. Conclusion: This study suggests that mASCs are anti-tumourigenic in pulmonary metastatic BC. Our findings emphasize the importance of considering the tumour microenvironment and employing relevant animal models when investigating the impact of MSCs on tumour progression.

Keywords: Mesenchymal stem cells, breast cancer, tumour growth, tumour progression, metastasis, in vivo

1. Introduction

Breast cancer (BC) is the most prevalent cancer in females and the leading cause of cancer deaths [1, 2]. In 2020, 2.3 million new female BC cases were diagnosed worldwide with over six hundred thousand deaths recorded. BC accounts for 1 in 6 cancer mortalities and 1 of every 4 cancer cases in women [2]. Current BC treatments, such as mastectomy or breast-conserving surgery, tumourectomy, chemotherapy, hormone therapy and radiotherapy, have all increased the 5-year relative BC survival rate [3]. However, these treatments are non-discriminatory as they also target healthy cells, in addition to not just cancerous cells. Furthermore, these treatments are not effective against advanced and metastatic BC [4]. It is thus clear that improved treatments are needed, especially treatments that

selectively target cancerous cells, such as cellular and immunotherapy [4]. Mesenchymal stromal/stem cells (MSCs) are a heterogenous population of cells that morphologically resemble fibroblastic cells [5-7], and can be isolated from most adult tissues, with adipose tissue (ASCs), bone marrow (BM-MSCs) and umbilical cord (UC-MSCs) being the most frequently used tissue sources [5]. Multipotent MSCs have the capacity to self-renew and are able to differentiate into adipocytes, osteoblasts, chondrocytes and other cell types [5, 6]. Due to the immunomodulatory and regenerative properties associated with these cells, *in vivo* studies have investigated the use of these cells to treat multiple conditions such as sclerosis, glioblastoma, acute kidney injuries, chronic kidney failure, osteoarthritis, obstructive pulmonary disease, myocardial infarction, inflammatory bowel syndrome and ischemic stroke [8]. MSCs are reported to "home" to the tumour microenvironment (TME) where they elicit an immune response that either promote (pro-tumorigenic) or suppress tumour (anti-tumorigenic) progression [9].

Adipose tissue is rich in MSCs, containing 500-fold more of these cells per gram of tissue than MSCs present in a similar volume of bone marrow [10]. Breast tissue is mainly composed of adipose tissue (fat cells) and serves as an important endocrine organ by secreting signalling molecules that regulate various cellular processes [10]. The secretory profiles of breast adipocytes of BC patients and healthy controls differ and it has been suggested that growth factors and other signalling molecules secreted by breast adipose tissue contributes to BC development and progression [11].

Numerous studies, both *in vitro* and *in vivo*, have demonstrated that MSCs from sources other than adipose tissue promote BC progression [12-15]. As mentioned earlier, adipose tissue is rich in ASCs. Several studies indicated that ASCs promote BC cell proliferation, migration and invasion [16-19]. Furthermore, several *in vivo* studies have demonstrated that ASCs induce primary tumour growth, epithelial to mesenchymal transition (EMT), angiogenesis and metastasis [8, 20-23]. ASCs secrete several cytokines, such as IL-6, IL-8 and VEGF, IL-10, TGFβ-1, MMPs, chemokine ligand 2 (CCL2) and CCL5 resulting in elevated levels of these cytokines in the TME and which are suggested to stimulate BC progression [18, 24-28]. Furthermore, metastatic lesions are often observed in the lungs and occasionally in the liver and spleen of BC patients [8, 21, 22]. Knockdown of leptin expression in obesity-altered ASCs (obASCs) in SCID mice decreased primary tumour volume and significantly reduced the number of metastatic lesions in the liver and lung [29, 30]. It also has been suggested that mutations in BC associated genes such as BRCA1 in ASCs promotes BC invasion and growth [27]. Mouse adipose mesenchymal stem cells (mASCs) have been observed to promote tumour growth and metastasis through increased secretion of insulin-like growth factor-1 [8, 20].

In contrast, numerous studies have suggested that ASCs inhibit BC progression [31-39], and ASCs significantly decrease BC cell proliferation, promote BC cell apoptosis, reduce BC cell invasion and tumour migration, reduce tumour mass and slow tumour growth rate [34, 35, 40]. Using immortalized BC cell lines (MDA-MB-231 and T47D), Clark et al. (2015) [39] observed *in vitro* that ASCs inhibit BC cell migration and invasion through the secretion of tissue inhibitor of metalloproteinases inhibitors, TIMP-1 and TIMP-2. ASCs also inhibit BC by downregulating EMT genes such as *TWIST1, CDH2, Snail1* and *Snail2* [37]. Exosomes produced by ASCs have been associated with decreased BC cells viability [40]. In summary, there is currently no consensus regarding the effect of ASCs on BC progression and more studies are needed to understand the interactions between ASCs and BC cells [27].

The contradicting findings of the effect of ASCs on BC tumour progression have been extensively reviewed by Oloyo et al. (2017 [41], who concluded that the significant variation in experimental design/approaches is one of the major contributors to the different outcomes observed. Study approaches range from using either primary human-derived ASCs isolated from mammary tissue or lipo-aspirates, human-derived ASCs isolated from breast tissue from mastectomies of BC patients [17, 22, 27, 36, 38], or mouse-derived primary ASCs [42-44] to investigate the effect of ASCs on human-

derived immortalized BC cell lines, such as MCF-7, MDA-MB-231, ZR-75-1, T47D, BT-474, CG5, SK-BR-3, HCC1937 and MDA-MB-435 [18, 19, 22, 23, 31, 36] or murine breast carcinoma cells such as E0771, 4T1 and Met-1 [8, 20].

*In vivo, b*oth xenograft and allograft experimental models have been used to investigate the effect of ASCs on BC development, progression and metastasis. Allograft experimental models have investigated the effect of mASCs on mouse mammary adenocarcinoma cells [20, 34, 35], while xenograft experimental models utilized human BC cells and human ASCs in experimental animal models [18, 19, 22, 23, 31, 36]. Moraes et al. (2016) and Li et al. (2020) used an allograft experimental design whereby BALB/c and C57BL/6J mice developed tumours after receiving 4T1 and E0771 BC cell lines injected into their fat pad. A week later these animals received mouse ASCs (mASCs) derived from gonadal adipose tissue and inguinal fat pads from C57BL/6J mice. The mice received ASCs with either $CD90^{high}$ or $CD90^{low}$ extracellular vesicles (EVs). In comparison to ASCs-$CD90^{high}$, ASCs-$CD90^{low}$ resulted in significantly reduced tumour mass and slower tumour growth rate [34, 35]. These studies demonstrated that ASCs (directly or indirectly) inhibits BC progression.

Investigating the impact of mASCs on BC progression and metastasis using an isogenic mouse model of spontaneous mammary tumour development is believe to yield more physiologically relevant observations that could result in improved translational to the human setting. This more accurately recapitulates what might occur in patients who incidentally have a small or latent tumour and who might receive autologous ASCs for a variety therapeutic purpose, unrelated to tumorigenesis per se, for example for regenerative medicine indications.

The effect of mASC treatment on BC progression and metastasis was investigated using an isogeneic FVB/N-Tg(MMTV-PyVT)634Mul/J mouse model. These animals contain the MMTV-PyVT (mouse mammary tumour virus- polyoma virus middle T antigen) transgene which induces spontaneous development of primary mammary tumours [45]. The mouse model used in this study demonstrates a progression of mammary gland tumours that mimics the stages of human ductal BC. These tumours exhibit similarities to luminal B subtype of human BCs, as revealed by gene expression profiling. Additionally, they share histopathological characteristics and express basal-like markers, resembling aggressive basal-like BC in humans [45]. Genes involved in tumour invasion, angiogenesis, and metastasis such as *cd36*, endoglin (*cd105*), transforming growth factor- beta 3 (*tgf-β3*), vascular endothelial growth factor receptor 1 (*vegfr1*) and metadherin (*mtdh*) [46-51] were also investigated. Immunohistochemical analysis of CD3 and M2-associated (CD163-positive) macrophages was likewise performed.

2. Materials and Methods

2.1. Animal studies

The study was approved by the Faculty of Health Sciences Research Ethics Committee (ethics reference no.: REC166-19) and the Animal Ethics Committee (ethics reference no.: 534/2019) of the University of Pretoria. Animal husbandry was conducted at the Onderstepoort Veterinary Animal Research Unit (OVARU). FVB-TgN(MMTV-PyVT) mice were purchased from Jackson Laboratory (The Jackson Laboratory; Bar Harbor, ME, USA) and were used for breeding and isolation of mASCs. To obtain heterozygous offspring, hemizygous males were bred with wild-type females. The resulting offspring were genotyped, and the heterozygous females were recruited into the study while the heterozygous males were used for breeding. A total of 20 heterozygous female mice (n = 10 for mASC-treatment group and n = 10 for control group) were used for this study.

2.2. Genotyping

The KAPA Mouse Genotyping Kit (KAPABIOSYSTEM, Cape Town, South Africa) was used for genotyping according to the manufacturer's instructions. Briefly, DNA was extracted from 2 mm mouse tail biopsies and placed in 0.2 mL microcentrifuge tubes. The forward primer 5-' CAAATGTTGCTTGTCTGGTG-3' and reverse primer 5'-GTCAGTCGAGTGCACAGTTT-3' specific for internal positive control and the forward primer 5'- GGAAGCAAGTACTTCACAAGGG-3' and reverse primer 5'- GGAAAGTCACTAGGAGC GGG-3' specific for the transgene was used in the PCR genotyping experiment. The two pairs of primer sequences were obtained from Jackson Laboratory website (The Jackson Laboratory; Bar Harbor, ME, USA). Amplification was done using a thermocycler (GeneAmp® PCR System 9700) for 35 cycles under the following conditions: Initial denaturation at 95˚C for 3 minutes, denaturation at 95˚C for 15 seconds, annealing at 60˚C for 15 seconds, and extension for 15 seconds for 2 minutes. The amplicons were separated on a 2% agarose gel containing ethidium bromide using electrophoresis. The purpose was to determine their size, and this was achieved by running the gel alongside a molecular marker (FastRuler™ low range DNA ladder (Thermo Scientific)) with a molecular mass ranging from 50 bp to 1500 bp. The electrophoresis process was conducted at 120V for 30 minutes.

2.3. mASC isolation and *in vitro* expansion

The inguinal white adipose tissue (ingWAT) excised from wild-type FVB/N mice under sterile conditions, was placed in a tissue culture dish and minced in a biosafety cabinet (ESCO, BSC class II). The minced tissue was transferred into 30 mL digestion medium (pre-prepared) that constituted of 0.8mg/mL collagenase II (Gibco,ThermoFisher, MA, USA,) dissolved in tissue medium ((1% fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich, Darmstadt, Germany)) dissolved in Hanks' balance salt solution (Sigma-Aldrich, Darmstadt, Germany), and placed in a water bath at 37˚C for 45 minutes (vortexed every 5 minutes). The digested tissue was then filtered through a 200 µm nylon mesh into a 50 mL tube containing 10 mL complete culture media (CCM; 20% foetal bovine solution (FBS) (Gibco, ThermoFisher, MA, USA), 2% Pen/Strep (Gibco, ThermoFisher, MA, USA), 1% glutamine (Sigma-Aldrich, Steinheim, Germany) and 0.2% amphotericin (Sigma-Aldrich, Steinheim, Germany) in DMEM/F-12 (Lonza, Whitesci, Switzerland). The filtrate was centrifuged (SL-16R, Thermo Scientific) at 500 g for 7 minutes after which the supernatant was aspirated leaving behind the pellet. The mASC pellet was resuspended in CCM, plated at $5x10^3$ cells/cm^3 in 75 cm^3 culture flasks and placed in a 37˚C/5% carbon dioxide (CO_2) incubator (Labotec, Thermo Scientific). The cell culture medium was changed twice a week and cells were passaged when they became confluent. At passage 5, mASCs were cryopreserved by resuspending dissociated cells in freezing medium (70% FBS, 20% Dulbecco's modified Eagle medium/F12 (DMEM/F12) supplemented with 10% dimethyl sulfoxide (DMSO)), transferred to cryovials (Lasec, Greiner Darmstadt, Germany) and stored in liquid nitrogen vapor (Statebourne biorack 4800, Thermo Scientific, Washington, UK). To thaw the mASCs, 700 µL of FBS was added to the cryovials and centrifuged at 500g for 7 minutes and then plated at $5x10^3$ cells/cm^3 in 75 cm^3 culture flask. The mASCs used for this study were at passages 6 to 8.

2.4. Characterisation of mASCs

The immunophenotypic profile and adipogenic and osteogenic differentiation capabilities of the mASCs were determined before the cells were used in *in vivo* experiments.

2.4.1. Flow cytometric analysis of mASCs

mASCs were immunophenotyped at passage 3 using a Cytoflex flow cytometer (Beckman Coulter, Florida, USA). Immunophenotyping was done as previously described with some modifications [52]. Briefly, mASCs were washed twice using PBS and dissociated by adding 7 mL trypsin (GIBCO, Life Technologies™, New York, USA), followed by incubation for 4 minutes in a 37 °C/5% CO_2 incubator. An equal volume of CCM was added to the dissociated cell suspension followed by centrifugation at 500g for 7 minutes. An aliquot of the cell suspension (100 µL) was transferred to a flow cytometry tube after which 5 µL of each antibody was added to cells and incubated for 15 minutes in the dark. The

anti-mouse antibodies used to stain the cells were CD45-Brilliant Violet 421 (30-F11) (Biocom, Biolegend, San Diego, CA, USA), CD90.2-APC (53-2.1), CD31-PE (390), CD29-FITC (HMb1-1), CD105-PE-C7 (MJ7/18) and CD106-PE-C7 (429) purchased from eBioscience, Invitrogen (San Diego, CA, USA). The cells stained with CD105 were processed in a separate tube because this marker has the same fluorochrome as CD106. The data was analysed using Kaluza Flow Cytometry analysis software 1.2 (Beckman Coulter, Miami, USA).

2.4.2 Adipogenic and osteogenic differentiation

For adipogenic differentiation, mASCs were plated at a density of 2000 cells/cm³ in a 6-well plate. The cells were differentiated into the adipogenic lineage as previously described [52] with slight modifications. Briefly, CCM was added to cells at passage 3 to serve as non-induced controls (3 wells); adipogenic induction cocktail consisting of Dulbecco's Modified Eagle's Medium (GIBCO, Life Technologies™, New York, USA) supplemented with 20% FBS, 2% Pen/Strep, 10 µg/mL insulin (Gibco, ThermoFisher, MA, USA), 0.5 mM 3-isobutyl-methlyxanthine (IBMX), 5 mM dexamethasone, 200 µM indomethacin. Indomethacin, IBMX and dexamethasone were purchased from Sigma-Aldrich, Darmstadt, Germany. After differentiation period of 21 days, cells were stained using 2.5 µg/mL 4', 6-diamino-2-phenylindole, dihydrochloride (DAPI) (Life Technologies, Oregon, USA) and 50 ng/mL Nile red (Life Technologies, Oregon, USA). Images for were captured at 10X magnification using a ZEISS Axio Vert.A1 inverted microscope (Carl Zeiss, Gottingen, Germany).

For osteogenic differentiation, 2000 cells/cm³ were plated in a 6-well plate at passage 3. The differentiation procedures were performed as described by Seavey et al. [53], with the exception that cells were differentiated for 21 days instead of 14 days as described by the authors. Osteoblast staining was performed as described by Koch et al. 2007 [54], with a few minor modifications. In summary, the culture media was removed, and 10% formalin was added to the cells followed by incubation for 1 hour in at room temperature (RT). After fixation, the cells were stained with 2% alizarin red for 45 minutes at RT. The solution was aspirated, and wells washed 4 times with dH_2O, and 1 mL PBS was added.

2.5. mASCs treatment and tumour measurements

Experiments done on both control (untreated) and treatment groups were performed on 10 mice each. Each mouse in the mASCs treatment group received $2x10^6$ mASCs suspended in 100 µL 0.9% SABAX saline solution (Adcock Ingram, South Africa) through intraperitoneal injection (IP) on days 30 and 37 (from time of birth). On day 44, the mice received $1.6x10^6$ mASCs; the adjustment was due the number of mASCs available and was made to ensure that all animals received the same number of cells. Each mouse in the treatment group thus received a total number of $5.6x10^6$ mASCs over a period of 44 days. Mice in the control group received 100 µL of saline solution at each time point; the administration route was the same. Palpable primary tumours were measured once a week until termination, using a calliper to determine tumour volume. Volume was calculated using the formula $L \times W^2 / 2$; L (length) and W (width). At termination, the mammary tumours were excised and weighed (Sartorius, Göttingen, Germany) to determine the tumour mass [in grams (g)] per animal.

2.6. Histology and Immunohistochemistry

Mammary and lung tissues were collected from mice in both (control and mASCs treated) groups after they were euthanized and fixed in 10% neutral buffered formalin (NBF). Haematoxylin and eosin staining was performed as previously described by Dhanraj et al. 2021 and Pitere et al. 2022 [55, 56]. To visualize CD163-positive macrophages, the tissues were processed and stained, with minor modifications, using a rabbit monoclonal anti-mouse antibody directed against CD163, as previously described [55, 56]. Briefly, 3-micron sections were cut from formalin fixed paraffin embedded (FFPE) tissue blocks and baked overnight in a 58°C oven. Slides were deparaffinized in xylene, hydrated with decreasing concentrations of alcohol and washed with distilled water. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 5 minutes at 37°C. Antigen retrieval was performed in a high

pH buffer (Dako Envision FLEX Retrieval solution high pH, Agilent Technologies, Denmark), after which the sections were rinsed in phosphate buffered saline (PBS) followed by blocking the background staining with protein block (Novolink™ Leica Biosystems, Newcastle Upon Tyne, UK) for 30 minutes at room temperature to reduce background staining. Sections were incubated overnight at 4°C in a 1:300 rabbit monoclonal anti-CD163 antibody [EPR19518] (ab182422) (Abcam, Cambridge, UK). Slides were once again rinsed in PBS and detection performed using anti rabbit Novolink™ Polymer Detection Kit (Leica Biosystems) for 25 minutes at RT. Slides were once again washed in PBS and chromogen detection performed (4 minutes at 37°C) using 3,3'-diaminobenzidine (DAB) (Novolink™ Polymer Kit). Sections were rinsed and counterstained in haematoxylin for 1 minutes. Dehydration in alcohol, clearing in xylene and mounting in DPX followed. Negative controls were performed substituting the anti CD163 antibody with PBS. For CD3 IHC, 3-micron sections were cut, and the process was performed similarly to CD163 with a few differences. Antigen retrieval was performed in a low pH buffer (Cell Conditioning Solution CC2, Ventana Medical Systems, Inc Arizona USA). Sections were incubated with a 1:100 rabbit monoclonal anti CD3 (Abcam ab16669 clone SP7) antibody at room temperature for 120 minutes. Slides were rinsed in PBS and detection performed using anti rabbit Polymer HRP IgG (Novolink™ Polymer Detection Kit, Leica Biosystems) for 30 minutes at room temperature. Negative controls were performed substituting the CD3 antibody with PBS. Images were captured at 40X magnification using a Leica AT 2 Aperio scanner (Leica Biosystems, Nussloch, Germany) and analyzed using Qupath software, version 0.2.3 (The Queens University of Belfast, Northern Ireland).

2.7. mRNA isolation and RT-qPCR

Total cellular mRNA was extracted from mammary and lung tissues of treated and untreated mice using the E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. The quality of mRNA was determined using the Nanodrop spectrophotometer (Inqaba, Biotec, South Africa). Complementary DNA (cDNA) was generated from mRNA using the SensiFAST™ cDNA synthesis kit (Meridian Bioscience®, USA) according to the manufacturer's instructions and was quantified using a Nanodrop spectrophotometer. TaqMan RT-qPCR was used to determine the expression of the following genes: *cd105* (Mm00468252_m1), *tgf-β3* (Mm00436960_m1), *vegfr1* (Mm00438980_m1), *mtdh* (Mm00482588_m1) and *cd36* (Mm00432403_m1). *gapdh* (Mm99999915_g1) was used as reference gene. For RT-qPCR, 15 µL master mix containing 10 µL TaqMan fast advanced master mix (2X), 1 µL TaqMan assay probe (20X), 4 µL nuclease-free water and 5 µL (30 ng/µL) cDNA (sample template) were added to wells in a 96-well plate. The plate was run in standard mode on a QuantStudio™ 6 Flex Real-time PCR (Applied Biosystems™, MA, USA) under the following conditions for 40 cycles: incubation at 50 °C for 2 minutes, polymerase activation at 95 °C for 10 minutes, denaturation at 95°C for 15 s, extension at 60 °C for 1 minute. The software of QuantStudio 6 and 7 measured the threshold limit (Ct value) and the comparative CT method was used to calculate gene expression fold changes using the formula: ΔCT = CT average mASCs – CT average reference gene, ΔCT = CT average control – CT average for reference gene; ΔΔCT= ΔCT mASCs - ΔCT control; Fold change = $2^{-\Delta\Delta CT}$.

2.8. Measurement of plasma cytokines

Approximately 800 µL of blood was collected in EDTA tubes through cardiac puncture and centrifuged at 14 000 rpm (28 766g) for 15 minutes. Approximately 250-300 µL of plasma was collected from each sample into 2 mL microcentrifuge tubes. The Legendplex mouse inflammation panel (13-plex) kit (Biolegend®, San Diego, CA, USA) was used for cytokine profiling. The assay was performed according to the manufacturer's instructions. The levels of the following 13 mouse cytokines which were determined: Interleukin-23 (IL-23), IL-1α, interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), IL-12p70, IL-1β, IL-10, IL-6, IL-27, IL-17A, IFN-β, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Cytokines levels (present in standards and samples) were measured using the Cytoflex flow cytometer (Beckman Coulter,

California, USA). Standard curves were generated according to manufacturer's instruction. Cytokine concentration levels were determined using the LEGENDples™ data analysis software [version 8.0; BioLegend, San Diego, USA (https://legendplex.qognit.com/)]. All the analysis was done using the Biolegend's data analysis software.

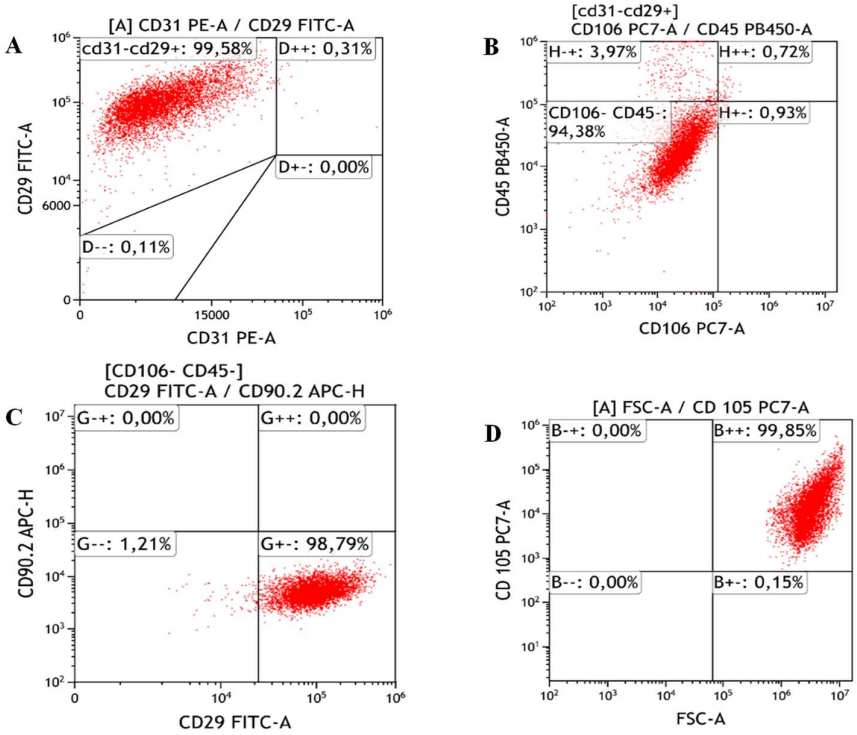
2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5; GraphPad Software Inc., San Diego, CA, USA). Data was expressed as mean ± standard error of mean (SEM). Two-tailed unpaired student T-test was used to compare means between two groups. A two-way ANOVA and multiple comparison test were used to compare means of more than two categories.

3. Results

3.1. mASCs characterisation

To confirm that the cells used were mASCs, cells were immunophenotyped using a panel of anti-mouse antibodies. mASCs are positive for CD29, CD105, and negative for CD31, CD106, CD45 and CD90.2. The markers, CD31- and CD29+ were identified in 99.58% cells, of which 94.38% lacked the expression of CD106 and CD45 (Figure 3A-B). Moreover, 98.79% of these cells co-expressed CD29 and lacked CD90.2 (Figure 3C). Moreover, 99.85% of the mASCs expressed CD105 (Figure 3D). In panels (E_H), the overlay dot plots clearly indicate the negative controls (isotypic controls) in green and the cells stained with the respective monoclonal antibodies in red. Adipogenic and osteoblastic differentiation were assessed to confirm the multipotent differentiation of these mASCs. Lipid droplet accumulation was observed in mASCs induced to undergo adipogenic, confirming successful adipogenic differentiation, while there was an absence of lipid droplets in the non-induced cells (Figure 4A-B). Osteogenic differentiation was observed with the presence of calcium deposits that are found in osteoblasts which are visibly red in the induced cells, while no differentiation occurred in the non-induced mASCs (Figure 4C-D).



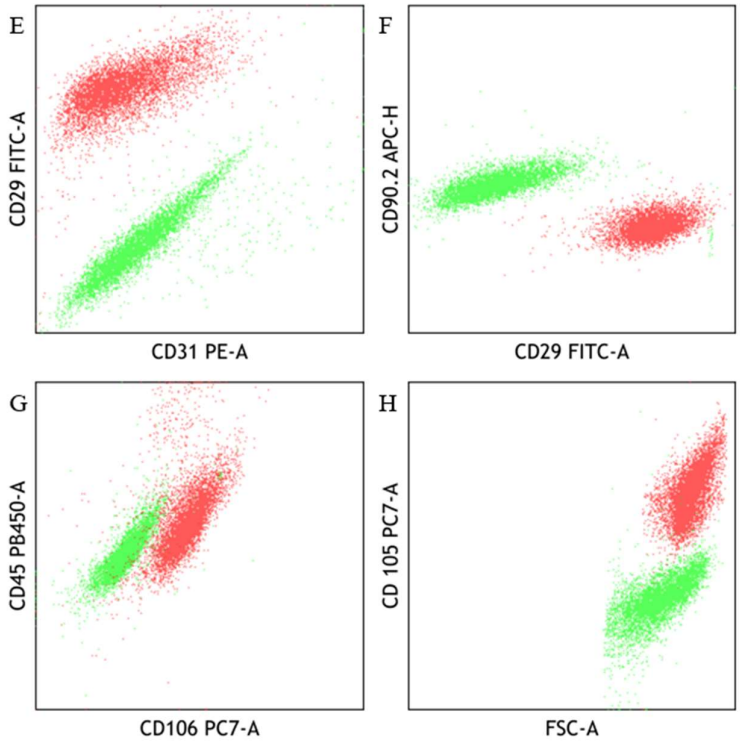


Figure 3: Representative flow cytometry two-parameter plots of mASCs immunophenotype: (A-D) Two-parameter flow cytometry plots indicate that mASCs were positive for CD29 and CD105 and negative for CD31, CD45, CD90.2 and CD106. Plots A – H were gated (region A) on viable, intact ASCs (E-H) Overlay dot plots to indicate the isotypic controls (negative control) (green) and cells that were stained with the respective monoclonal antibodies (red).

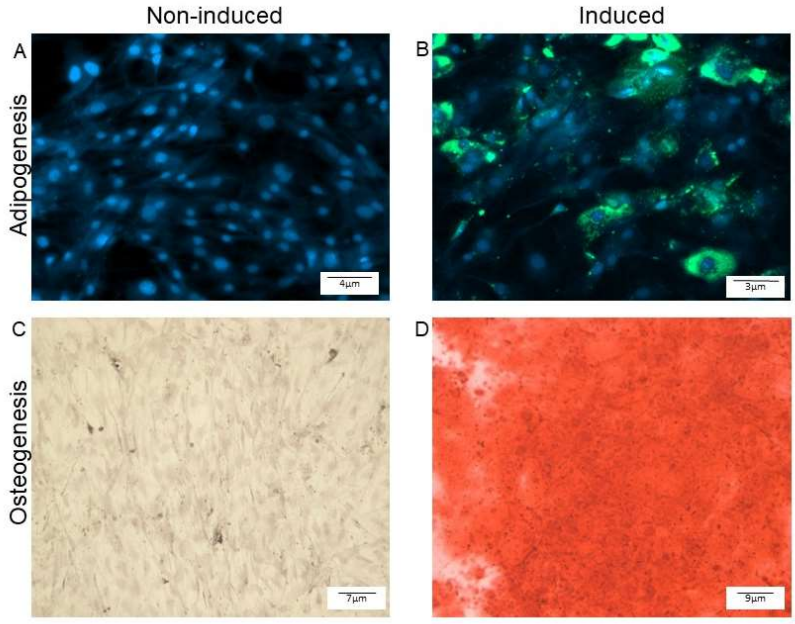


Figure 4: Adipogenic and osteogenic differentiation of mASCs: Representative microscopy images of mASCs induced to undergo adipogenic differentiation; (A) non-induced mASCs stained with DAPI

and Nile red (no visible lipid droplet accumulation observed, indicating no differentiation). (B) Nile red-stained lipid droplets were observed in mASCs induced to undergo adipogenic differentiation, confirming successful adipogenic differentiation. C – D; Osteogenic differentiation of mASCs; (C) non-induced mASCs stained with Alizarin red showed no calcium deposits present, (D) mASCs successfully differentiated into osteoblast that contain calcium deposits that stain red. All images taken at 10X magnification. Scale bar represented by the horizontal line indicates length of 4µm, 2µm 7µm and 9µm for panels A, B, C and D, respectively.

3.2. mASCs effect on tumour volume and mass

To test for the effect of mASC treatment on tumour growth and progression, the size of mammary tumours was measured once a week for 4 weeks using a calliper. Measurements commenced after mice received the last dose of mASCs. Each mouse in the mASC group received $2x10^6$ cells on days 30, 37 and 1.6 $x10^6$ cells on day 44; Therefore, the mASCs treated group received a total of 5.6 x 10^6 cells over a 14-day period. On week 3 after treatment, a larger number of mice (60%) that received mASC treatment developed palpable tumours earlier compared to 30% in the control group (Figure 5A). This observation suggests that the administration of mASCs resulted in earlier tumour initiation. At the point of termination, tumours were removed, measured (size), volume calculated, and weighed to determine tumour mass. On average, no change was observed in tumour volume (*p*-value =-0.7782) and mass (*p*-value = 0.6411) between the mASC treated and untreated groups (Figure 5B-C). Our results therefore suggest that mASCs may have contributed to tumour initiation but not progression.

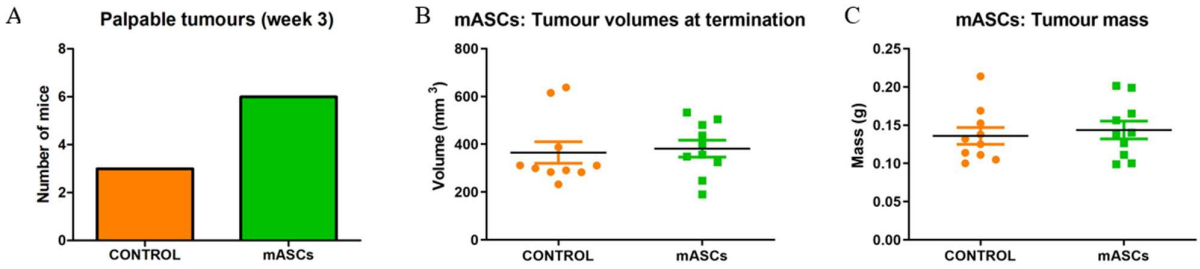


Figure 5: Tumour volumes and mass. (A) A larger number of mice in the mASC group developed tumours earlier. (B) On average at termination, mammary tumour volume and, (C) mass were similar in both groups (N=10 in each group).

3.3. Histopathological analysis of tumours from mammary and lung tissues

Five tumour and lung tissue sections from the mASC treated and control groups (5 sections per group) were selected for H&E staining. Boundaries were drawn manually around necrotic areas and another boundary was drawn around the entire tissue section (Figure 6A-B). The degree (level) of tumour necrosis was measured by dividing the total sum of the necrotic areas with the area of the entire tissue section to determine the percentage of necrosis observed. Tumour necrosis (*p*-value = 0.3486) was higher in the control group compared to the mASCs treated group (Figure 6C). However, the mASC treated mice displayed higher levels of necrosis in the lung tissue (Figure 6D). Although the result was not statistically significant, the *p*-value of 0.0882 suggest that mASC treatment promotes tumour cell necrosis. In summary, our results suggest that mASC treatment inhibited necrosis in primary mammary tumours but enhanced necrosis in the lungs.

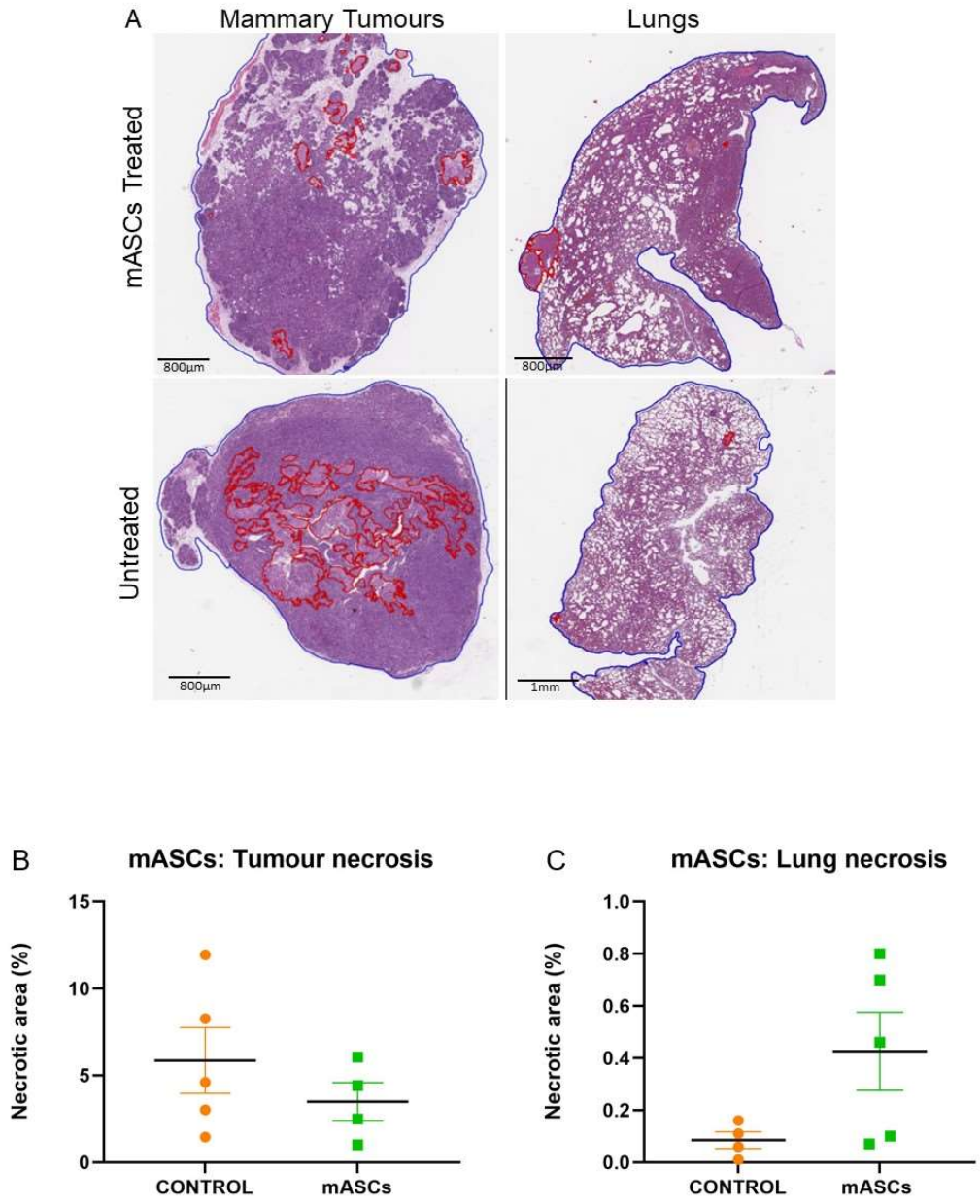


Figure 6: H&E sections of tumour and lung sections . (A) Red boundaries encircle necrotic tissues, while a blue boundary surrounds the entire tissue on mammary tumour and lung tissue. Scale bar represented by the horizontal line indicates lengths of 800µm and 1mm for mammary and lung tissues. (B) Tumour necrosis was lower in the mASC treated group. (C) Higher levels of necrosis were observed in the lungs of the mASC treated group (N=5 in each group).

3.4. Immunohistochemical analysis of CD163-positive M2 macrophages in tissues

Five lung and mammary FFPE tissue sections from mASC treated and untreated mice were randomly selected for immunohistochemistry processing The percentage of positively stained cells was determined by dividing the total number of positive cells by the total number of all macrophages multiplied by 100. In mammary tumours, the number of CD163+ M2-associated anti-inflammatory macrophages (dark

brown stained cells) (Figure 7A) was greater in the mASC treated group (Figure 7B). However, in the lung tissue, there were fewer CD163+ macrophages in the mASC treated group. (Figure 7C). Despite these observations, none of the results reached statistically significance. The *p*-value for lung tissue was 0.0770 and in tumour tissue, it was 0.8763. In summary, our findings suggest that mASCs contribute to a higher anti-inflammatory response in primary mammary tumours but not the lungs.

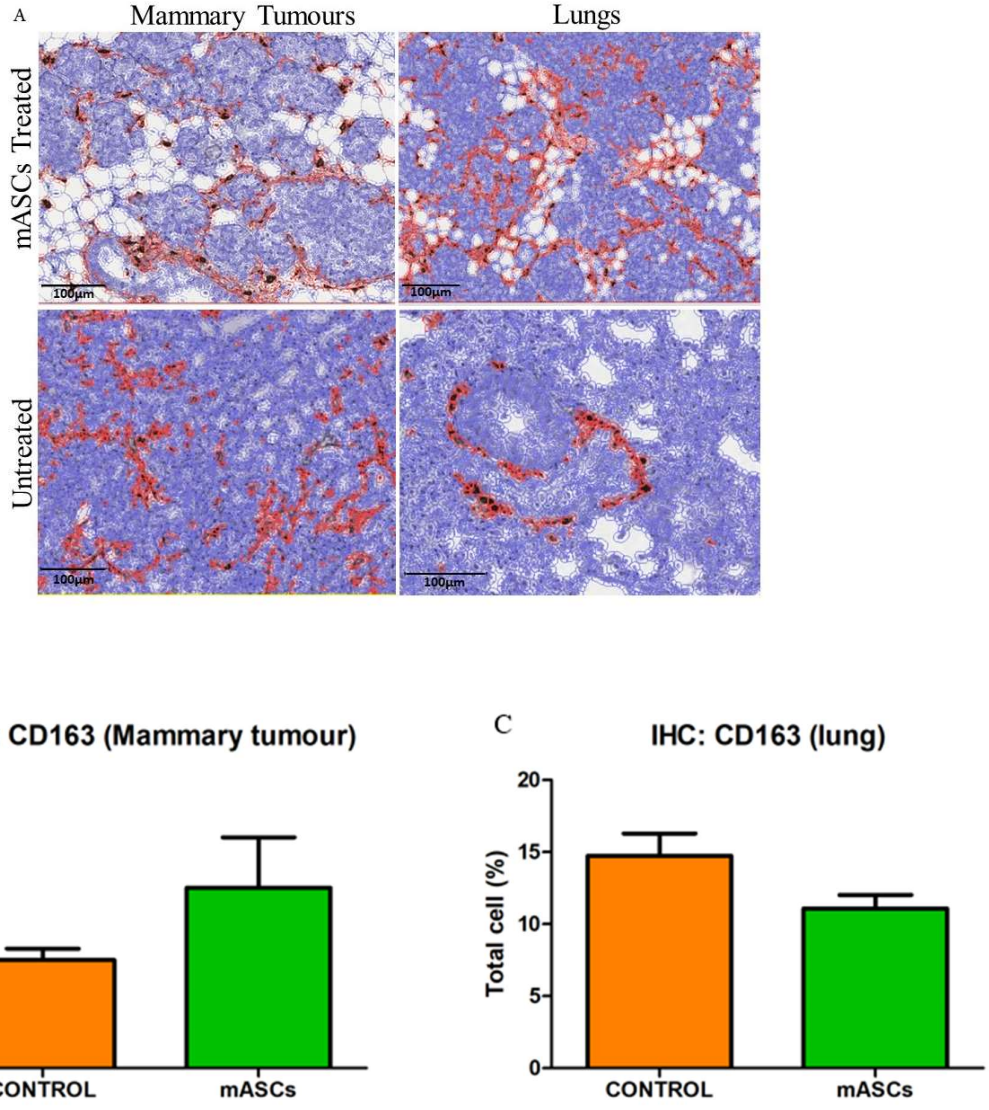


Figure 7: (A) Representative Immunohistochemistry images of M2 involvement in primary mammary tumour and lung. CD163+ cells stained dark brown. Scale bar represented by the horizontal line indicates a length of 100µm for all tissues. (B) and (C) Quantification of CD163+ cells in the primary tumours and lungs, respectively. (B) Anti-inflammatory CD163+ cells were more frequent in the mASC treated group. (C) Fewer CD163+ cells were observed in the mASC treated group B & C: N=5 in each group.

3.5. Immunohistochemical analysis of CD3 positive T cells in tissues

Five randomly selected lung and mammary tissue sections from both the treated and untreated groups were stained with a monoclonal antibody directed against the pan T-cell marker, CD3. A greater

number of CD3+ T-cells (stained dark brown; Figure 8A) were observed in the mammary tumours and lung tissues of mASC treated mice (Figure 8B and 8C); however, the differences observed between the treated and untreated groups were not significant (mammary tumours p-value = 0.3025, lung tissue *p*-value = 0.0938).

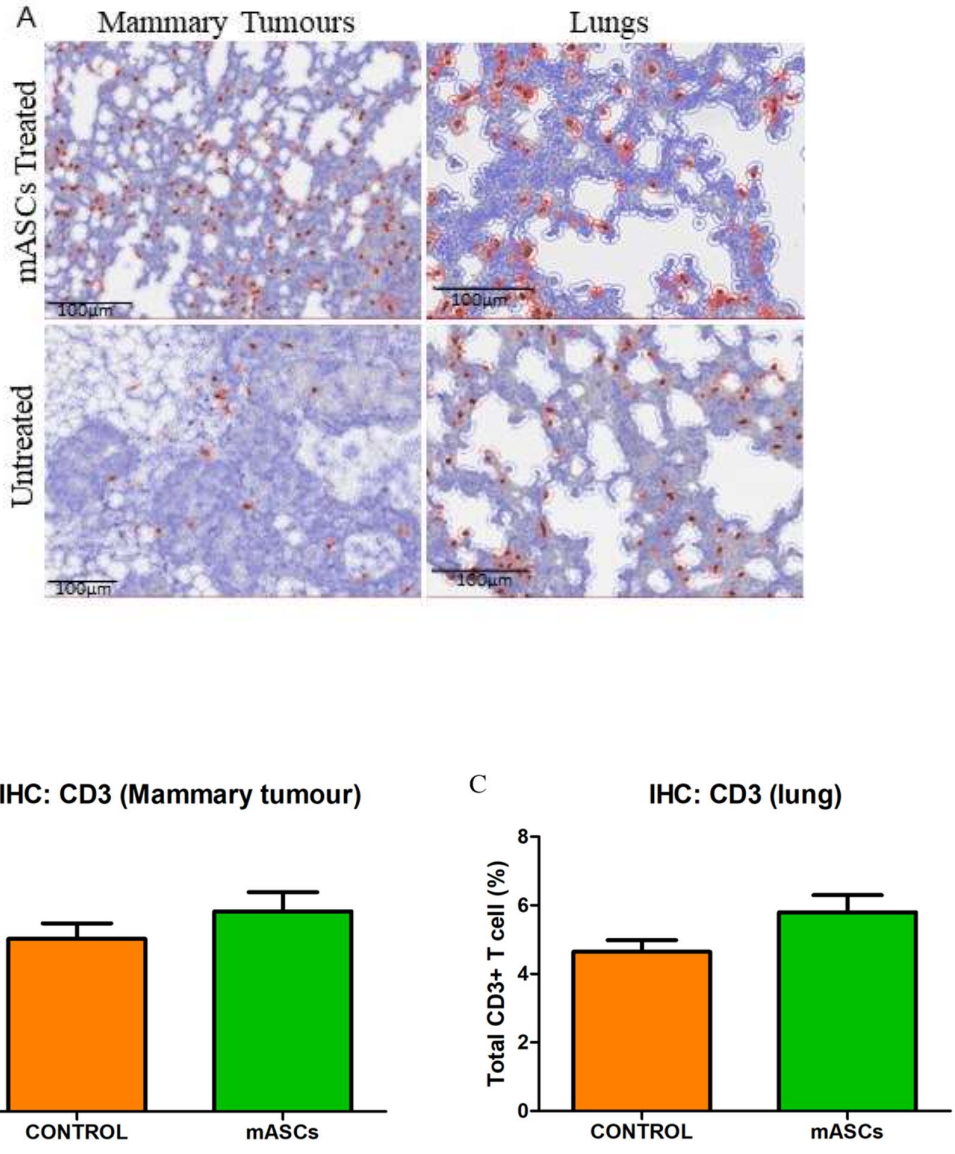


Figure 7: Representative immunohistochemistry images of CD3 in primary mammary tumours and lung tissue. The horizontal line on the image served as a scale bar, representing uniform length of 100µm. (A) CD3+ T-cells appear dark brown. More CD3+ T-cells were observed in mammary tumours (B) and lung tissue (C) of mASCs treated mice.

3.6. Effect of mASCs treatment on gene expression in mammary and lung tissues

Five genes [c*d36*, *tgf-β3*, *vegfr1*, *eng* (*cd105*), and *mtdh*], previously reported to be important in BC growth and progression, were investigated. GAPDH (*gapdh*) was used as the reference gene. The expression of *tgf-β3*, *vegfr1* and *cd105* were all downregulated in both primary mammary tumours and lungs of

mASC-treated mice (Figure 9A-C). The expression of *mtdh* was similar to controls in mammary tumours (Figure 9D) and *cd36* expression was similar to controls in lung tissue (Figure 9E).

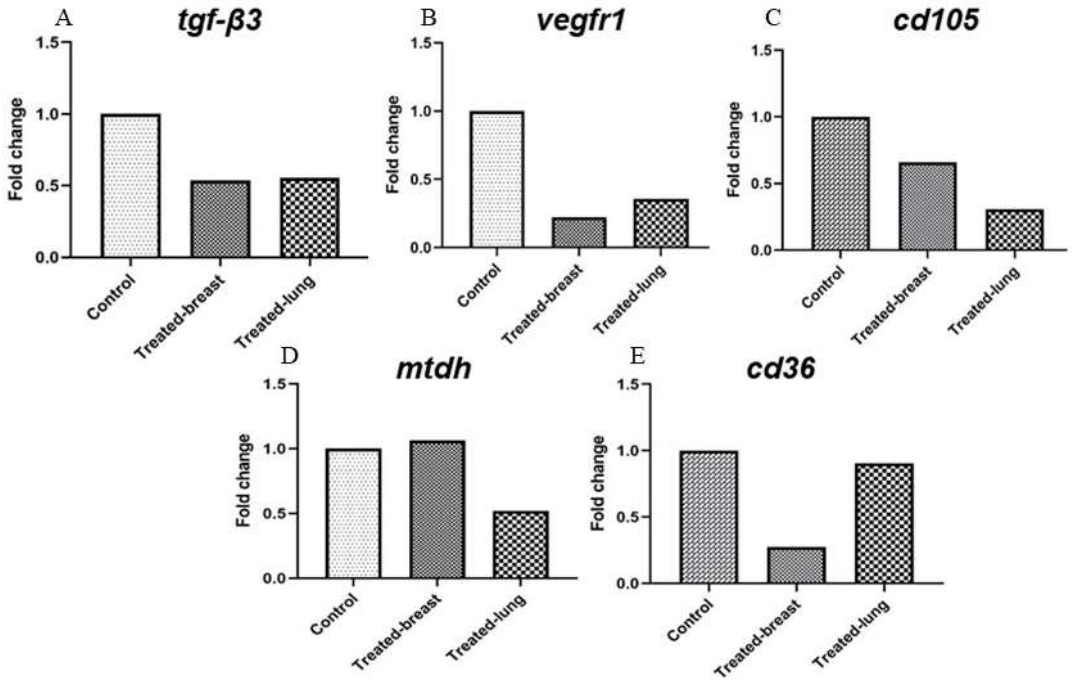


Figure 8: Fold change in gene expression within mammary and lung tissues of mice treated with mASCs, demonstrating SD<2%.(N=10 in each group).

3.7. Cytokine concentrations

The plasma concentrations of all the cytokines measured were higher in the mASC-treated group except for IL-27 ($p = 0.4463$) where no change was observed between untreated and treated groups. Although not statistically significant, the following cytokines were notably higher in the mASC-treated group when compared to controls (untreated): IFN-γ ($p = 0.3464$), IL-1α $p = 0.5614$), IL-1β ($p = 0.2559$), IL-6 ($p = 0.2237$), IL-10 ($p = 0.2332$), IL-12p70 ($p = 0.5308$), IL-17A ($p = 0.2501$), and TNF-α ($p = 0.1399$). Additionally, MCP-1 ($p = 0.2405$), IL-23 ($p = 0.8053$) and IFN-β ($p = 0.3051$) also showed increased levels in the mASC-treated group (Figure 10).

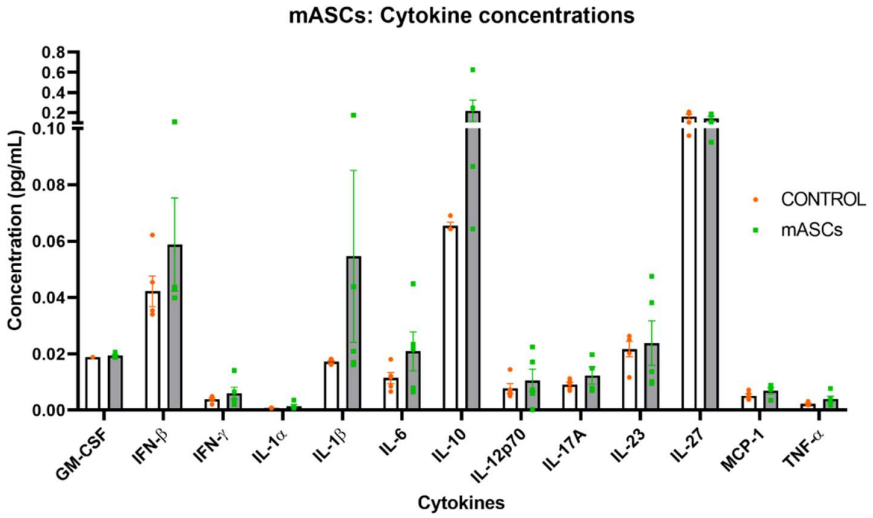


Figure 9: Plasma cytokine concentrations in control and mASC-treated groups: The concentration of all cytokines measured was higher in mice in the mASC group compared to those in the control group (N=5 in each group). White bars represent cytokines levels observed in the control group. Grey bars represent cytokine levels observed in the mASC-treated group.

4. Discussion

In this study, The influence of mASCs on breast cancer development and progression was investigated using a mouse model of spontaneous mammary carcinogenesis. This model is believed to more accurately reflect expected outcomes when BC patients (humans) receive ASCs as cellular therapy. The isogeneic experimental design used in this study limits genetic variability, which has been a major limitation in previous studies, and is therefore, we believe likely to result in a more accurate reflection of the (human) clinical setting. Heterozygous female mice that spontaneously develop mammary tumours with lung metastasis were given either saline or mASCs intraperitoneally over a 14-day period (3 different time points, 7 days apart) and were terminated 28 days after receiving the last dose. There was no difference in mammary tumour volumes and mass between the untreated and treated groups. However, twice as many mice in the mASC treatment group developed palpable mammary tumours between days 60-65 from the time of birth compared to the control group. Lengyel et al. (2018) suggest that adipocytes and ASCs present in adipose tissue in close proximity to mammary glands secrete extracellular matrix molecules such as collagen IV and have been implicated in BC progression [57]. Furthermore, ASCs are known to secrete several growth factors, chemokines and cytokines such as platelet-derived growth factor (PDGF-BB), chemokine ligand 5 (CCL5), VEGFA, VEGFB, stroma-derived factor 1α (SDF-1α), stem cell factor (SCF) and hepatocyte growth factor (HGF) that support BC cells proliferation in the TME [58]. Another study showed that WAT ASCs are recruited by tumours and promote growth [42]. BC xenograft studies have also reported an increase in pro-inflammatory cytokines that causes BC progression by stimulating growth in a paracrine manner [17, 29, 59]. We also observed a notable increase, although not significant, in pro-inflammatory cytokines (IFN-γ, IL-1α, IL-1β, IL-6, IL-17A, IL-12p70, and TNF-α) in the mASC treatment group. In this study, all 13 cytokines measured were higher in the mASC treatment group. Interleukin 10 which was also high is a potent anti-inflammatory cytokine [60]. Eterno et al. (2014) using a xenograft model suggested that ASC-associated pro-inflammatory cytokines cause an increase in BC proliferation but do not maintain tumour growth [24]. ASCs are, therefore, not necessarily tumorigenic but rather exacerbate tumorigenic behaviour by creating an inflammatory environment [24]. The earlier detection of palpable tumours in

the mASC-treatment group with no difference in tumour growth (volume and mass) observed in this study suggests that mASCs only exacerbate tumour initiation but not tumour progression.

Chemotherapeutic drugs eradicate cancer cells via necrosis [61]; therefore the presence of necrotic tissue is an indicator of anti-tumour activity. More CD163+ macrophages and less necrotic tissue were observed in the mammary tumours of mASCs treated mice. However, lower numbers of M2-associated CD163+ macrophages and more necrotic tissue were observed in the lung of mASC treated mice. The expression of CD163+ macrophages was used as a prognostic indicator for predicting BC recurrence-free survival [62]. An in vivo study found that high CD163 expression increased metastatic ability and tumorigenicity [62]. An increased number of CD163+ macrophages has been linked to reduced overall survival of BC patients, metastases, early recurrence and increased production of TGF-β1, IL-10 and VEGF [63, 64]. Moreover, Shabo et al. (2008) demonstrated that CD163+ macrophages are present in greater numbers in the TME of advanced histological grade BC [62, 64]. Therefore, an increase in the number of CD163+ macrophages is detrimental in BC, and this was only observed in mammary tumours and not the in lungs of mASC treated mice compared to controls. However, CD3+ T-cell numbers were greater in mASC treated mice than in controls. High CD3 T numbers is associated with a decreased risk of relapse, favourable outcome, and increased survival [65, 66]. Thus, despite the higher number of CD163+ macrophages in mammary tumours, the high number CD3+ T-cells in the mammary TME shows that there potentially could be an anti-tumour effect if the number of days of life was allowed to increase.

In the primary mammary tumour of mASC treated mice, *cd36* was downregulated but no change was observed in its expression in the lungs. A pro-tumour marker, *cd36* is a transmembrane receptor involved in angiogenesis, apoptosis, adipocyte differentiation, immune signalling and TGF-β activation [49, 50, 67]. Studies on a variety of human BC cell lines demonstrated that downregulation of *cd36* expression supports the progression of an aggressive, metastatic and invasive tumour cell type while upregulated *cd36* was observed in non-aggressive BC cells [68, 69]. The mouse FVB/N-Tg(MMTV-PyVT)634Mul/J model contains PyVT oncogene that activates numerous pathways that lead to an aggressive tumour phenotype [70]. This may explain the downregulation of *cd36* expression in mammary tumours and no change in expression in the lungs of mASC treated mice. Therefore, the more aggressive a tumour, the lower the *cd36* level thus supporting tumour development and an increased likelihood of having metastatic potential [67, 69].

The expression of *tgf-β3* and *vegfr1* (FTL1) were downregulated in both primary mammary tumours and in the lungs in mASC treated mice. In BC, these two genes are mainly involved in angiogenesis [71]. Downregulation of TGF-β3 has been associated with the early development of BC in ductal carcinoma in situ [72]. Furthermore, downregulated TGF-β3 is correlated to TGF-β3 gene loss of heterozygosity in human breast cancer [73]. TGF-β3 restoration in mice inhibits tumour invasion, metastasis, and angiogenesis [73, 74]. Moreover, tumour growth, metastasis and angiogenesis were inhibited in a BC xenograft nude mouse model treated with TGF-β3 alone *in vivo* [74-76]. An *in vivo* study by Hank et al. (2020) demonstrated the lack of TGF-β3 expression created an immunotolerant TME by increasing TGF-β signalling in the dendritic cell (DC) population and upregulated CCL22 myeloid DCs and indoleamine 2,3-dioxygenase (IDO) DCs [77]. Treg infiltration was mediated by these DCs, hence promoting BC development in 4T1 murine BC model [77]. This study shows that mASC treatment downregulates *tgf-β3* possibly creating a pro-tumour effect. Vascular endothelial growth factor interacts with *vegfr1* to stimulate angiogenesis and is involved in BC development and metastasis [47]. Generally, *vegfr1* is absent in healthy breast tissue but expressed in BC cells [78]. The expression of *vegfr1* maintains the survival of BC cells and is associated with a poor prognosis in BC patients [78, 79]. As long as *vegfr1* is expressed, there is metastatic potential but the degree of *vegfr1* expression determines its tumorigenic and metastatic involvement. Various studies have reported that elevated expression of *vegfr1* is linked to metastasis of multiple cancers including BC and a shorter survival time

[78, 80-83]. The result of this study shows that mASC-treatment exert an anti-tumour effect by downregulating *vegfr1* expression.

The expression of *cd105* was also downregulated in both mammary tumour and in the lungs. Lack of or low expression of *cd105* in primary mammary tumours is linked to gene methylation and poor clinical outcome in BC [84, 85]. It has also been demonstrated that *cd105* downregulation leads to increased invasion and tumorigenicity [86, 87]. Additionally, *cd105* overexpression decreases migration and metastasis [84, 85]. Thus, mASC treatment may promote a pro-tumour effect by downregulating *cd105* expression.

Mammary tumours did not show any change in the expression of *mtdh* which was downregulated in pulmonary metastasis. Metadherin is an oncogenic protein that promotes metastasis, cancer progression and chemoresistance in mammary carcinoma [51, 88]. Primary mammary tissues highly express *mtdh* compared to normal tissue [89]. Overexpression of *mtdh* correlates with increased risk of relapse and poor disease-free survival [90]. Thus, this study suggests mASC treatment has an anti-tumour effect in pulmonary metastasis by downregulating *mtdh* expression.

The effect of mASC treatment on primary mammary and pulmonary metastatic tumours produced pleiotropic effects on the different molecular factors investigated. However, the combination of these varied molecular events resulted in a measurable phenotypic outcome which suggests a pro-tumour and anti-tumour effect of mASCs on mammary and pulmonary tumours, respectively. The pro-tumour effect exerted by mASC treatment include the following; (i) double the number of mice in the mASC treatment group developed tumours early; (ii) more CD163+ macrophages were observed in mammary tumours of the mASCs treated mice, and (iii), downregulation of *cd36, tgf-β3, and cd105*, all of which are constituents that outweigh the anti-tumour effect exerted by the downregulation of *vegfr1,* thereby contributing to less necrosis observed in mammary tumours of mASC treated mice. Although the number of CD3+ T-cells was higher in mASC treated mice, this was not enough to render an anti-tumour effect. On the contrary, the anti-tumour effect produced by mASC treatment on pulmonary metastasis includes the following: (i) the lower number of CD163+ macrophages; (ii) the higher number of CD3+ T-cells; and (iii) the downregulation of *mtdh* and *vegfr1* which is suggestive to be strong enough to outweigh the pro-tumour activity resulting from the downregulation of *cd105* and *tgf-β3,* thereby resulting in an increase in tumour necrosis. Furthermore, it is possible that the strong anti-tumour effect by mASCs observed in the lungs, but not in primary mammary tumours may be as a result of mASCs being trapped in the lungs [91]. This could suggest that there were fewer cells in the primary mammary tumour to render a net anti-tumour effect. It will be interesting to explore a different mode of administration such as direct injection of mASCs into the mammary gland to ensure that mASCs are present in that locale and investigate the effect on BC progression.

5. Conclusion

In an attempt to recapitulate the clinical scenario of individuals with BC receiving MSC treatment and to investigate the possible outcomes of such treatment, an isogenic experimental design was used to investigate the effect of mASCs on BC progression in a transgenic MMTV-PyMT mouse model that spontaneously develops mammary tumour with pulmonary metastasis. This study suggests that mASC treatment produced a pleiotropic effect on BC progression by demonstrating pro-tumour activity on primary mammary tumours and anti-tumour activity on pulmonary metastatic tumours, resulting in less and more tumour necrosis respectively at these sites.

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Ambele; Supervision, Michael Pepper and Melvin Ambele; Writing – original draft, Kimberly Peta; Writing – review & editing, Chrisna Durandt, Michael Pepper and Melvin Ambele.

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Chapter 3

This chapter has been presented in the final manuscript version submitted for publication (appendix F) submitted to the Cell Biochemistry & Function journal (Manuscript number CBF-23-0192), which is currently under review.

Effect of 2-methoxyestradiol treatment on early- and late-stage breast cancer progression in a mouse model

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ABSTRACT

BACKGROUND: The prevalence of breast cancer (BC) continues to increase and is the leading cause of cancer deaths in many countries. Numerous *in vitro* and *in vivo* studies have demonstrated that 2-methoxyestradiol (2-ME) has antiproliferative and antiangiogenic effects in BC thereby inhibiting tumour growth and metastasis. The effect of 2-ME was compared between early and late-stage BC using a transgenic mouse model – FVB/N-Tg(MMTV-PyVT) – of spontaneously development of aggressive mammary carcinoma with lung metastasis. **METHODS**: Mice received 100 mg/kg 2-ME treatment immediately when palpable mammary tumours were identified (early-stage BC; experimental group 1) and 28 days after palpable mammary tumours were detected (late-stage BC; experimental group 2). 2-ME was administered via oral gavage three times a week for 28 days after initiation of treatment, while control mice received the vehicle containing 10% dimethyl sulfoxide (DMSO) and 90% sunflower oil for the same duration as the treatment group. Mammary tumours were measured weekly over the 28-day period and at termination, blood, mammary and lung tissue were collected for analysis. Mice with a tumour volume threshold of 4000mm^3 were euthanized before the treatment regime was completed. **RESULTS**: 2-ME treatment of early-stage BC led to lower levels of mammary tumour necrosis, while tumour mass and volume were increased. Additionally, necrotic lesions and anti-inflammatory CD163 expressing cells were more frequent in pulmonary metastatic tumours in this group. In contrast, 2-ME treatment of late-stage BC inhibited tumour growth over the 28-day period and resulted in increased CD3+ cell number and tumour necrosis. Furthermore, 2-ME treatment slowed down pulmonary metastasis, but did not increase survival of late-stage BC mice. Besides late-stage tumour necrosis, none of the other results were statistically significant. **CONCLUSION**: This study demonstrates that 2-ME treatment has an antitumour effect on late-stage BC, however with no increase in survival rate, while the treatment failed to demonstrate any benefit in early-stage BC.

Keywords: 2-methoxyestradiol, breast cancer, tumour growth, metastasis, *in vivo*

1. Introduction

Female breast cancer (BC) incidence has surpassed lung cancer with about 2.3 million new cases in 2020 [1]. BC incidence is based on new cases documented in 159 of 185 countries and is the leading cause of death in 110 countries [1]. Although current treatments increase survival rate, they target both cancer and healthy cells [2, 3]. Moreover, some of these treatments may be ineffective for late-stage metastatic BC [3]. Given the limitations of current treatments, other therapies have been investigated. One such therapy is a promising anti-cancer agent called 2-methoxyestradiol (2-ME), a natural endogenous steroid that is a metabolite of 17β-estradiol (E2) [4-6]. E2 is generated by O-methylation of estradiol at 2-position and sequential hepatic hydroxylation [6, 7]. 2-ME is anti-angiogenic and antiproliferative and induces apoptosis of actively dividing cells *in vitro and in vivo* [8, 9]. The apoptotic nature of 2-ME effectivity extends to oestrogen-independent and oestrogen-dependent cell lines

[10]. 2-ME targets dividing cells during the mitosis (G2/M) cell cycle phase and spares quiescent cells [11, 12]. This drug binds to the colchicine binding site on tubulin, inducing microtubule depolymerisation and inhibiting microtubule assembly [13, 14], consequently inhibiting proliferation and inducing apoptosis [15]. However, 2-ME does not impact the extent of tubulin assembly but impedes the rate [16].

Due to the anti-angiogenic and antiproliferative effects of 2-ME, numerous studies have investigated its effect on BC. Many *in vitro* studies have demonstrated that 2-ME inhibits tumour initiation, tumour growth, metastasis, and induces apoptosis in various BC cells in a dose-dependent manner [17-20]. This is achieved by inhibiting microtubule turnover which leads to cell cycle arrest and apoptosis [7, 21]. Furthermore, 2-ME decreases cell viability with increased 2-ME concentrations and exposure time [17]. LaVallee *et al*. (2008) exposed the MDA-MB-231 BC cell line to 2-ME analogues and found that the analogues induced G2-M cell cycle arrest and apoptosis after 4-16 hours and 16-24 hours respectively [22]. Many *in vivo* studies have also demonstrated the antitumour effect of 2-ME [14, 18, 22-24]. However, some studies have suggested that the antiproliferative effect of 2-ME is limited [17, 18]. These studies suggested that 2-ME may not inhibit, but rather slow the rate of tumour growth, and that if 2-ME is administered for a longer period of time before tumours appear, it may actually increase tumour growth. [17, 18]. Other studies suggested that lack of antitumour activity may be due to suboptimal 2-ME concentrations which exhibit stimulatory effects, but not the inhibitory effect of 2-ME which is dose-dependent [17, 23]. The treatment dosages varied from 20 mg/kg to 150mg/kg given for varying numbers of days. All these studies have xenograft models except for one allograft study where C3(1)/Tag transgenic mice developed spontaneous oestrogen receptor-negative mammary carcinoma and were treated with 150mg/kg/day with 2-ME for 6 weeks [18]. Treatment was given orally for two different periods before tumours formed at 12 weeks and after 18 weeks of age when palpable tumours were 0.5cm in diameter [18]. 2-ME decreased tumour growth and burden in both treatment periods [18].

A xenograft study with a similar treatment design, whereby 2-ME treatment at a concentration of 150mg/kg/day was given orally for 33 days when tumours reached 0.5cm in diameter, revealed that 2-ME inhibited angiogenesis and tumour growth from implanted MBA-MB-231 cell lines [23]. This study showed that a higher concentration of 2-ME given for a longer period can induce antitumour effects. However, a higher concentration (150mg/kg/day) did not always result in antitumour activity. In another xenograft study, 2-ME was given intraperitoneally and orally at 150mg/kg/day and 75mg/kg/day respectively for 19 days after palpable tumours had developed. Treatment with 2-ME showed no antitumour activity but, instead, increased tumour growth in mice inoculated with oestrogen receptor-negative MBA-MB-435 cells and oestrogen-dependent MCF-7 cells [25]. Klauber et al. (1997) suggested an optimal concentration of 75mg/kg/day is needed to avoid toxic effects such as weight loss, diarrhoea, hair loss and lethargy [14]. Despite the contradicting reports, most studies have clearly shown that prolonged administration of 2-ME renders an anti-tumour activity after palpable tumours have developed.

Cytokines are involved in various stages of BC and play a crucial role in either inhibiting or stimulating BC invasion and proliferation [26, 27]. Interferons, interleukins (IL) such as IL-12 and IL-18 inhibit BC while IL-6, IL-1, transforming growth factor β (TGFβ) and IL-11 stimulate BC [26]. These cytokines are secreted by immune cells such as macrophages and T cells contributing to the inflammatory tumour microenvironment (TME) [27, 28]. BC cells secrete factors that differentiate macrophages toward the M2 phenotype [29]. M2-associated CD163+ macrophages are a prognostic marker for BC and metastasis and an increased number of CD163+ macrophages are associated with decreased patient survival [30-32]. In contrast, higher levels of CD3+ cells are associated with good prognosis biomarkers such as CD8 and CD20, and are associated with increased survival [33-35].

Many studies have demonstrated an anti-tumour effect of 2-ME on BC progression, most of which are xenograft models. However, no study has investigated the distinct effect of 2-ME treatment on early- and late-stage BC progression. In this study a transgenic mouse model (FVB/N-Tg(MMTV-PyVT)634Mul/J) that spontaneously develops mammary tumours with lung metastasis (exhibiting an aggressive phenotype of BC) [36] was used to investigate and compare the effect of 2-ME treatment on early- and late-stage BC progression. Moreover, the cytokine profile and immunohistochemistry of some prognostic biomarkers were also investigated.

2. Materials and Methods

2.1 Animal studies

This study was approved by the Faculty of Health Sciences research ethics committee (ethics reference no.: REC166-19) and the animal ethics committee (ethics reference no.: 534/2019) of the University of Pretoria. The FVB-TgN(MMTV-PyVT) mouse model was obtained from Jackson Laboratories (The Jackson Laboratory; Bar Harbor, ME, USA) and mice were bred to obtain heterozygous offspring by crossing hemizygous males with wild-type females. All offspring were genotyped, and only heterozygous females were included in the study.

2.2 Animal genotyping

Mouse genotyping was performed using the KAPA Mouse Genotyping Kit (KAPABIOSYSTEM, Cape Town, South Africa) according to the manufacturer's instructions. Briefly, a 2 mm mouse tail biopsy was placed in 0.2 mL microcentrifuge tubes and deoxyribonucleic acid (DNA) was extracted. For polymerase chain reaction (PCR) genotyping experiments, two pairs of primer sequences obtained from the Jackson Laboratory website (The Jackson Laboratory; Bar Harbor, ME, USA) were used. The forward primer 5'-GGAAGCAAGTACTTCACAAGGG-3' and reverse primer 5'- GGAAAGTCACTAGGAGC GGG-3' were specific for the transgene and the forward primer 5-' CAAATGTTGCTTGTCTGGTG-3' and reverse primer 5'-GTCAGTCGAGTGCACAGTTT-3' were specific for internal positive control. A thermocycler (GeneAmp® PCR System 9700, CA, USA) was used to amply DNA under the following conditions: initial denaturation at 95˚C for 3 minutes, denaturation at 95˚C for 15 seconds, annealing at 60˚C for 15 seconds, and extension for 15 seconds for 2 minutes for 35 cycles. The sizes of the amplicons were determined using ethidium bromide-stained 2% agarose gel electrophoresis.

2.3 2-ME treatment and tumour measurements

Treatment was divided into two experimental groups: one for early-stage BC and the other for late-stage BC. The early-stage BC (Ex. 1) treatment commenced immediately when palpable mammary tumours were felt. The late-stage BC (Ex. 2) treatment began 28 days after palpable tumours were felt. In both experiments, mice received 100mg/kg of 2-ME in a vehicle made up of 90% sunflower oil (Sunfoil, South Africa) and 10% DMSO given three times per week via oral gavage for four weeks followed by euthanization of animals. The control mice received the vehicle. Late-stage BC mice on average received treatment 8 times in both (control and treatment) groups. Mice that reached the mammary tumour volume threshold of approximately 4000 mm^3 were terminated to avoid suffering as a result of tumour burden. Mice in the early-stage BC group received 2-ME treatment a total of 12 times.

A 2-ME concentration of 100mg/kg administered 3 times a week was chosen based on literature to avoid adverse effects in mice. During the duration of the treatment, palpable mammary tumours were measured once a week and at termination using a calliper. Tumour volume was calculated using the formula $L \times W^2 / 2$; L (length) and W (width) [37]. At termination, mammary tumours were excised, and the mass measured in grams (g) on a scale (Sartorius, Göttingen, Germany). A light microscope (OLYMPUS, SC 100, Dubai, UAE) was used to identify and physically count the number of metastatic lesions on the surface of the lungs. Images were also captured using the CellSens dimension imaging software (XV imaging, product version 3.9, Hague, Netherlands). A total of 18 heterozygous female mice (9 for 2-ME and 9 for control) were used for each of the experimental procedures.

2.4 Histology and immunohistochemistry

Lung and mammary tissues were collected from euthanized 2-ME treated and control group mice and fixed in 10% neutral buffered formalin. Haematoxylin and eosin (H&E) staining was performed as previously described [38, 39]. Immunohistochemical analysis for CD163 and CD3 was also performed according to previously described protocols [38, 39] with slight modifications. Briefly, formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut into 3-micron sections and baked in a 58 °C oven overnight. Xylene was used to deparaffinize slides whereafter they were hydrated with decreasing concentrations of alcohol to distilled water. A 3% hydrogen peroxide solution was used to quench endogenous peroxidase for 5 minutes at 37°C. Antigen retrieval was performed using a high pH buffer retrieval solution (Dako Envision FLEX Retrieval solution high pH, Agilent Technologies, Denmark), washed in phosphate buffered saline (PBS) and background staining was subsequently blocked with a protein block (Novolink Leica Biosystems, Newcastle Upon Tyne, UK) for 30 minutes at room temperature (RT). The sample sections were incubated overnight at 4°C in a 1:300 anti-CD163 antibody [EPR19518] (ab182422) (Abcam, Cambridge, UK) and washed in PBS. Detection of the antigen-antibody binding site was performed with Novolink™ Polymer Detection Kit (Leica Biosystems) as recommended by the manufacturer. Slides were rinsed in PBS and incubated with 3,3′-Diaminobenzidine (DAB) (Novolink™ Polymer

Kit) for chromogen detection. Sections were washed and counterstained in haematoxylin for 1 minute, dehydrated with increasing concentration alcohol solutions, cleared in xylene and mounted with dibutylphthalate polystyrene xylene (DPX). CD3 IHC was performed on 3-micron sections in a manner similar to CD163 but with a few differences. The antigen was retrieved using a low pH buffer (Cell Conditioning Solution CC2, Ventana Medical Systems, Inc Arizona USA). At RT, sections were incubated in a 1:100 rabbit monoclonal anti CD3 (Abcam ab16669 clone SP7) antibody for 120 minutes. Slides were rinsed in PBS and detected for 30 minutes at RT with anti-rabbit Polymer HRP IgG (Novolink™ Polymer Detection Kit, Leica Biosystems). The negative controls were prepared by staining with PBS instead of CD163 or CD3 antibody. The Leica AT 2 Aperio scanner (Leica Biosystems, Nussloch, Germany) was used to capture images at 40X magnification and Qupath software (https://github.com/qupath/qupath/releases/tag/v0.3.2), version 0.3.2 (The Queens University of Belfast, Northern Ireland) was used for analyses. Each scanned tissue section was imported into the software, and the image type was set to brightfield (H-DAB). A boundary was drawn around tissue section limits in order to focus only on the cells within the section. To distinguish between positive and negative cells, the estimation stain vector was set to automatic. The software was trained by identifying positively stained cells and indicating to the software what was viewed as positive cell detection. The detection image parameter was set to "optical density sum", and the scan was initiated. After completion, the result showed the number of positive, negative, and total cell counts. The number of positive cells was divided by the total number of cells to obtain the percentage of CD163 and CD3 positive cells.

2.5 Measurement of plasma cytokines

Mice were euthanized with isoflurane (Isofor; Piramal I Healthcare, Mumbai, India) and blood (800 µL) was collected via cardiac puncture into ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 28487 g for 15 minutes. Plasma (250-300 µL) was aliquoted in 2 mL microcentrifuge tubes. For cytokine profiling the Legendplex mouse inflammation panel (13-plex) kit (Biolegend®, San Diego, CA, USA) was used. The kit tested for 13 mouse cytokines which are monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IFN-β, interleukin-1 alpha (IL-1α), IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23 and IL-27. The assay was performed according to manufacturer's instructions. Briefly, increasing standard concentrations (standard supplied with kit) were prepared (in duplicate) in a 96-well plate, and data acquired on a Cytoflex flow cytometer (Beckman Coulter, California, USA). A standard curve was generated. The plasma samples were diluted two-fold, and staining was performed according to the manufacturer's instructions. The samples were analysed using a Cytoflex flow cytometer and the respective cytokine concentrations calculated using Biolegend's data analysis software https://legendplex.qognit.com/.

2.6 Statistical analysis

GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. To compare means between two groups, a one-tailed unpaired *t*-test was used. Data were presented as mean ± standard error of the mean (SEM). To compare the means of more than two categories, a multiple comparison test and a two-way ANOVA was used.

3. Results

3.1 Effect of 2-ME on tumour volume and mass

Heterozygous female mice were genotyped and those that had the MMTV-PyVT transgene were included in the study (Fig 11). Tumour mass is the measure of tumour weight, and tumour volume is the calculated volume based on tumour diameter [40]. In early-stage BC (Ex 1), there was no statistically significant difference in tumour volumes between both groups at termination while there was an increase in tumour mass in the 2-ME treated mice (Fig 11a-b). The mice in the 2-ME treated mice had continuously higher tumour volumes throughout the 4-week period although this was not statistically significant (Fig 11c). In late-stage BC (Ex 2), tumour volume and mass were lower in the 2-ME group at termination (Figure 12a-b). Tumour volumes of both groups were similar at weeks 1 and 2, but in weeks 3 and 4, lower tumour volumes were observed in the 2-ME group (Fig 12c), although this was not statistically significant.

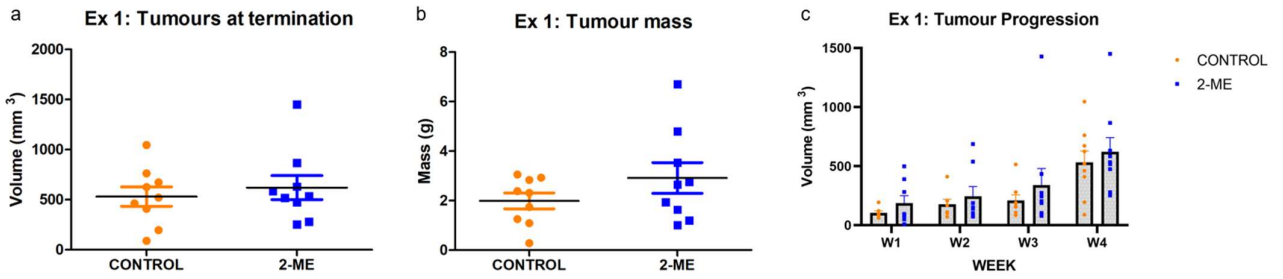


Figure 10: 2-ME treatment of early-stage BC. (a) Average tumour volumes were the same in both groups at termination ($p = 0.2847$). (b) The average tumour mass was higher in the 2-ME treated group ($p = 0.1004$). (c) Throughout the 4-week period, tumour volumes of 2-ME treated mice were higher (N=9 in each group).

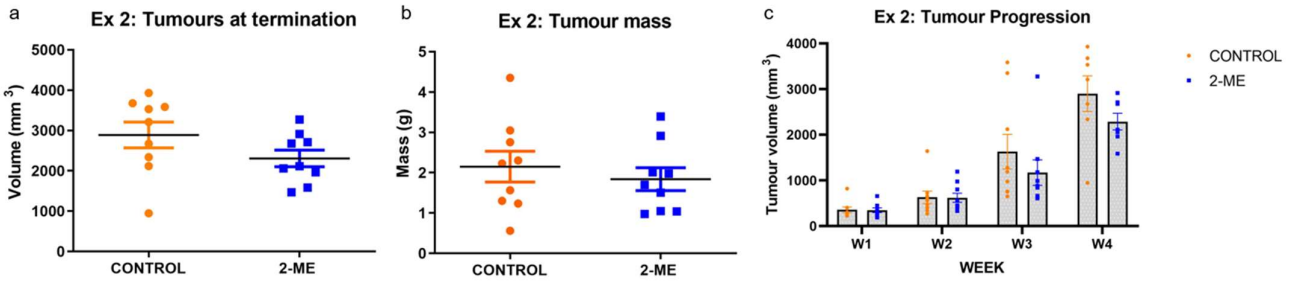


Figure 11: 2-ME treatment of late-stage BC. (a) Tumour volume ($p = 0.0729$) and, (b) tumour mass ($p = 0.2624$) was higher in the control group. (c) Equivalent tumour volumes were observed in weeks 1 and 2 but higher tumour volumes were observed in the control group in weeks 3 and 4 (N=9 in each group).

3.2 Effect of 2-ME on pulmonary metastasis

Mouse lungs were examined for pulmonary lesions at termination. Pulmonary lesions which appeared as nodules were identified and counted. The lesions in early-stage BC were smaller compared to late-stage BC lesions which were larger with varying sizes (Fig 13a). In early-stage BC treated mice, the number of pulmonary lesions was higher in the 2-ME group compared to the control group with a p-value of 0.1169 (Fig 13b). Likewise, in late-stage BC treated mice, a greater number of pulmonary lesions were observed in the 2-ME treated mice (Fig 13c) although this was not statistically significant.

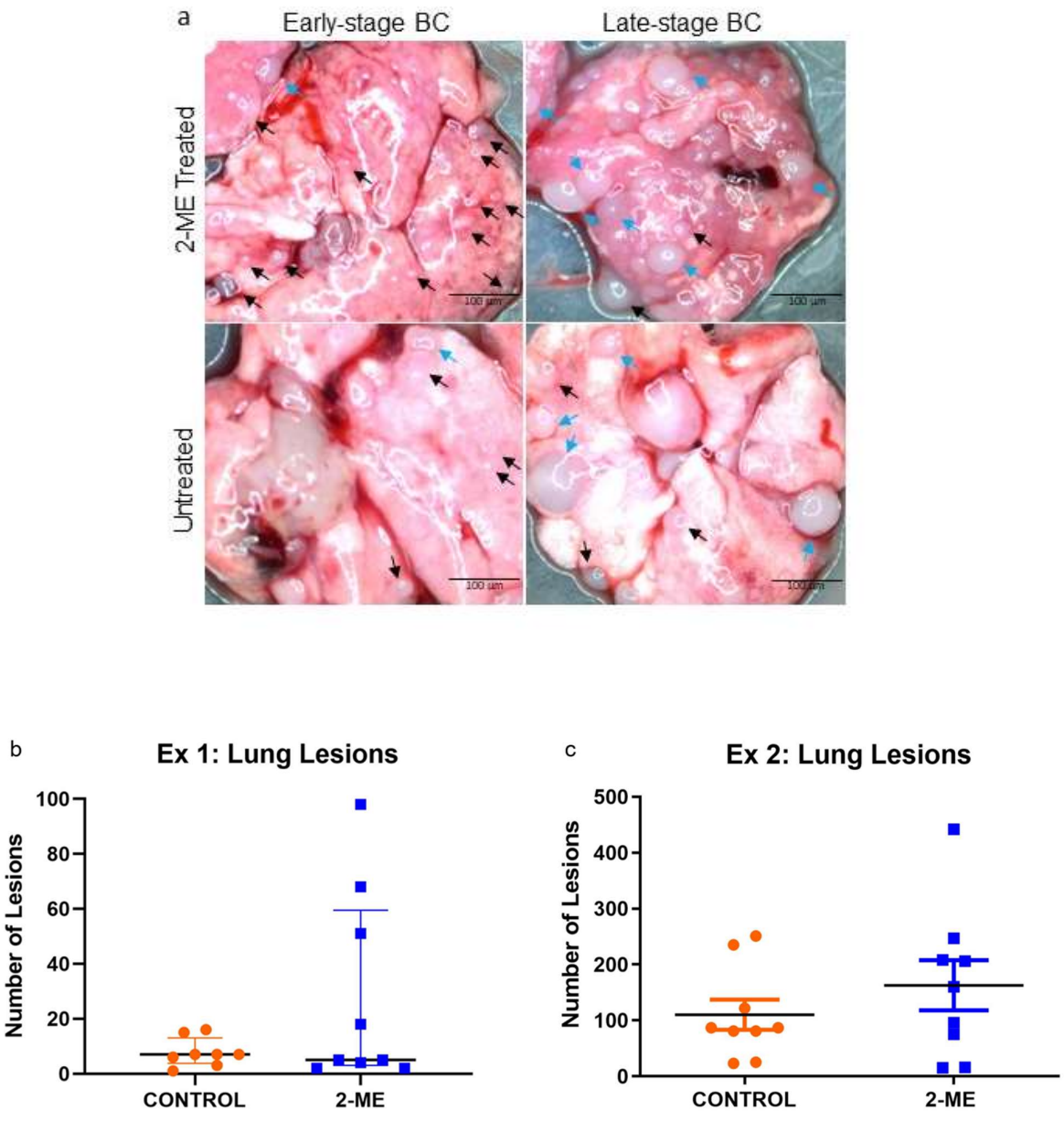
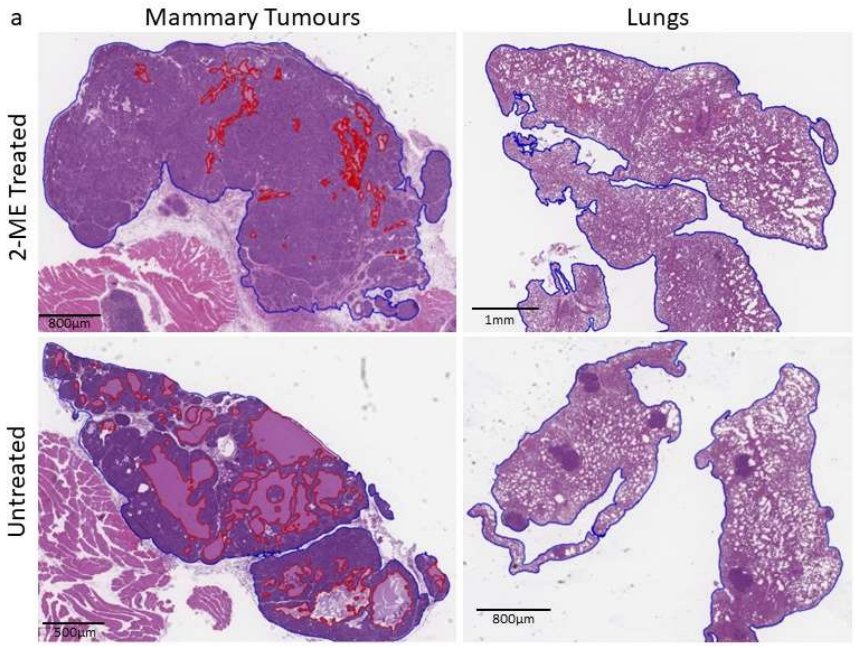


Figure 12: (a) Pulmonary lesions of early-stage BC were smaller (arrows) and a combination of small (black) and large (blue) lesions were observed in late-stage BC. The horizontal line on the image serves as a scale bar, indicating a uniform length of 100µm for all tissues. (b) A greater number of pulmonary lesions were observed in both the early-stage and (c) late-stage (p = 0.1654) 2-ME treated mice (N=9 in each group).

3.3 Histopathological analysis of tumours from mammary and lung tissues

The H&E images taken were analysed by drawing red lines around the necrotic regions and a blue line around the entire tissue (Fig 14a). No necrotic pulmonary regions were observed for early-stage BC. To calculate the proportion of necrotic tissue, the total sum of the necrotic regions was divided by the area of the entire tissue. Mammary tumour necrosis was lower in the 2-ME group (p = 0.3176) in early-stage BC (Fig 14b). Only 1 mouse in the 2-ME group had pulmonary necrosis. In late-stage BC, tumour necrosis was significantly (p=0.0169) higher in the 2-ME group than in the control group (Fig 15a-b). Pulmonary necrosis was lower in the 2-ME group, albeit not significant (p = 0.3480) (Fig 15c).



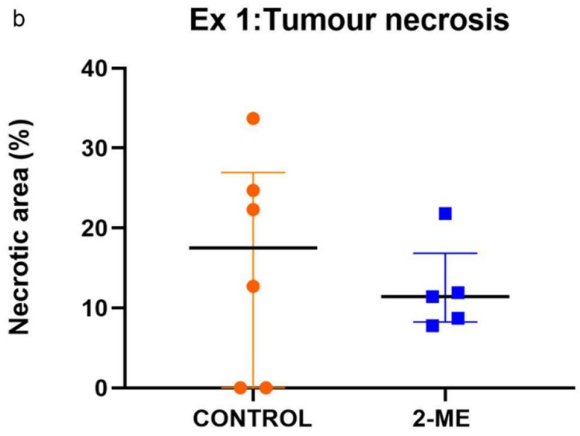


Figure 13: Histopathological analysis of mammary tumour and lungs in early-stage BC mice treated with 2-ME. (a) Early-stage tumour and pulmonary tissues labelled with red boundaries representing necrotic regions. Mammary tumour and lung tissue sections with varying scale bars representing 500µm, 800µm and 1mm.(b) Greater tumour necrosis was observed in the control group compared to the 2-ME group (Control N=6, 2-ME N=5).

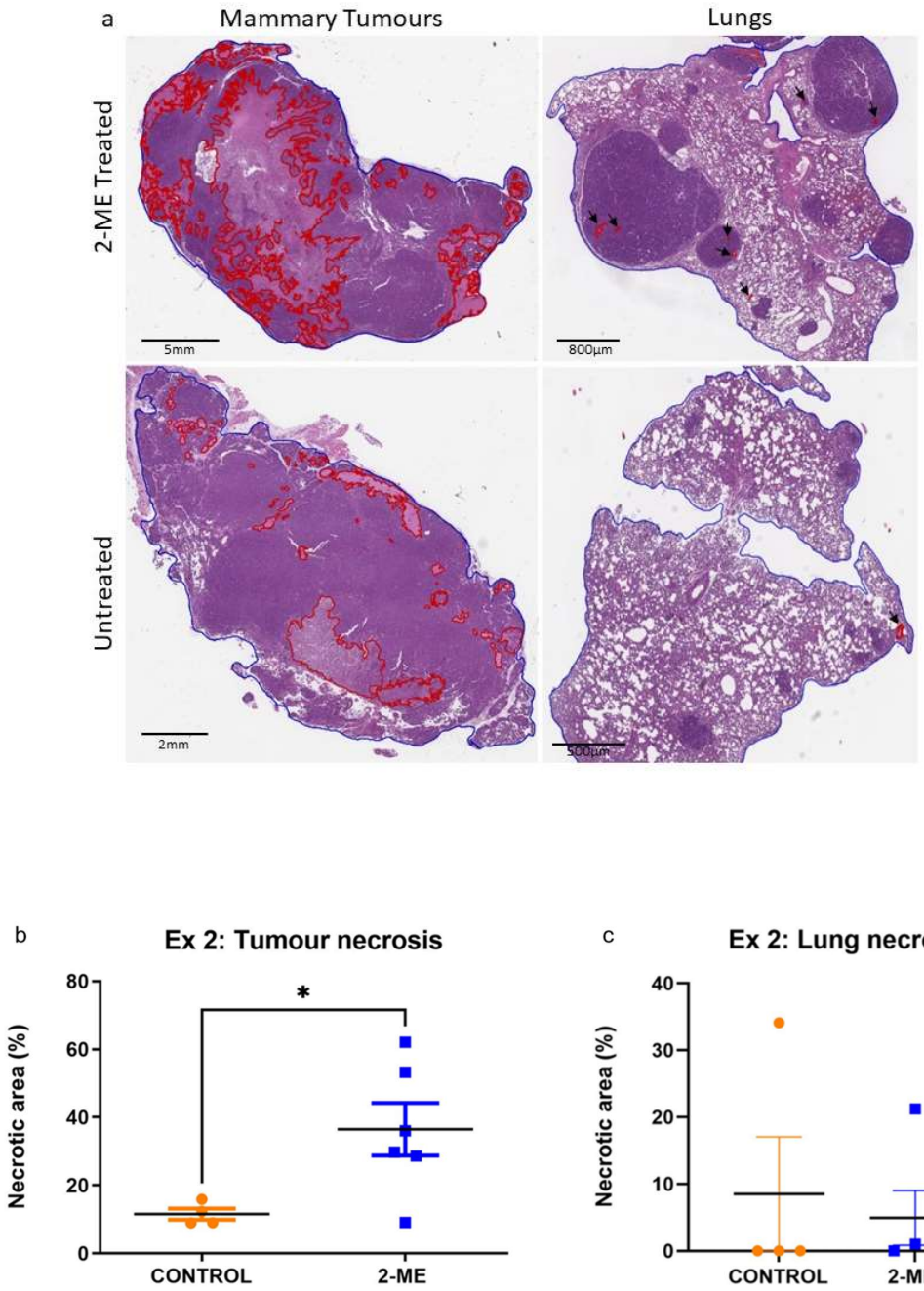
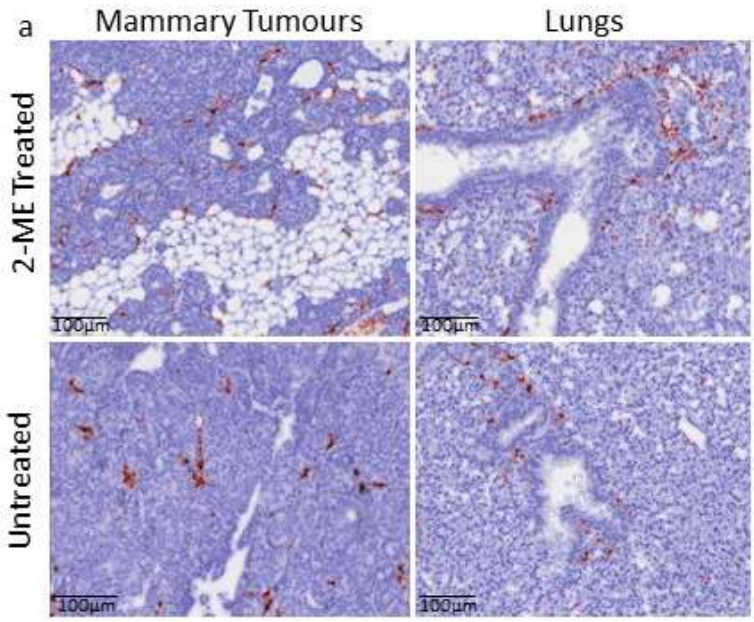


Figure 14: Histopathological analysis of mammary tumour and lung tissues from late-stage BC mice treated with 2-ME. (a) Red boundaries surrounding necrotic areas of late-stage mammary tumours and pulmonary tissues are shown with arrows. Mammary tumour and lung tissue sections with showing different scale bars denoting sizes of 500µm, 800µm, 1mm and 5mm. (b) Tumour necrosis was significantly higher in the 2-ME treated mice, p=0.0169 (Control N=4, 2-ME N=6). (c) Control group pulmonary necrosis was higher than in the 2-ME group (Control N=4, 2-ME N=5).

3.4 Immunohistochemical analysis of CD163 positive M2 macrophages

Dark brown stains represent CD163+ macrophages (Fig 16a). In early-stage BC, the number of CD163+ macrophages in 2-ME treated mice were lower in mammary tumour tissues but higher in lung tissue (Fig 16b-c). In late-stage BC, there was no difference in the number of CD163+ macrophages between the 2-ME treated and the control group both in mammary and lung tissues (Fig 17a-c).



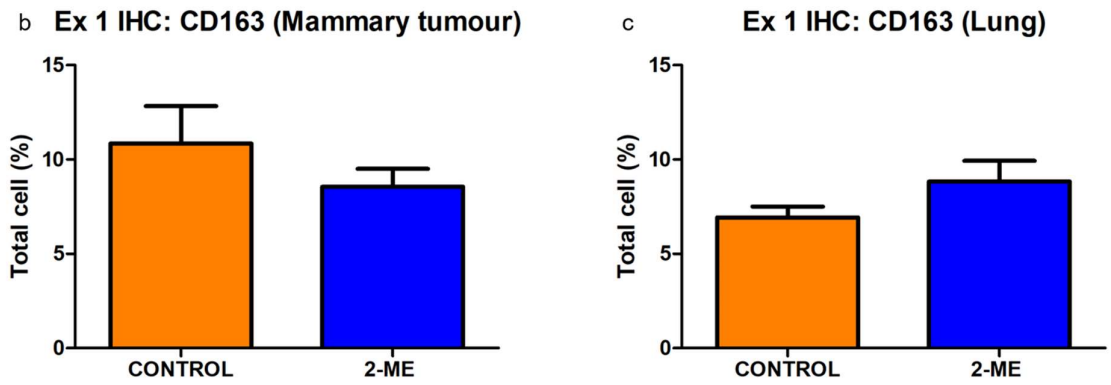
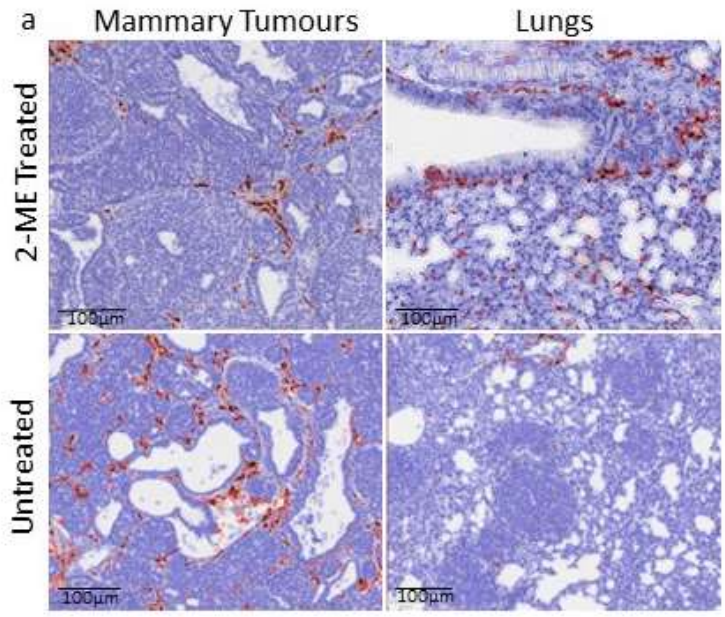


Figure 15: Immunohistochemistry for CD163 staining in mammary tumour tissue and lung of early-stage BC treated with 2-ME. (a) In early-stage mammary and lung tissue, brown staining represents CD163+ macrophages. IHC images of mammary and lung tissue sections, each with a scale bar indicating 100µm. (b) Early stage 2-ME treated mice had a lower number ($p = 0.1617$) of CD163+ macrophages in mammary tissue and (c) a higher number ($p = 0.0811$) of CD163+ macrophages in lung tissue (N=5 in each group).



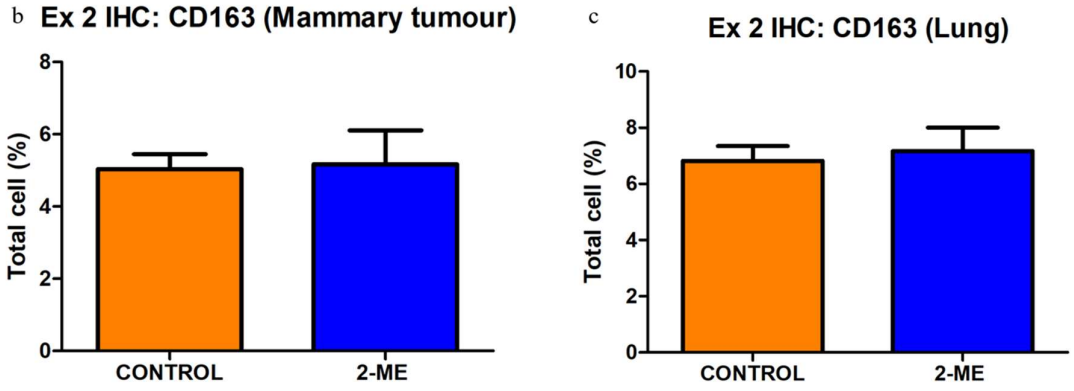


Figure 16: Immunohistochemistry for CD163 staining (brown-stained cells) in mammary tumour tissue and lung of late-stage BC treated with 2-ME. (a) Late-stage mammary and lung tissue immunohistochemistry. IHC images displaying mammary and lung tissue sections, each with a scale bar of 100µm (b) A similar number of CD163+ macrophages was noted in the mammary (p =0.4546) and (c) lung tissues in both groups (p = 0.3729) (Control N=3 and 2-ME N=5).

3.5 Immunohistochemical analysis of CD3 positive cells

Stained (brown) cells represent CD3+ T-cells (Fig 18a and 19a). No difference was observed between groups for mammary tumours and pulmonary metastasis in early-stage BC (Fig 18b-c). However, in late stage-BC, the number of CD3+ T-cells was higher in the mammary tumours, but lower in the lung tissue of 2-ME treated mice (19b-c). None of the results were significant.

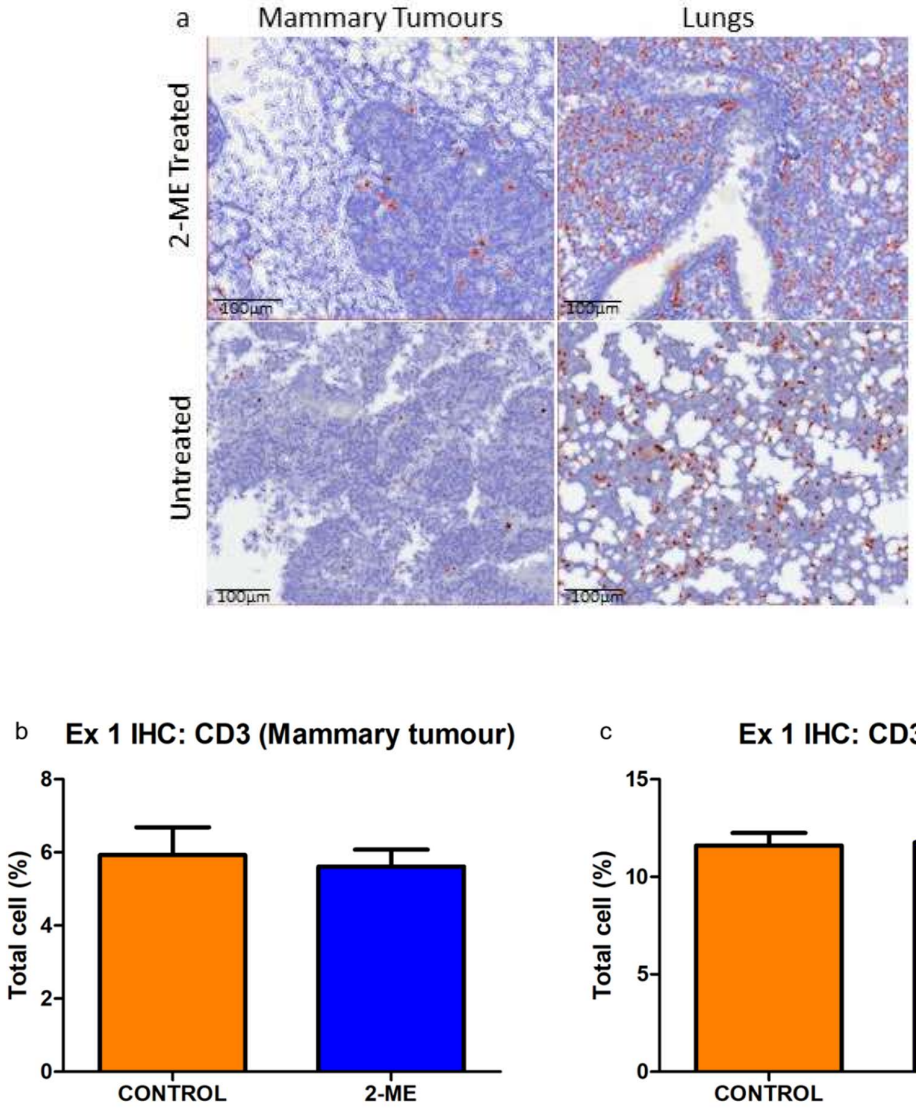
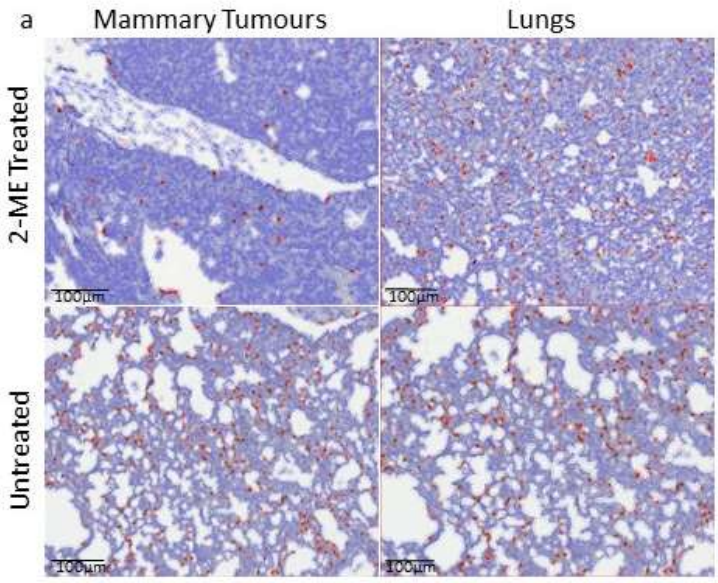


Figure 17: (a) CD3+ T-cells are stained brown and encircled in red. IHC images of mammary and lung tissue sections, with a scale bar of 100µm indicating the length. (b-c) No difference was observed in early-stage BC (p = 0.3665) for both mammary tumours and pulmonary tissue (p = 0.4040). (Control N=3 and 2-ME N=5).



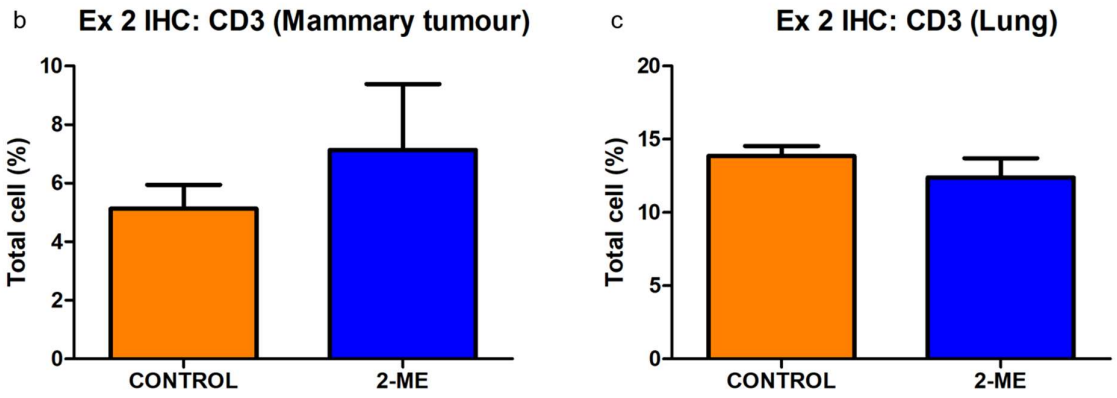


Figure 18: (a) CD3+ T-cells are encircled in red. IHC staining if mammary and lung tissue sections, each with a scale bar of 100µm. (b) In late-stage BC, a higher number of CD3+ cells were observed in mammary tumours (p = 0.3018) and (c) fewer CD3+ cells were detected in the pulmonary tissue (p = 0.2243) of 2-ME treated mice (Control N=3 and 2-ME N=5).

3.6 Effect of 2-ME on the longevity of late-stage BC

The number of days mice in the late-stage BC experimental group lived before termination was evaluated to assess the effect of 2-ME on survival. Importantly, mice with mammary tumours that reached a volume of approximately 4000 mm^3 were terminated due to heavy tumour burden that impaired quality of life. The decision to terminate was determined by a qualified veterinarian. On average, mice in the 2-ME group lived for fewer days compared to mice in the control group (Figure 20). However, as previously stated, the tumour volumes of the 2-ME group were lower at the point of termination.

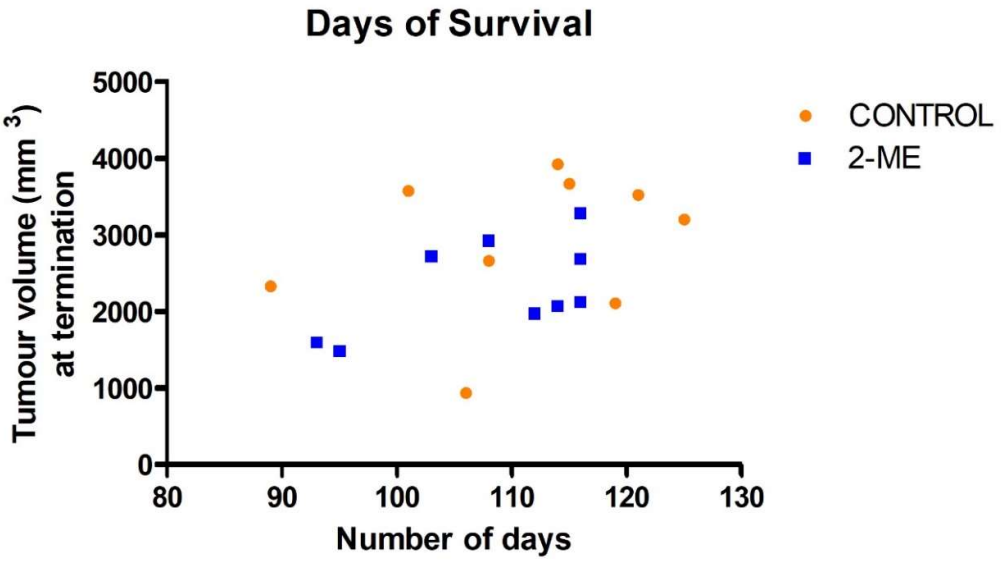


Figure 19: Days of survival of late-stage BC mice. At termination, the number of days of survival of mice in the control group exceeded that of the 2-ME treatment group (N=9).

3.7 Cytokine concentrations associated with early stage BC

Cytokines associated with inflammation were measured in the plasma 2-ME treated and untreated mice in early-stage BC. The following cytokine concentrations were similar in both groups: IL-1α (p = 0.0892), IL-1β (p = 0.4336), IL-12p70 (p = 0.4179), IL-17A (p = 0.3907), and GM-CSF (p = 2354). Cytokines that were higher in the 2-ME treated group were IFN-β (p = 0.0731), IFN-γ (p = 0.1864), IL-10 (p = 0.2279), IL-23 (p = 0.3353), MCP-1 (p = 0.103) and TNF-α (p = 0.1063), with IFN-β, IFN-γ, IL-10 and MCP-1 notably high, but not statistically significant. IL-6 (p = 0.1569) and IL-27 (p = 4232) levels were lower in the 2-ME group (Figure 21).

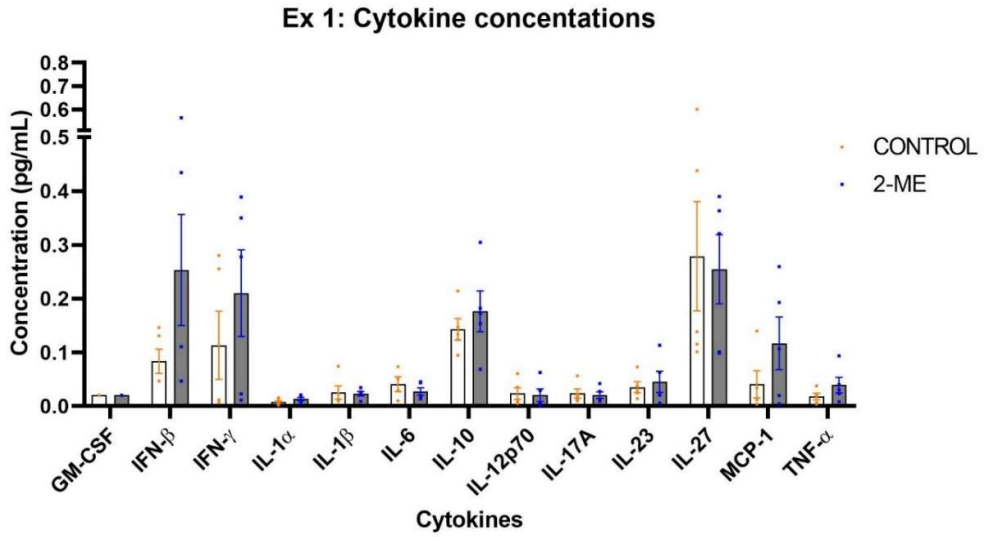


Figure 20: Plasma cytokine concentrations of the control and 2-ME (Ex 1) groups: Cytokine concentrations that were notably higher in the 2-ME group were IFN-β, IFN-γ, IL-10 and MCP-1 while IL-6 and IL-27 were lower. The other cytokines were present at equivalent levels in both *groups* (N=5 in each group).

4. Discussion

The effect of 2-ME on early- and late-stage BC was investigated using a transgenic mouse model that represents an aggressive form of spontaneous mammary carcinoma. The study aimed to simulate a clinical scenario in which 2-ME treatment is given early (when a palpable tumour first appears) or late (28 days after a palpable mammary tumour first appears), depending on the stage at diagnosis. The mice were given 100 mg/kg 2-ME treatment orally 3 times a week for 4 weeks. Thus, the total number of doses administered in the early-stage BC group was 12. Due to the decision to terminate earlier due to excessive tumour burden in the late-stage BC group, the total number of doses administered in both the 2-ME treated and control groups was 8.

In early-stage BC, mammary tumour volumes in the 2-ME treated and control groups were the same, but tumour progression was more rapid and tumour mass increased. The latter observation is supported by the lower degree of mammary tumour necrosis seen in the 2-ME treated group, as increased cancer cell necrosis indicates an anti-tumour effect [41]. However, the number of CD163+ macrophages was lower in mammary tumours, which does not support the findings considering the phenotypic characteristics of higher tumour progression and increased tumour mass. The number of CD3+ T-cells in the mammary TME was similar in both groups. These phenotypic results may account for the similar tumour volumes at the point of termination, since large tumours are associated with fewer CD3+ T-cells [42], and CD163+ macrophages are associated with median-sized tumours [43]. Although mammary tumour volumes in the 2-ME treated group were higher in the week preceding termination, this was not the case at termination. Due to the drug's prolonged exposure, it appears that 2-ME inhibits tumour growth. Longer 2-ME exposure has previously been shown to cause BC cell apoptosis [17], suggesting that if 2-ME is given for a longer period it may have an anti-tumour effect. Furthermore, pulmonary lesions were more common in the 2-ME treated group, as evidenced by a higher number of CD163+ macrophages detected, but there was no discernible difference in the number of CD3+ T-cells. An *in vivo* study demonstrated that increased CD163+ macrophages led to enhanced metastatic ability and tumorigenicity [32], and we observed similar trends. Taken together, our findings suggest that 2-ME did not cause an anti-tumour effect. A study by Huh *et al.* on the late intervention of 2-ME which correlates with the early-stage BC group in this study, found that there was a in 60% decrease tumour volume in 2-ME treated C3(1)/Tag transgenic mice compared to controls, and suggested that a high dose of 150 mg/kg can decrease tumour volume and inhibit angiogenesis [18]. This result is contrary to what we found in this study and could be because of the increased dosage of 150 mg/kg/daily for 6 weeks. Similarly, another study reported that 2-ME is anti-tumorigenic, but with a high dose of 150 mg/kg/day for a period of 33 days [44]. There are several other studies with a similar experimental design to the early-stage group i.e., treatment was initiated after detecting the presence of palpable tumours. However, the dose and duration of treatment varied with the dose ranging from 50 mg/kg/day for 16 days to 75 mg/kg/day for 30 days [14, 24, 45]. Despite the concentration variations all studies reported an anti-tumour effect for 2-ME. Oral administration of a concentration of 25 mg/kg/day has proven effective against metastasis [46]. What stands out between previous studies and this study is that 2-ME was administered daily in most studies as opposed to the approach used in this study, which used a spaced-out treatment (thrice per week for 4 weeks). In hindsight, the dosage schedule should be reconsidered in future studies as it seems that anti-tumour effect of 2-ME is based on its consistent bioavailability to the TME. This hypothesis is supported by a study on the pharmacokinetics of 2-ME which reported that the bioavailability of 2-ME at 10 mg/kg was low after 24 hours in the plasma of mice [47]. Furthermore, the authors reported that oral administration of 20 mg/kg/day for 28 days showed no statistically significant effect on tumour growth [47]. Taken together, the results suggest that 2-ME should be given daily at a dose higher than 20 mg/kg/day to cause an anti-tumour effect.

Notably, 2-ME-treated mice had higher levels of the inflammatory cytokines, IFN-β, IFN-γ, IL-10, and MCP-1, while IL-6 and IL-27 were lower. Studies have shown that MCP-1 is elevated in BC and has been implicated in BC progression [27, 48]. Moreover, MCP-1 is involved in cancer initiation and activates monocytes that promote pulmonary metastasis in BC [49, 50]. Interferons are anti-tumourigenic and inhibit BC cells' capacity to form mammospheres [51, 52]. Interleukins 6 and 10 are anti-inflammatory cytokines that are also protumorigenic [27, 53]. Significantly elevated IL-27 levels have been observed in BC patients and are associated with tumour growth [54]. Despite the fact that elevated levels of interferons appear to indicate an anti-tumor effect, most of these other notable cytokines indicate that 2-ME may have a pro-tumour effect in early-stage BC.

Contrary to early-stage BC, the 2-ME effect in late-stage BC suggests anti-tumour activity. Tumour volume, mass, and tumour progression were lower in the 2-ME group. This observation was supported by tumour necrosis which was significantly higher in 2-ME-treated mice, as was the number of CD3+ T-cells. CD3+ T-cell number is associated with increased survival, and patients with low levels of CD3+ T-cells had an elevated risk of relapse in BC [55, 56]. However, there was a similar number of CD163+ macrophages in mammary tumours of the 2-ME-treated and control groups. Furthermore, pulmonary lesions were higher and pulmonary necrosis was lower in the 2-ME-treated group. This phenotypic finding is further supported by the presence of fewer CD3+ T-cells in the pulmonary tissue. Also, despite having less pulmonary necrosis on average in the 2-ME group, more mice in this group had pulmonary necrosis. This suggests that 2-ME slowed down pulmonary metastasis which is supported by the lack of difference in the number of CD163+ macrophages observed between the 2-ME and the control groups. Taken together, our data suggests that 2-ME rendered an anti-tumour effect on mammary tumours, but not on pulmonary metastases. These results demonstrate that 2-ME treatment is not effective in inhibiting metastasis in mice that receive treatment late. Generally, treating advanced BC with current therapies is challenging [57, 58], and advanced BC treatments are aimed at prolonging and improving quality of life [59, 60]. Studies have shown that 2-ME treatment inhibits tumorigenesis in advanced BC and increases the overall survival rate [18]. An anti-tumour effect was also observed on late-stage BC, but 2-ME failed to increase overall survival. In previous studies, 2-ME treatment was given to mice either on the day of inoculation [24], or after the appearance of palpable tumours [14, 18, 44]. To our knowledge, this is the first *in vivo* study that used this late-stage BC treatment strategy. Further studies are needed to understand the effect of 2-ME on advanced mammary carcinoma from a mechanistic perpective. Furthermore, the role of 2-ME in the TME, the pharmokinetic profile of the drug and its effect on leucocytes should be investigated. This could lead to the development of optimal 2-ME treatment strategies capable of eliminating BC cells at every stage.

5. Conclusion

Our data suggests that 2-ME has the potential to be an effective treatment for late-stage BC, demonstrating anti-tumour activity, whereas for early-stage BC, most of the evidence suggests a pro-tumour effect. In late-stage BC, 2-ME inhibited tumour growth, increased tumour necrosis, and slowed pulmonary metastasis. In early-stage BC, pulmonary metastasis was associated with increased tumor volume and a higher number of CD163+ macrophages. Because the effect of 2-ME on the two BC stages differed, future research should focus on the mechanism and influence of 2-ME in the TME of the various BC stages for the treatment to be an effective cancer therapy.

Disclosure

The funders had no role in the preparation of the manuscript and the decision to publish.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Supplementary Figures

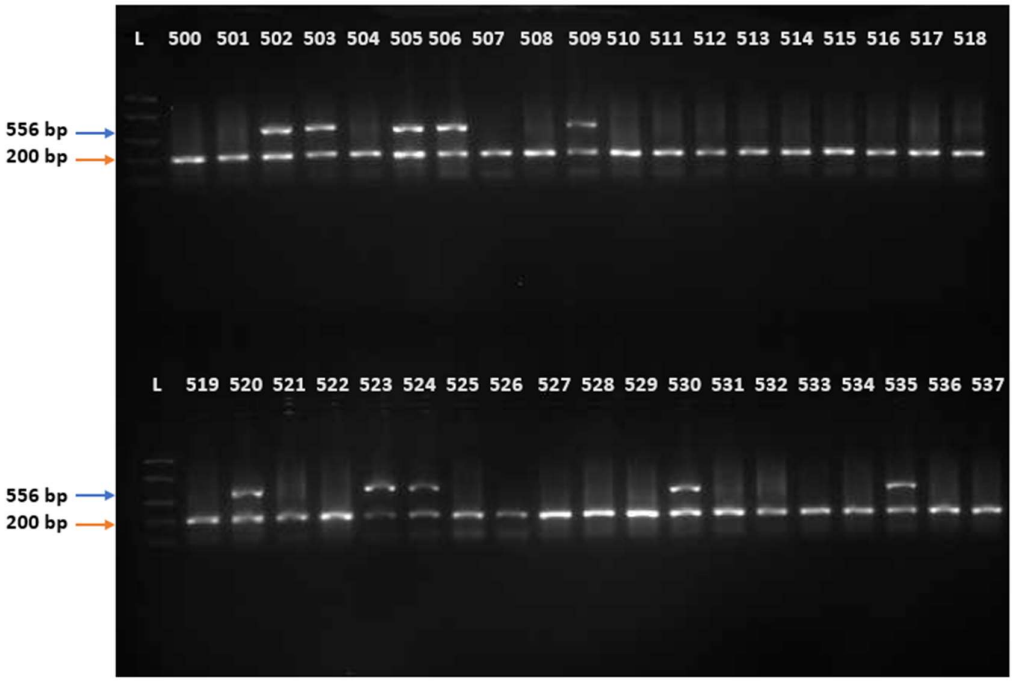

Figure 21: PCR amplicons. The ruler ladder (L) indicates the sizes of the amplicons. The transgene bands were 556 bp (blue arrow) and the internal positive control bands were 200 bp (blue arrow). The numbers 500-537 were utilized to identify the mice.

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Chapter 4

This has been presented in the final manuscript version submitted for publication (appendix G).

Effect of 2-methoxyestradiol on mammary tumour initiation and progression

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ABSTRACT

BACKGROUND: The anti-cancer agent 2-methoxyestradiol (2-ME) has been shown to have anti-proliferative and anti-angiogenic properties. Previously, the effect of 2-ME on early- and late-stage BC was investigated *in vivo* using a transgenic mouse model (FVB/N-Tg(MMTV-PyVT)) of spontaneous mammary carcinoma. Anti-tumour effects were observed in late-stage BC with no effect on early-stage BC. Given the contrasting results obtained from the different BC stages, we have now investigated the effect of 2-ME when administered before the appearance of palpable tumours. **METHODS:** Each mouse received 100 mg/kg 2-ME on day 30 after birth, twice per week for 28 days, while control mice received vehicle only. Animals were terminated on day 59. **RESULTS:** 2-ME increased tumour mass when compared to the untreated animals (p=0.0139). The pro-tumorigenic activity of 2-ME was accompanied by lower CD3+ T-cell numbers in the tumour microenvironment (TME) and high levels of the pro-inflammatory cytokine interleukin (IL)-1β. Conversely, 2-ME-treatment resulted in fewer CD163+ cells detectable in the TME, increased levels of tumour necrosis, and increased IL-10 plasma levels, low IL-6 and IL-27. **CONCLUSION:** Taken together, these findings suggest that 2-ME promotes early-stage BC development.

Keywords: 2-methoxyestradiol, breast cancer, in vivo, tumour growth, metastasis

3.2 Introduction

Breast cancer (BC) is one of the most frequently occurring cancers (Alwan 2016; Kreiter et al. 2014). It is the most prevalent cancer among women worldwide, accounting for nearly 25% of all cancer cases in women according to the World Health Organization (WHO) (Alwan 2016). Surgery is usually the first treatment option for and can include either lumpectomy or mastectomy (Rubino et al. 2003; Tesarova 2013; Giordano et al. 2005). Radiation therapy is often used to eliminate cancer cells that have escaped surgery (Rubino et al. 2003; Tesarova 2013; Giordano et al. 2005). Hormonal therapy is used to prevent oestrogen from promoting the growth of certain types of BC (Rubino et al. 2003; Tesarova 2013; Giordano et al. 2005). Chemotherapy is a systemic treatment that primarily inhibits DNA synthesis and mitosis leading to apoptosis in rapidly driving cancer cells (Senapati et al. 2018). Depending on the stage of the cancer, chemotherapy may be administered before or after surgery (Rubino et al. 2003; Tesarova 2013; Giordano et al. 2005).

Advances in the development of chemotherapy include more targeted treatments that specifically targets proteins involved in cancer cell growth and progression in cancers including BC, ovarian cancer, and prostate cancer (Senapati et al. 2018). 2-Methoxyestradiol (2-ME) targets the colchicine-binding site in tubulin and alters polymerization kinetics hindering tumour vascularization and growth (Eichenlaub-Ritter et al. 2007; Pérez-Pérez et al. 2016). Numerous studies in BC, both *in vivo* and in *vitro,* have demonstrated that 2-ME has anti-angiogenic and anti-proliferative properties (Huh et al. 2006; Mabjeesh et al. 2003; LaVallee et al. 2008; Snoeks et al. 2011; Klauber et al. 1997; Vorster and Joubert 2010; Tang et al. 2020; Tevaarwerk et al. 2009). Moreover, the use of 2-ME has been shown to enhance the effects of chemotherapy in the treatment of BC (El-Zein, Thaiparambil, and

Abdel-Rahman 2020). For example, combining 2-ME with paclitaxel, a widely used chemotherapy drug, has been shown to improve treatment outcomes in preclinical models of BC (El-Zein, Thaiparambil, and Abdel-Rahman 2020). Despite its promising anti-tumour properties, more research is required to fully comprehend the potential benefits and risks of 2-ME in BC treatment.

Cytokines are generated by different cell types present in the TME and contribute to a complex, dynamic system by facilitating crosstalk between the different cell types. The majority of the cytokines are produced by macrophages and infiltrating T-helper (Th) cells (Chavey et al. 2007; Hong et al. 2013). The Th subsets, Th1 and Th2 are antagonistic to each other and produce cytokines that initiate different activities (Chavey et al. 2007; Hong et al. 2013). Th1 cells effectively generate an anti-tumour immune response by secreting granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), IL-2, IL-12 and TNF-α that stimulate cytotoxic lymphocytes and macrophages (M1) thereby promoting inflammation and cellular immunity (Chavey et al. 2007; Hong et al. 2013). Th2 cells produce IL-4, IL-5, IL-2, chemokine ligand 2 (CCL2) also referred to as monocyte chemoattractant protein 1 (MCP-1), CCL7, and CCL11 which stimulate antibody production by B cells and M2 macrophage polarization that is essential in mediating a humoral immune response and which has been reported to enhance mammary tumour development (Chavey et al. 2007; Hong et al. 2013). A recent study has revealed that B and T lymphocytes can indirectly exert pro-tumour activity by regulating the bioactivity of myeloid cells such as monocytes, macrophages and mast cells leading to metastasis and resistance to endocrine therapies (DeNardo et al. 2011). Chemotherapy drugs act non-specifically on various cell types in the TME, influencing cytokine production by these cells, hence affecting tumour growth and progression. For example, paclitaxel, a chemotherapeutic drug, is a microtubule destabiliser that is used to treat BC (White et al. 1998). Paclitaxel increases IL-1β (IL-1β), IL-10, IL-6, and IL-8 in the plasma (White et al. 1998; Pusztai et al. 2004). However, this drug decreased the level of tumour necrosis factor alpha (TNF-α) (Panis et al. 2012). Furthermore, paclitaxel increases T cell (CD3+) activation in BC patients and increased T-cell clones inside tumours (Melichar et al. 2001; Chun et al. 2022). This indicates that multiple cytokines and leukocytes are present in the BC tumour microenvironment (TME) (Chavey et al. 2007; DeNardo et al. 2011).

The number of CD3+ T-cells and M2-associated CD163 macrophages in the TME are prognostic factors in BC patients (Celepli et al. 2022). Following antigen recognition, CD3 (T-cell receptor) initiates a signalling cascade that activates both CD4+ and CD8+ T cells (Brown et al. 2014), which are referred to as tumour infiltrating lymphocytes (TILs) and are involved in eliminating tumour cells (Rathore et al. 2014; Zhou et al. 2020). Increased numbers of CD3+ cells in the TME are associated with small tumour size, decreased lymph node metastasis and increased overall BC patient survival (Rathore et al. 2014; Barbosa et al. 2021; Mukherjee et al. 2020). Conversely, increased numbers of CD163+ macrophages are associated with poor prognosis, large tumour size, metastasis, distant recurrence, tumour progression, and decreased survival (Maisel et al. 2022; Fortis et al. 2017; Jamiyan et al. 2020; Shabo et al. 2009).

The effect of 2-ME on early- and late-stage mammary carcinoma was previously investigated in a transgenic mouse model (FVB/N-Tg(MMTV-PyVT)) that spontaneously develops mammary carcinogenesis. 2-ME treatment was initiated (i) as soon as palpable tumours appeared for early-stage BC investigation or (ii) on day 28 after the appearance of palpable tumours for late-stage BC investigations. 2-ME treatment of late-stage BC inhibited mammary tumour growth and slowed pulmonary metastasis while a pro-tumour effect was observed in early-stage BC. This apparently contrasting effect of 2ME on early- and late-stage BC may suggest that the anti-tumour effect of 2-ME may be dependent on the stage of mammary carcinoma, thus prompting the initiation of this study. This study therefore aimed to investigate the effect of 2-ME on tumour initiation in FVB/N-Tg(MMTV-PyVT transgenic mouse model. This was done to investigate the BC stage-specific effect of 2-ME treatment and to further explore the potential of 2-ME as an effective preventive treatment in a scenario where BC development is evident such as in hereditary BC.

2. Material and Methods

2.1 Animal studies

This study was approved by the University of Pretoria Faculty of Health Sciences Research Ethics Committee (ethics reference no.: REC166-19) and the Animal Ethics Committee (ethics reference no.: 534/2019). The FVB-TgN(MMTV-PyVT) mouse model was obtained from Jackson Laboratory (Bar Harbor, ME, USA), and mice were bred to produce heterozygous offspring by crossing hemizygous males with wild-type females. All offspring were genotyped, and only heterozygous females were used in the study.

2.2 Animal genotyping

The KAPA Mouse Genotyping Kit (KAPABIOSYSTEM, Cape Town, South Africa) was used to genotype mice according to the manufacturer's instructions. A 2 mm mouse tail biopsy was placed in 0.2 mL microcentrifuge tubes, and DNA was extracted. Two primer pair sequences obtained from the Jackson Laboratory website (The Jackson Laboratory; Bar Harbor, ME, USA) were used for polymerase chain reaction (PCR) genotyping experiments. The forward primer 5'-GGAAGCAAGTACTTCACAAGGG-3' and reverse primer 5'-GGAAAGTCACTAGGAGG-3' were specific for the transgene, while the forward primer 5-'CAAATGTTGTCTGGTG-3' and reverse primer 5'-GTCAGTCGAGTGCACAGTTT-3' were specific for the internal positive control. A thermocycler (GeneAmp® PCR System 9700, CA, USA) was used to amplify DNA under the following conditions: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 3 minutes, denaturation at 95°C for 3 minutes, denaturation at 95°C for 3 minutes, denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 2 minutes for 35 cycles. The amplicon sizes were determined using 2% agarose gel electrophoresis stained with ethidium bromide (10 mg/mL).

2.3 2-ME treatment and tumour measurements

In this experiment, mice (30 days of age) were given 100mg/kg of 2-ME in a vehicle consisting of 90% sunflower oil (Sunfoil, South Africa) and 10% dimethyl sulfoxide (DMSO) two times per week via oral gavage for four weeks before being euthanized. The vehicle alone was given to control mice. Treatment was administered a total of 8 times and began on day 30 after birth, which is before palpable tumours appeared. On average, palpable tumours appeared at day 50 after birth as determined in our laboratory (data not shown). Mammary tumours were excised, and the mass measured on a scale (Sartorius, Göttingen, Germany) at termination in grams (g). A light microscope (OLYMPUSSC 100, Dubai, UAE) was used to identify and count the number of lesions on the lung surface. CellSens dimension imaging software (XV imaging, product version 3.9, Hague, Netherlands) was used to process images. Eighteen heterozygous female mice (9 for 2-ME-treated and 9 for control) were used.

2.4 Histology and immunohistochemistry

Lung and mammary tissues were collected from both euthanized 2-ME treated and control group mice and fixed in 10% neutral buffered formalin. Haematoxylin and eosin (H&E) staining was performed as previously described (Dhanraj et al. 2021; Pitere et al. 2022), and immunohistochemical analysis for CD163 and CD3 staining was conducted with minor modifications as previously described (Dhanraj et al. 2021; Pitere et al. 2022). Formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut into 3-micron sections and baked overnight at 58°C. After deparaffinizing the slides in xylene, they were hydrated with decreasing concentrations of alcohol to distilled water. Endogenous peroxidase was quenched for 5 minutes at 37°C with a 3% hydrogen peroxide solution. After antigen retrieval with a high pH buffer retrieval solution (Dako Envision FLEX Retrieval solution high pH, Agilent Technologies, Denmark), background staining was blocked for 30 minutes at room temperature with a protein block (Novolink Leica Biosystems, Newcastle Upon Tyne, UK).The sample sections were washed in phosphate buffer saline (PBS) after being incubated overnight at 4°C in a 1:300 anti-CD163 antibody solution [EPR19518] (ab182422) (Abcam, Cambridge, UK). The antigen-antibody binding site was identified using the NovolinkTM Polymer Detection Kit (Leica Biosystems) as directed by the manufacturer. For chromogen detection, the slides were washed in PBS and incubated with 3,3′-diaminobenzidine (DAB) (NovolinkTM Polymer Kit). Sections were washed and counterstained in haematoxylin for 1 minute before being dehydrated in increasing concentrations of alcohol, cleared in xylene, and mounted with dibutylphthalate polystyrene xylene (DPX). CD3 IHC was performed on 3-micron sections in the same manner as CD163, but with a few differences. Using a low pH buffer, the antigen was retrieved (Cell Conditioning Solution CC2, Ventana Medical Systems, Inc Arizona USA). Sections were incubated in a 1:100 rabbit monoclonal anti CD3 (Abcam ab16669 clone SP7) antibody solution for 120 minutes at room temperature. Slides were rinsed in PBS and detected with anti-rabbit Polymer HRP IgG for 30 minutes at RT (NovolinkTM Polymer Detection Kit, Leica Biosystems). PBS was used as a negative control instead of CD3 or CD163 antibody. The Leica AT 2 Aperio scanner (Leica Biosystems, Nussloch, Germany) was utilized to capture images at 40X magnification, and Qupath software (The Queens University of Belfast, Northern Ireland), version 0.3.2, was used for analysis. The software was used to import each scanned tissue section, and the image type was set to brightfield (H-DAB). To focus solely on the cells within the section, a perimeter was drawn around the tissue section. The estimation stain vector was set to automatic to distinguish between positive and negative cells. The software was trained by identifying positively stained cells and indicating to it what constitutes positive cell detection. The detection image parameter was changed to "optical density sum," and the scan was initiated. The final result displayed the number of positive, negative, and total cell counts. The percentage of CD163 and CD3 positive cells was calculated by dividing the number of positive cells

by the total number of cells. The magnification of the images was 100µm (immunohistochemistry), 500µm and 800µm (histology).

2.5 Measurement of plasma cytokines

Mice were humanely euthanized with isoflurane (Isofor; Piramal I Healthcare, Mumbai, India), and 800 µL of blood was collected via cardiac puncture in ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 28766g for 15 minutes. Aliquots of plasma (250-300 µL) were placed in 2 mL microcentrifuge tubes. The Legendplex mouse inflammation panel (13-plex) kit (Biolegend®, San Diego, CA, USA) was used for cytokine profiling. The kit tests for 13 mouse cytokines MCP-1, GM-CSF, TNF-α, IFN-γ, IFN-β, IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23 and IL-27. The assay was carried out in accordance with the manufacturer's instructions. In summary, increasing standard concentrations (supplied with kit) were prepared (in duplicate) in a 96-well plate to generate a standard curve. Analyses were done using a Cytoflex flow cytometer (Beckman Coulter, California, USA), 'and Biolegend's data analysis software (https://legendplex.qognit.com/) was used to calculate cytokine concentrations.

2.6 Statistical analysis

For statistical analysis, GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) was used. A parametric one-tailed unpaired *t*-test was used to compare means between two groups. Data is presented as the mean plus standard error of the mean (SEM). A multiple comparison test and a two-way ANOVA were used to compare the means of more than two categories.

3. Results

3.1 Effect of 2-ME on the rate of tumour appearance

Seven mice in the 2-ME group and 3 mice in the control group developed palpable tumours earlier (between day 45 and day 52), while 2 mice and 6 mice in the 2-ME and control groups respectively, developed tumours later (between day 53 and day 59) (Figure 23).

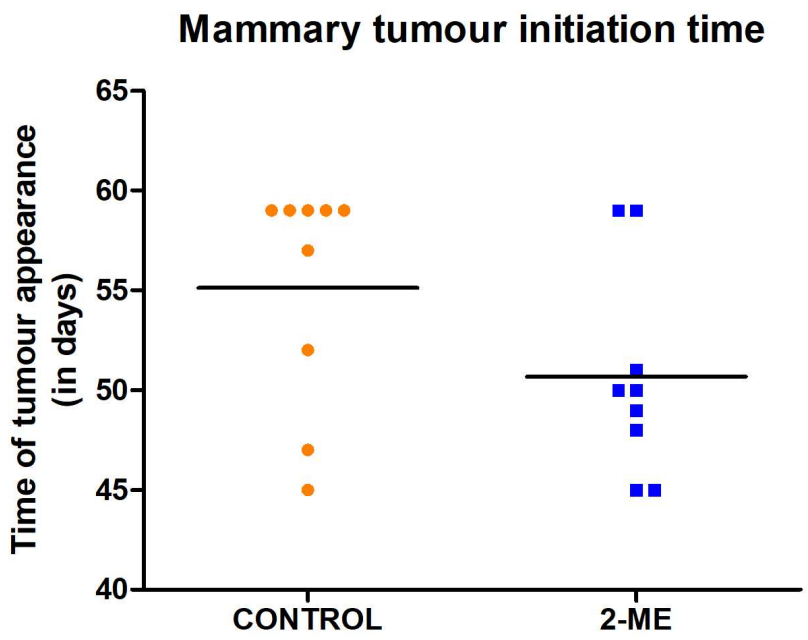


Figure 22: The time taken for mice to develop palpable mammary tumours. In the 2-ME and control groups, seven mice as opposed to three mice in the control group developed tumours earlier, respectively.

3.2 Effect of 2-ME on tumour volume and mass

The volume and mass of mammary tumours were measured at the time of termination. On average, no difference was observed in tumour volumes (Figure 24a), but tumour mass was significantly higher (p=0.0139) in the 2-ME treated group compared to the control group (Figure 24b). Importantly, the tumour mammary volumes and

masses of each mouse were totalled; the plotted dots thus represent the total tumour volumes and masses of each mouse.

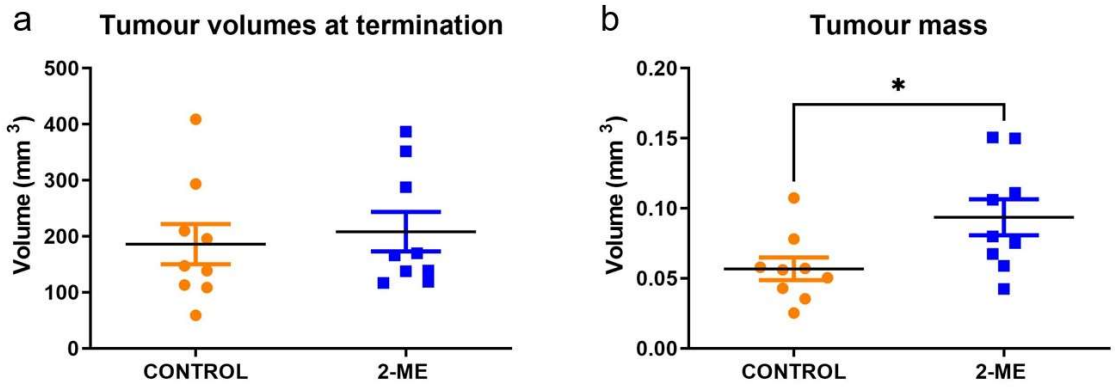
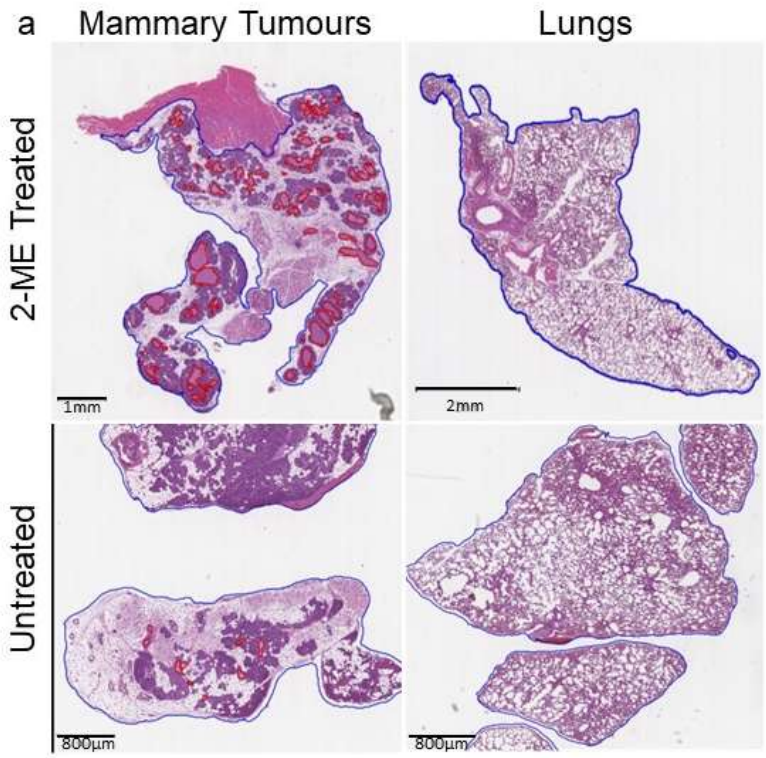


Figure 23: (a) No difference was observed in tumour volumes in the 2-ME treated and control groups ($p = 0.3319$). (b) A significantly greater tumour mass was observed in 2-ME treated mice ($p=0.0139$; $p<0.05$) (N=9 in each group).

3.3 Histopathological analysis of mammary and lung tissues

Mammary tumour and lung tissues from 2-ME treated and control groups were stained with H&E. Blue boundaries were drawn around the entire tissue region while red lines surround necrotic regions, and the arrow heads point to smaller necrotic regions (Figure 25a). Tumour necrotic tissue was greater in the 2-ME treated group compared to the control group (Figure 25b). The result was however not statistically significant. Pulmonary necrosis was minimal and was detected in 2 mice from each group with similar necrotic area percentages. The lack of difference observed was likely due to premature termination, i.e. animals were terminated before pulmonary metastasis had time to occur.



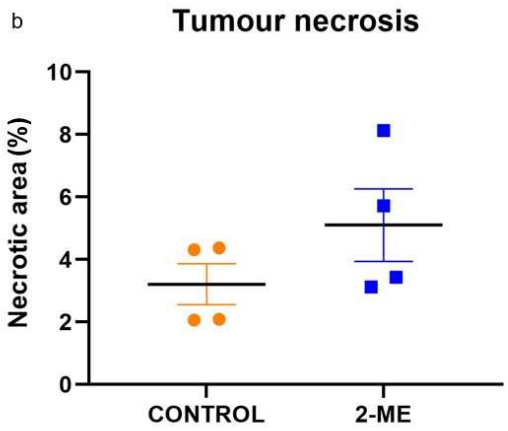
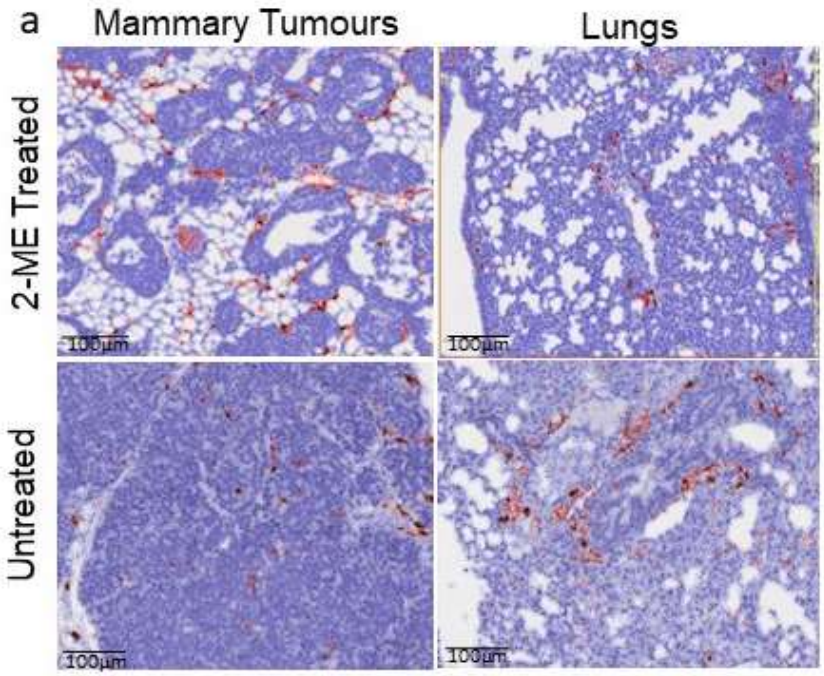


Figure 24: (A) Blue boundaries surround the tumour and necrotic regions are surrounded by red boundaries and arrows. Histology images showcasing various sizes (1mm, 2mm and 800µm) along with a scale bar. (B) More extensive ($p = 0.1031$) necrotic regions were observed in mice that were treated with 2-ME (N=4).

3.4 Immunohistochemical analysis of M2 associated CD163 macrophages

Immunohistochemistry was performed on tumours from mammary and lung tissues in each group (2-ME treated and control group). The CD163+ cells stained dark brown (Figure 25a). A lower number of CD163+ cells were detected in the mammary tumours of 2-ME treated mice compared to controls, while no change was observed in the lungs (Figure 25b-c). The result was not statistically significant.



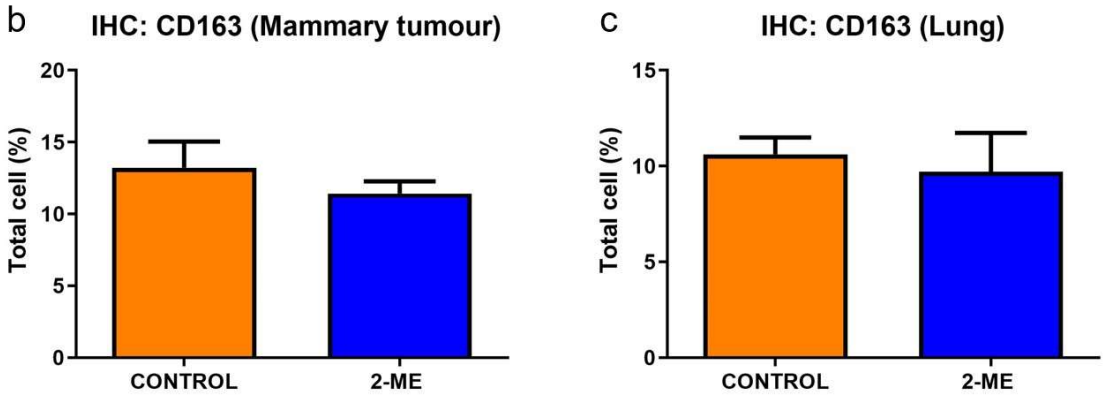
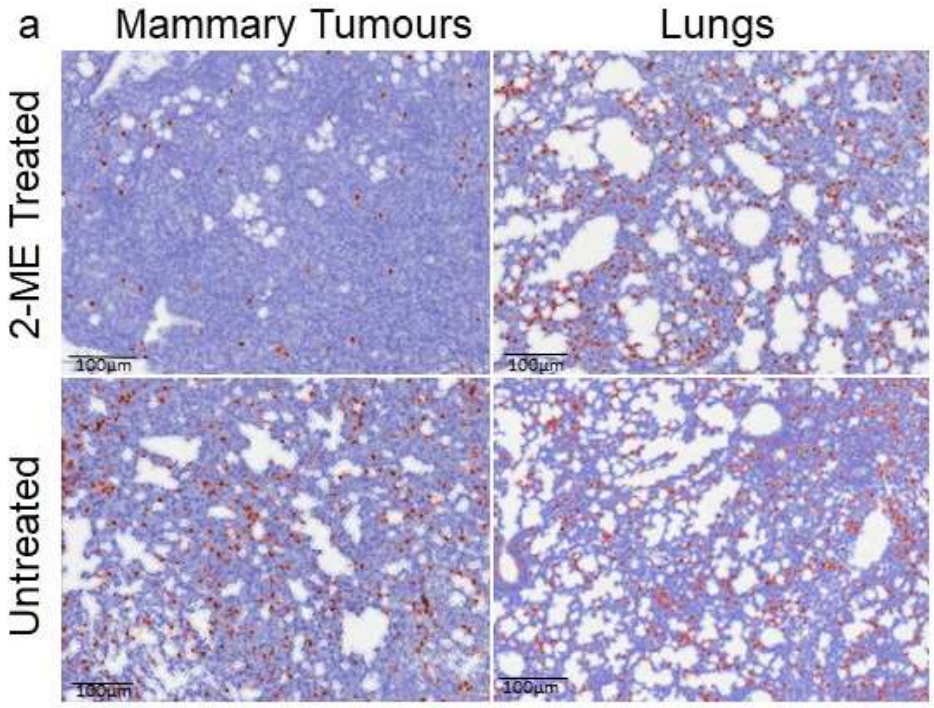


Figure 25: (a) CD163+ cells stained dark brown with red boundaries. (b) A lower number of CD163+ cells (p = 0.1965) were detected in the 2-ME group while no change was observed in the lungs (p = 0.3450) (c) (N=5 in each group). IHC images accompanied by a 100µm scale bar for reference.

3.5 Immunohistochemical analysis of CD3 positive cells

The dark brown stained cells indicate CD3+ cells (Figure 26a). The number of CD3+ cells was significantly lower in the mammary tumours of the 2-ME group (p=0.0217; Figure 26b). Similarly, fewer CD3+ cells, although not significantly different compared to the control group, were observed in the lungs of the 2-ME-treated group (Figure 26c).



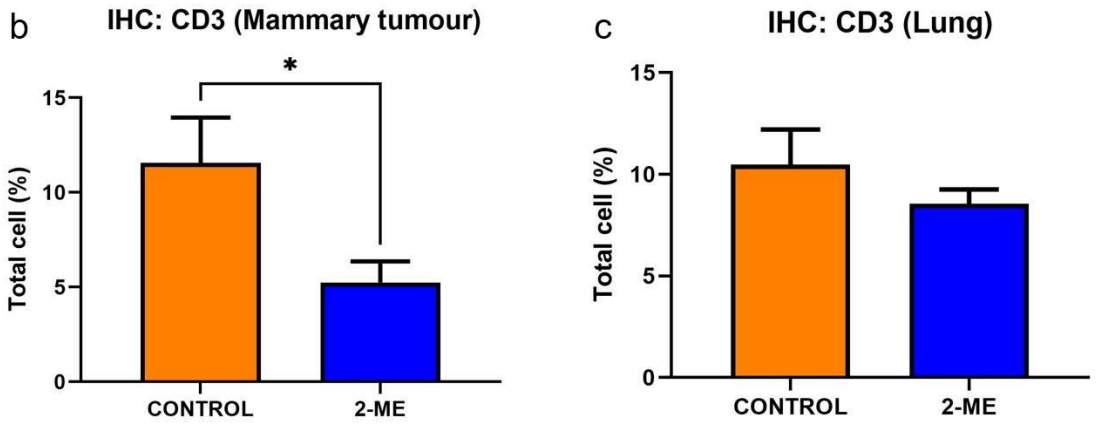


Figure 26: (a) CD3 immunohistochemistry in mammary and lung tissue. IHC images with a scale bar representing 100µm. Significantly lower number of CD3+ cells were observed in mammary tissue in the 2-ME group ($p=0.0217$) ($p=0.05$) (b) and lower number of CD3+ cells were detected in the lung tissues of the same group (c) (N=5 in each group).

3.6 Cytokine profile

Plasma cytokine levels were measured in both 2-ME treated and untreated mice. The cytokine concentrations that were similar in both groups were IL-1α (p = 0.2063), IFN-γ (p =0.4562), TNF-α (p = 0.1206), IL-17A (p = 2408) and GM-CSF. Cytokine levels that were lower in the 2-ME treated group include IL-23 (p = 0.1914), IL-12p70 (p = 0.0776), IL-10 (p = 0.3183) and IFN-β (p =0.3231). The 2-ME treated group had higher levels of IL-1β (p = 0.2182). Notably, high levels of IL-6, and IL-27 were observed in the control group (Figure 28).

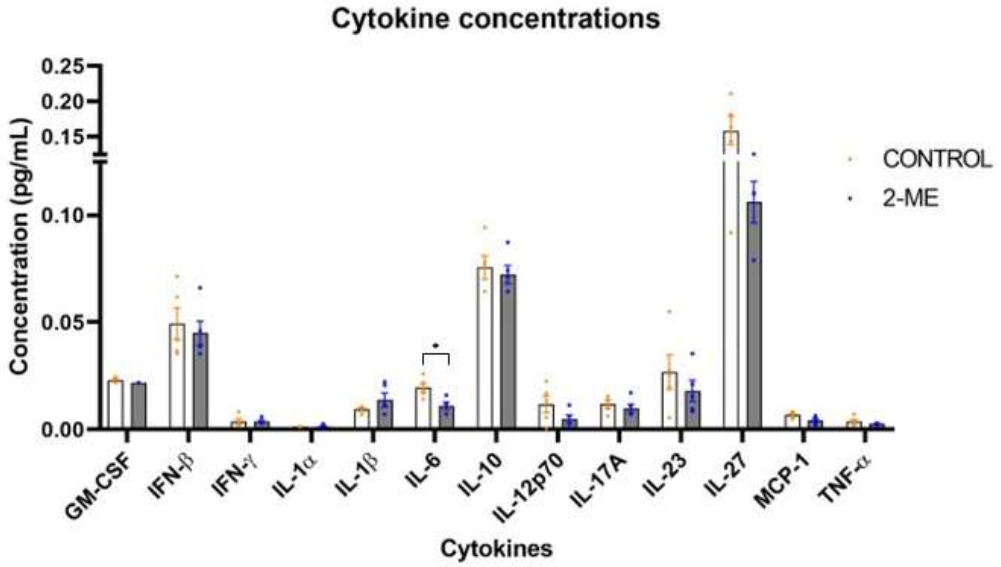


Figure 27: Cytokine profiles of 2-ME treated and control group (N=5 in each group). IL-6 was significantly higher (p = 0.0057) (p = 0.05), and IL-27 (p = 0.3317) were notably higher in the control group.

4. Discussion

In this study the effect of 2-ME was investigated when administered before the development of mammary carcinoma in a MMTV-PyVT transgenic mice that spontaneously develop palpable tumours. The mice received

2-ME (100mg/kg) treatment via oral gavage twice per week for a period of 4 weeks while the control animals received saline. By day 51, more mice in the 2-ME group (n = 7) compared to the control group (n = 3) developed early palpable mammary tumours. The findings suggest that 2-ME may be involved in promoting early mammary tumour development. In the prevention study, Huh et al. reported a similar finding whereby enhanced tumour multiplicity and growth were observed after 2-ME treatment (Huh et al. 2006). The significantly lower number of CD3+ T cells observed in the 2-ME group (p=0.0217) may have contributed to the significant (p=0.0139) tumour mass increase. High intertumoral CD3 is associated with good prognosis because of its cytotoxic activity (Rathore et al. 2014; Singh, Dees, and Grewal 2021): therefore, the low CD3+ T cell number indicates a protumour effect. 2-ME decreases CD3+ T cell proliferation, but does not affect the cytokine production of T cells (Luc et al. 2015). Tumour necrosis was observed in the 2-ME group which could be as a result of the lower CD163+ cell numbers present in the TME. A low number of CD163+ cells is associated with greater overall patient survival (MIURA et al. 2017). It is also possible that the high levels of necrosis observed in the tumours of mice given 2-ME may be more related to the drug's anti-angiogenic effects previously reported (Huh et al. 2006), rather than the low number of CD163+ cells. 2-ME may have reduced the blood supply to the tumour by inhibiting angiogenesis, resulting in hypoxia and subsequent necrosis (Pribluda et al. 2000). However, the expression of angiogenic markers was not investigated in this study and should be considered in the future. It is also plausible that together with 2-ME activity, other immune cells such as neutrophils may be implicated in necrosis (Yee and Li 2021).

Pulmonary metastasis was also investigated. The 2-ME group had no lung lesions, while two mice in the control group had one lung lesion each. This is likely because mice were terminated early. Based on the Jackson laboratory reports, about 94% of female mice develop pulmonary metastasis by three months (Guy, Cardiff, and Muller 1992). Another study observed that pulmonary metastasis in this model occurred after 10 weeks (Shishido et al. 2013). Only 2 mice, 1 from each group, had similar levels of necrotic tissue in the lungs. Both groups had a similar number of M2-associated CD163+ cells. The most plausible explanation is that the termination timepoint on day 59 is shorter than when pulmonary metastasis is anticipated in this model. The number of CD3+ T cells were fewer, although not significantly, in the lungs of the 2-ME group when compared to the control group. As previously mentioned, 2-ME inhibits CD3+ T cell proliferation. (Luc et al. 2015) Interestingly, Cimino-Mathews et al found that fewer CD3+ T cells are associated with metastatic BC, a finding that is also aligned with our observation (Cimino-Mathews et al. 2013). In this study, animals were terminated at the beginning stages of metastasis (before 10 weeks) resulting in a few cancerous cells migrating to the lungs and subsequently prompt the recruitment of CD3+ T cells to the lungs. However, these findings should be interpreted with caution, and it is recommended that future studies should allow the experimental animals to live for at least 4 months in order to adequately assess the effect of 2-ME on pulmonary metastasis when treatment is initiated before the development of primary mammary tumour.

Among the thirteen cytokines investigated, IL-6 plasma levels were significantly lower in the 2-ME group, while IL-27 exhibited notably reduced levels in the treated group. Both are pleiotropic cytokines (Yoshimoto et al. 2015; Kishimoto 2006), and an elevated level of expression is associated with BC tumour progression, therapeutic resistance, and poor prognosis (Manore et al. 2022; Gyamfi et al. 2018; Sullivan et al. 2009; Khodadadi et al. 2014; Lu et al. 2014). Low levels of IL-10, IL-12p70, IL-23, and IFN-β were observed. These cytokines are all pro-inflammatory cytokines with the exception of IL-10 which is anti-inflammatory (Xie et al. 2016). These pro-inflammatory cytokines have anti-tumour effects such as inhibiting BC proliferation (Stanilov et al. 2009; Doherty et al. 2017; Nicolini, Carpi, and Rossi 2006). Anti-inflammatory IL-10 is associated with poor prognosis, and in animal models, inhibiting IL-10 signalling hinders tumour growth (Wang et al. 2022). Additionally, IL-6 functions as an anti-inflammatory cytokine by promoting potent IL-10 cytokine production by T cells (Jin, Han, and Yu 2013). Moreover, Yasukawa et al showed that activated monocytes and macrophages produce IL-10 in response to IL-6 (Yasukawa et al. 2003). Both IL-6 and IL-10 were low in the 2-ME group, and this could possibly account for the low CD3+ and CD163+ cell numbers observed, resulting in the lower levels of these cytokines. Although, one of the outcomes is beneficial and the other pathogenic, this shows that 2-ME influences multiple immune cells that exhibit contradictory effect on BC progression. The 2-ME treated group had higher levels of pro-inflammatory IL-1β compared to controls. IL-1β is a pro-inflammatory cytokine that promotes BC growth and is associated with poor prognosis (Tulotta et al. 2021; Holen et al. 2016). Taken together, the low levels of IL-12p70, IL-23, and IFN-β combined with high level of IL-1β are likely to contribute to the tumour mass increase observed. However, the differences in these cytokines considering both the 2-ME and control groups is not noticeable let alone significant. Therefore, when considering the notably low (IL-6 and IL-27) and high (IL-10) level of cytokines as well as lower number of CD163+ cells, 2-ME may have rendered anti-tumour effects such as the greater tumour necrosis.

The findings of this study supported the findings of our initial investigations where we found that 2-ME treated mice also had higher tumour mass in early-stage BC. However, this observation was not made when 2-ME was administered to late-stage BC animals. This finding is supported by the observation of fewer CD3+ T cells in the TME, although in early-stage BC, CD3+ T cells were similar in both control and 2-ME treated groups. Late-stage BC had lower tumour mass and higher CD3+ T cell numbers. These results suggest that the number of CD3+ T cells in the TME may be important. There was also a significant increase in tumour necrosis observed in late-stage BC. Numerous studies have reported that 2-ME is anti-tumorigenic, decreasing or slowing tumour growth (Mallick, Paul, and Banerjee 2015; Klauber et al. 1997; Mabjeesh et al. 2003; Cicek et al. 2007), although in these studies, 2-ME treatment was initiated after palpable tumours were present. Additionally, the dosage and duration of 2-ME varied, ranging from 25mg/kg/day to 150mg/kg/day and from 16 days to 29 days (Mallick, Paul, and Banerjee 2015; Klauber et al. 1997; Mabjeesh et al. 2003; Cicek et al. 2007). Our results suggest that 100mg/kg of 2-ME treatment administered eight times may be optimal for promoting necrosis.

Based on the findings of this study, it is still unclear whether 2-ME can be used in individuals who are predisposed to BC. Additionally, the mechanism of action of 2-ME, especially in the different stages of BC, is not fully elucidated, and more studies are required to understand the pleiotropic effect of 2-ME in BC stages over a longer period. However, our findings do suggest that 2-ME contributes to earlier development of mammary carcinoma but the effect of tumour progression in this transgenic mouse model needs further investigation. If more mice were included in the study for a longer time to allow for pulmonary metastasis, a more conclusive result might have been observed. The significant increase in tumour mass could be the result of significantly fewer CD3+ T cells. However, there were fewer CD163+ cells and greater tumour necrosis in 2-ME treated mice. Moreover, there were no other noticeable changes in the lungs except for fewer CD3+ T cells in the 2-ME group, that could be a result of 2-ME inhibiting T cell proliferation. However, since the time was shorter than when pulmonary metastasis is expected in this model, this result should be interpreted with caution. Considering the above, 2-ME has a pro-tumour effect that has the potential to change into an anti-tumour effect if treatment is initiated early before tumour development and for a longer time (more than 28 days) before mice are terminated (between day 100 to 110 in this model) as reported in many studies (Mabjeesh et al. 2003; Huh et al. 2006; LaVallee et al. 2008). The best anti-tumour outcome was observed in late-stage BC in terms of mammary tumours, but not metastasis and longevity. Moreover, 2-ME administered with hormone and chemotherapeutic drugs such as paclitaxel, tamoxifen and doxorubicin, led to an enhanced anti-tumour effect (Nair et al. 2007; Mueck, Seeger, and Huober 2004; Azab et al. 2008). This combination may render anti-tumour effects in various stages of BC and should be considered in future experimental design.

5. Conclusion

In this study, we found that 2-ME treatment promote early palpable mammary tumour development and progression. The significant tumour mass increase occurred in response to pro-tumour cellular events, such as fewer CD3+ cells and signalling changes, such as higher IL-1β levels and lower levels of IL-12p70, IL-23 and IFN-β, in the TME. Despite this, there was evidence of anti-tumour activity such as the low number of CD163+ cells, greater necrosis, high levels of IL-10 and low levels of IL-6 and IL-27. Therefore, 2-ME promoted initiation of early tumour development while also providing anti-tumour activity at the molecular level.

Disclosure

The funders had no role in the preparation of the manuscript and the decision to publish.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Chapter 5

General discussion and conclusion

Breast cancer (BC) is the leading cancer in females and there are several treatments that increase survival including chemotherapy, radiotherapy and hormone therapy [1-2]. These treatments, however, are non-discriminatory, attacking both healthy and cancer cells, and are ineffective for BC at advanced/or metastatic stages [3]. These constraints necessitate a new therapeutic approach that will be more specific in targeting only malignant cells, such as immunotherapy and cell therapy. Mesenchymal stromal/stem cells (MSCs) have been shown to have potential as a form of cell therapy for the treatment of many diseases including cancer. In cancer studies, MSCs are shown to elicit both anti- and pro-tumorigenic immune responses in the tumour microenvironment (TME) [4]. Furthermore, Oloyo *et al*. performed an extensive systematic review on the role of MSC in tumour growth and reported that there are contradicting findings on the effect of MSCs in the TME [5]. One of the key contributors to these contradictory outcomes was the immense variation in experimental design/approach (as mentioned in chapter 2) that include xenogeneic and allogeneic models, introducing an element of genetic incompatibility which limits clinical translation. In order to overcome the genetic incompatibility associated with these models, an isogenic/syngeneic approach was used that would reduce any genetic barrier and provide better translation of the findings to the human context. 2-Methoxystradiol (2-ME) is an anti-proliferative and anti-angiogenic drug that has been proven to be effective against BC in numerous *in vitro and in vivo* studies [6] (referenced in chapter 3). However, no research study has explored the effect of 2-ME therapy on the different stages of BC development *in vivo*. Therefore, the aim of the study was to investigate the influence of mASCs and 2-ME treatment on tumour progression and metastasis *in vivo* using FVB/N-Tg(MMTV-PyVT)634Mul/J mice at different stages of BC development. This mouse model was chosen because it spontaneously develops mammary carcinoma and progression to lung metastasis.

The results from mASC treatment of BC showed there was no difference in mammary tumour volume and mass between the treated and untreated control mice. Moreover, more necrotic lesions were seen in lung but not mammary tumour in the mASC-treated mice. Furthermore, immunohistochemistry showed there were fewer CD163+ anti-inflammatory macrophages in

lung tissue but not in mammary tumour in the mASC-treated mice. CD3+ T cells were elevated in both mammary tumour and lung tissue in mASC-treated mice when compared to untreated control mice. The cytokine profile revealed that mASC-treated mice produced increased levels of circulating pro-inflammatory cytokines. The gene expression profile showed that transforming growth factor beta (*tgf-3β*), vascular endothelial growth factor receptor 1 (*vegfr1*), and endoglin (*cd105*) were downregulated in both mammary tumour and lung tissue of mice treated with mASCs. However, in mASC-treated mice, *cd36* and *metadherin (mtdh)* were only downregulated in mammary tumour and lung tissue, respectively. Downregulation of *vegfr1* and *cd105* is known to be anti-tumorigenic, while downregulation of *cd36* and *tgf-3β* is considered to be pro-tumorigenic. The downregulation of *vegfr1*, *cd105*, and *mtdh* in lung tissue is likely to produce an anti-tumour effect, which could be due to the presence of mASCs trapped in the lungs as has been shown in another study [7]. These results suggest that mASC therapy has a pleiotropic effect on BC progression *in vivo*, with pro-tumour activity in primary mammary tumours and anti-tumour activity in pulmonary metastatic tumours, resulting in reduced tumour necrosis and higher lung necrosis, respectively.

The results of 2-ME treatment in early- and late- stage BC were different. Early-stage BC treatment denotes the commencement of treatment as soon as palpable tumours were detected, whereas late-stage BC denotes advanced BC with metastases, which is 28 days after the detection of palpable mammary tumour. 2-ME therapy in early-stage BC reduced mammary tumour necrosis while promoting tumour progression. Furthermore, there were more necrotic lesions and anti-inflammatory CD163+ M2 macrophages in metastatic lung tumours of the 2-ME group compared to the untreated group. The inflammatory cytokines IFN-β, IFN-γ, IL-10, and MCP-1 were increased in mice treated with 2-ME, but IL-6 and IL-27 were decreased. Even though higher interferon levels appear to imply an anti-tumour effect, when taken together the cytokine profile suggests that 2-ME may have a pro-tumour effect in early-stage BC. On the other hand, 2-ME treatment of late-stage BC suppressed tumour development during a 28-day follow-up period, resulting in increased CD3+ T cell number and mammary tumour necrosis. In addition, 2-ME therapy delayed pulmonary metastasis but did not reduce mortality in mice with late-stage BC. Our findings suggest that 2-ME has the potential to be of benefit in late-stage BC as a result of its anti-tumour activity, while the data in early-stage BC points to a pro-tumorigenic effect.

Given the results observed for early- and late-stage BC, the effect of 2-ME on tumour initiation and progression. The findings demonstrated that administering 2-ME prior to tumour development in mice accelerated the growth of mammary carcinoma, resulting in a statistically significant (*p=0.0139*) increase in tumour mass. Furthermore, the decrease CD3+ T-cell number in the TME combined with high levels of the pro-inflammatory cytokine interleukin (IL)-1, are in line with 2-ME's observed pro-tumour activity. 2-ME treatment, on the other hand, resulted in fewer CD163+ M2 macrophages recruited into the TME, enhanced tumour necrosis, elevated plasma IL-10, and reduced plasma IL-6, and IL-27. Overall, the findings indicate that 2-ME promotes tumour formation throughout the early phases of cancer development while also providing anti-tumour activity at the molecular level, albeit in a limited capacity.

The lack of statistical significance in the study may be attributed to the study design. If the mice in the mASC group, the 2-ME early-stage BC group, and the 2-ME initiation experiment group were allowed to remain for a longer duration, it is possible that the results would have shown significant differences. Furthermore, the administration of 2_ME injection, instead of oral administration, might have been hindered by low bioavailability, thus impacting its effectiveness. In order to access the potential effects, alternative administration methods could be explored. Despite these considerations, further investigations are warranted to draw conclusive findings and access the true impact of 2-ME in these contexts.Comparing the effect of 2-ME on tumour initiation, early- and late-stage BC, the results obtained demonstrated that 2-ME enhances mammary tumour initiation and progression, and thus may not be suitable for treatment in people with familial BC or at early stages of BC development since tumour mass increased in both the tumor initiation and early BC stages. The significant increase in tumour mass in the initiation stage can be attributable to the TME having significantly fewer CD3+ T cells. However, in late-stage BC, increased infiltrating CD3+ T cells in the TME and enhanced tumour necrosis contributed to reduced tumour mass and volume compared to the control. Regarding pulmonary metastasis in all three stages, no significant anti-tumour effect was detected. Taken together, anti-tumour benefits of 2-ME treatment were demonstrated primarily in late-stage BC.

The limitations of this study includes;

I. The mASCs administered into mice were not tracked *in vivo* to assess if the observed effect on tumour growth was due to the presence of mASCs at the tumour site or due to a MSC-associated paracrine effect.

II. More in depth investigations of the various immune cells present in the TME would have provided information on the effect of the different treatments (mASCs or 2-ME) on immune cell composition or regulation in the TME, and should be considered in future studies.

III. The sample size for each experiment was 18 (9 control, 9 treated). Increasing the sample size could have significantly improved the study's power in multiple ways. It would enhance statistical precision, increase sensitivity to detect real effects, and improve the generalizability of results. Additionally, larger sample sizes lead to more reliable and robust findings, higher statistical power, and potential subgroup analysis. Therefore, a larger sample size in experiments can lead to more reliable, accurate, and generalizable results, improving the overall quality of the study and its potential impact on the scientific community.

Based on the limitations and observations of this study, recommendations for future studies includes;

I. In settings where MSC tracking technologies are not available, injecting mASCs directly into breast tumours to ensure their availability in the TME should be considered to reduce the likelihood of the stem cells becoming trapped in the lungs, and will provide a better understanding of the effect of MSCs in the TME on BC progression.

II. Regarding the 2-ME treatment of the early-BC and tumour initiation stages, mice should be allowed to live for about 35 and 56 days, respectively. That will allow for the collection of more data points to more accurately evaluate survival post-treatment.

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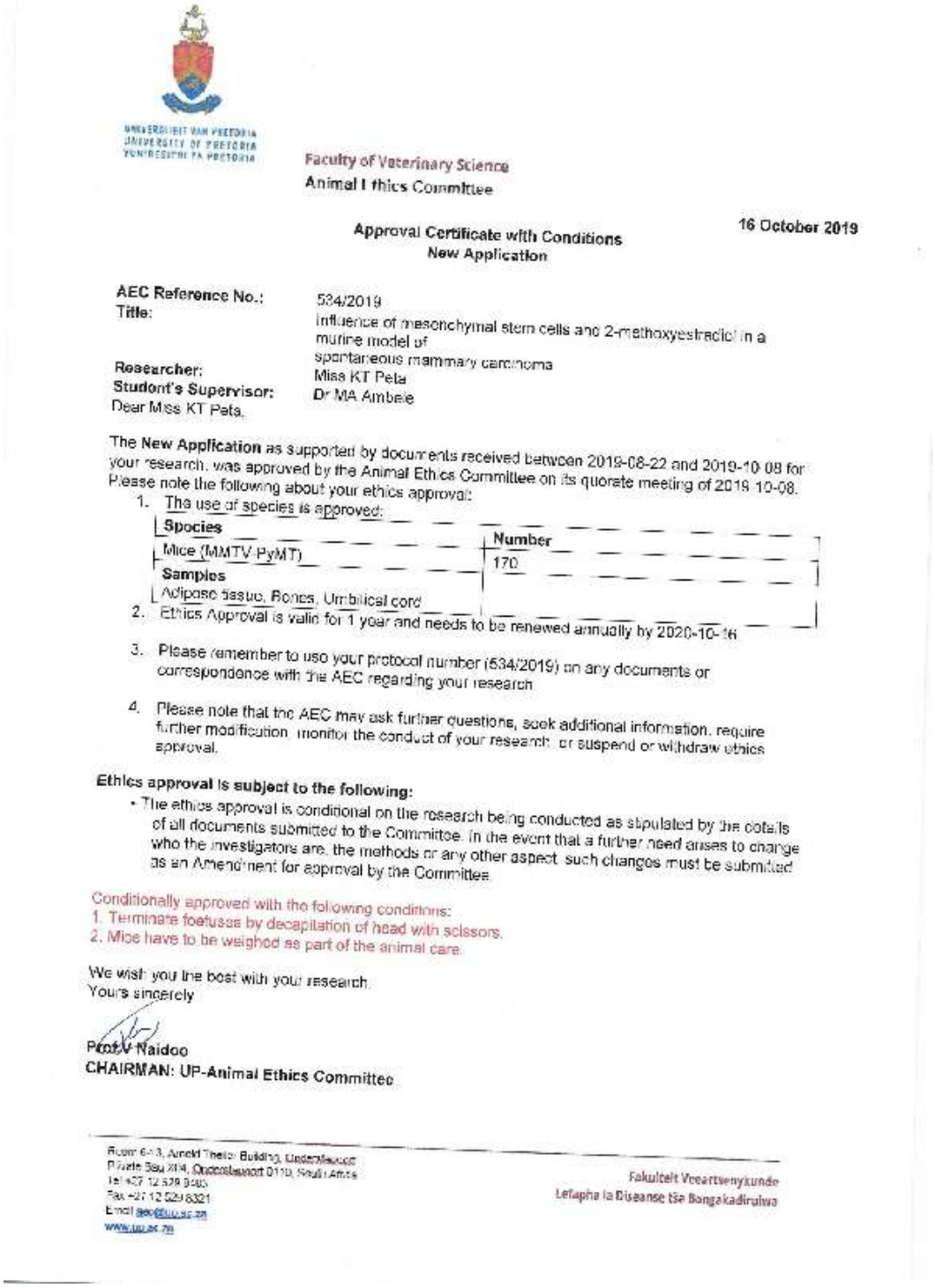
Appendix A: Ethics approval letters



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Faculty of Veterinary Science
Animal Ethics Committee

Approval Certificate with Conditions
New Application

16 October 2019

AEC Reference No.: 534/2019
Title: Influence of mesenchymal stem cells and 2-methoxyestradiol in a murine model of spontaneous mammary carcinoma
Researcher: Miss KT Pela
Student's Supervisor: Dr MA Ambele

Dear Miss KT Pela,

The **New Application** as supported by documents received between 2019-08-22 and 2019-10-08 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-10-08.
Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Mice (MMTV-PyMT)	170
Samples	
Adipose tissue, Bones, Umbilical cord	

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-10-16
3. Please remember to use your protocol number (534/2019) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Conditionally approved with the following conditions:
1. Terminate foetuses by decapitation of head with scissors.
2. Mice have to be weighed as part of the animal care.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 4-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8485
Fax +27 12 529 8321
Email [redacted]
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved on 22 May 2002 and Expires 03/20/2022.
- IRB 0000 3235 IORG0001762 Approved on 22/04/2014 and Expires 09/14/2020

16 August 2019

Approval Certificate
New Application

Ethics Reference No.: REC166-19
Title: The Effect of Mesenchymal Stromal Cells on Tumor Progression in a Mouse Model of Spontaneous Mammary Carcinoma

Dear Dr MA Ambele

The **New Application** as supported by documents received between 2019-07-18 and 2019-08-14 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-08-14.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-08-16.
- Please remember to use your protocol number (REC166-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4.00, Level 4, Tswelopele Building
University of Pretoria, Private Bag X323
Arcadia 0007, South Africa
Tel +27 (0)12 356 3084
Email deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix B: PhD approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

2 July 2019

Dr MA Ambele
Department of Medical Immunology
Faculty of Health Sciences

Dear Dr MA Ambele

STUDENT: PETA K (PhD MEDICAL IMMUNOLOGY)

TITLE: Influence of mesenchymal stem cells and 2methoxyestradiol in a murine model of spontaneous mammary carcinoma

The above-mentioned student's protocol has been approved by the PhD committee.

We wish the student all the best with her studies.

Kind regards

PROF V STEENKAMP
CHAIR: PhD COMMITTEE

Pharmacology Dept., BMS Building
University of Pretoria, Private Bag X323
Arcadia 0007, South Africa
Tel +27 (0)12 319 2321
Email: vanessa.steenkamp@up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix C: Statistics approval

Date: 18 / 6 /2019

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that,

Name(s): Ms Kimberly T Petin

from the University of PRETORIA

discussed with me the study titled Influence of mesenchymal stem cells and 2-methoxyestradiol in a murine model of spontaneous mammary carcinoma

I hereby confirm that I am aware of the project and also undertake to assist, if possible, with the Statistical analysis of the data generated from the project.

The analytical tool(s) that will be used is (are) Descriptive statistics by treatment. Two-factor ANOVA for two 2x2 factorial and for one 3x2 factorial designs. Post hoc pair-wise comparison. Possible non-parametric analysis based on ANOVA by ranks. Pls also refer to section on Statistical Considerations in protocol.

to achieve the objective(s) of the study.

Name: PJ Becker (Tel: 012-319-2203)

Signature

Research Office,
Faculty of Health Sciences, UP

BIOSTATISTICS
Faculty of Health Sciences
Research Office
2019 -06- 18
UNIVERSITY OF PRETORIA

Appendix D: Research outputs

Poster Presentations

14th International Congress of Human Genetics (ICHG); 2023; Cape Town, South Africa

Effect of 2-methoxyestradiol treatment on early and late-stage breast cancer progression in a mouse model

Kimberly T. Peta[1], Chrisna Durandt[1], Marlene B. van Heerden[2], Anna M. Joubert[3], Michael S. Pepper[1], Melvin Ambele[1,2]
1 Institute for Cellular and Molecular Medicine, Department of Immunology, South African Medical Research Council, Extramural Unit for Stem Cell Research and Therapy, Faculty of Health Sciences, University of Pretoria, Private Bag X323, Arcadia, 0007, South Africa
2 Department of Oral and Maxillofacial Pathology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, PO Box 1266, Pretoria 0001, South Africa
3 Department of Physiology, School of Medicine, Faculty of Health Sciences, University of Pretoria, Private Bag 323, Gezina, 0031, Pretoria, South Africa

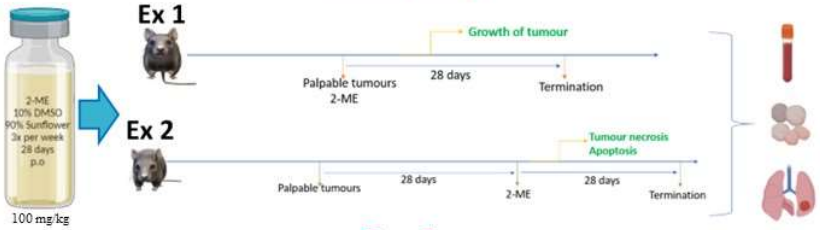
Background

Breast cancer (BC) is the leading cause of cancer deaths worldwide. *In vitro* and *in vivo* studies have shown that 2-methoxyestradiol (2-ME) has anti-angiogenic and antiproliferative effects against BC cells limiting tumour growth and metastasis.

Aim

To investigate the effect of 2-ME in early (Ex 1) and late-stage (Ex 2) BC development in transgenic mouse model (FVB/N-Tg(MMTV-PyVT)) that spontaneously develops mammary carcinoma with lung metastasis.

Methodology



Results

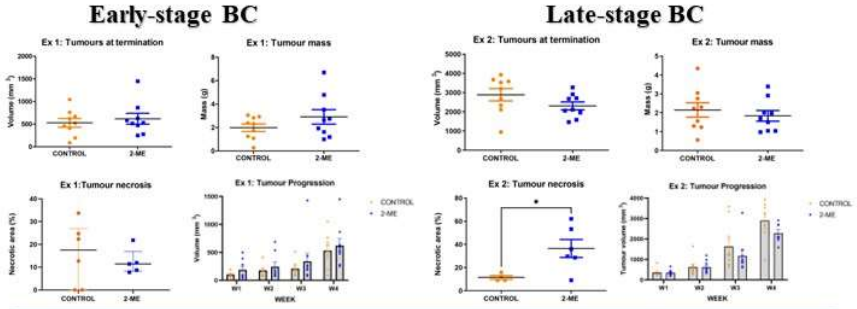


Figure 1: The 2-ME treatment inhibited tumour growth and progression in late-stage BC, but not in early-stage BC. Moreover, 2-ME significantly increased tumour necrosis (p=0.0169, p=0.05) in late-stage BC (Control N=9, 2-ME N=9).

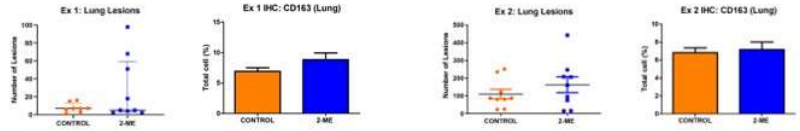


Figure 2: A higher number of pulmonary lesions observed in the 2-ME treated groups. Anti-inflammatory CD163 positive cells were higher in pulmonary metastatic tumours in early-stage BC. Although 2-ME did not increase survival of late-stage BC (Control N=9, 2-ME N=9).

Conclusion

This study demonstrates that 2-ME treatment has an anti-tumour effect on late-stage BC with no increase in survival rate, while the treatment failed to demonstrate any benefit on early-stage BC.

CRI-ENCI-AACR Sixth International Cancer Immunotherapy Conference; 2022; New York, USA

Effect of adipose mesenchymal stromal cells on mouse mammary and lung metastatic tumour formation

Kimberly T. Peta[1], Chrisna Durandt[1], Marlene B. van Heerden[2], Michael S. Pepper[1], Melvin A. Ambele[1,2]

1 Institute for Cellular and Molecular Medicine, Department of Immunology, SAMRC Extramural Unit for Stem Cell Research and Therapy, Faculty of Health Sciences, University of Pretoria, Private Bag X323, Arcadia, 0007, South Africa

2 Department of Oral Pathology and Oral Biology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, PO Box 1266, Pretoria 0001, South Africa

BACKGROUND

Breast Cancer (BC) is the most frequent cancer among women worldwide with 2.3 million new diagnosis in 2020. BC treatments such as chemotherapy, radiotherapy and surgery are non-discriminatory as it is also targeting normal cells. Thus, cellular therapy like mesenchymal stromal cells (MSC) which are suggested to home to tumour sites are considered to be a viable alternative option. However, research on the effect of MSC on BC development and metastasis has yielded many contradictory findings mainly due to sub-optimal experimental designs. For this reason, we opted for an isogenic experimental design to investigate the effect of MSC on BC *in vivo*.

AIM

Investigate the effect of mouse adipose-derived mesenchymal stem cells (mASCs) on tumour progression in vivo using an isogenic mouse model.

METHODOLOGY

Case and control study using FVB/N-Tg(MMTV-PyVT)634Mul/J transgenic mice. Mouse ASCs (mASCs) were isolated from wild type mice from the same strain.

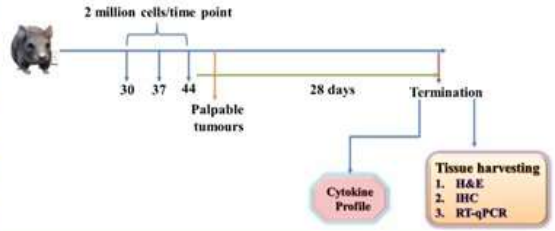


Fig. 1: Methodology flow chart

RESULTS

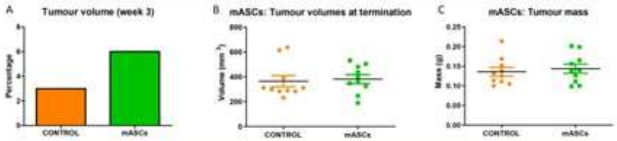


Fig. 2: mASC treated mice developed tumours notably earlier (60%) but no change was observed in mammary tumour volume and mass between both groups (n=10).

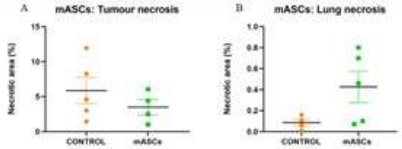


Fig. 3: mASC treated mice showed lower levels of necrosis in mammary tumours but higher necrosis in lung tumours (n=5).

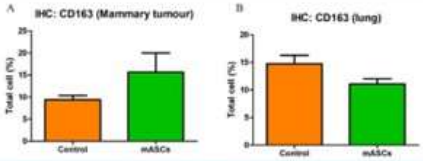


Fig. 4: The number of CD163+ stained M2-associated macrophages was higher in mammary tumours but lower in the lung tumours of mASC treated mice (n=5).

CONCLUSION

This study suggests an anti-tumour effect of mASCs in pulmonary metastatic BC but not for primary mammary tumour.

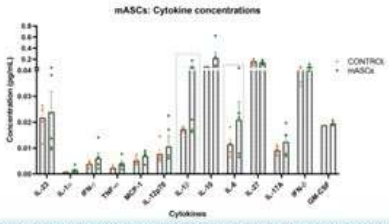


Fig. 5: All 13 cytokines measured were higher in the mASC treated mice except for IL-27. Notably, IL-1α, IL-6, IFN-γ, TNF-α, IL-12p70, IL-1β, IL-10 and IL-17A were pro-inflammatory cytokines higher in mASC treated mice.

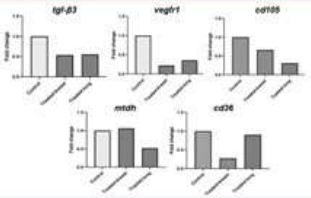


Fig. 6: The *tgf-β3*, *vegfr1* and *cd105* are all downregulated in both mammary and lung tumours of mASC treated mice. The *mtdh* and *cd36* expression in mammary and lung tissue was similar in both groups, while MMP9 was not expressed in either mammary or lung tissues in both groups.

DISCUSSION

Primary mammary tumours: mASC treatment showed no change in tumour volume and mass but led to tumour developing earlier with increased number of CD163+ cells and lower levels of necrosis necrosis compared to untreated mice, which suggests a pro-tumour effect on primary mammary tumours.

Lung metastasis: mASC treatment seems to increase necrosis and decreased in the number of CD163+ anti-inflammatory macrophages suggesting an anti-tumour effect.

A057

Appendix E: Chapter 2 manuscipt submission

[Pharmaceutics] Manuscript ID: pharmaceutics-2439502 - Submission Received

Editorial Office <pharmaceutics@mdpi.com>
to me, Kimberly, Chrisna, Marlene, Michael, Melvin

Dear Ms. Peta,

Thank you very much for uploading the following manuscript to the MDPI submission system. One of our editors will be in touch with you soon.

Journal name: Pharmaceutics
Manuscript ID: pharmaceutics-2439502
Type of manuscript: Article
Title: Effect of adipose mesenchymal stromal cells on mouse mammary and lung metastatic tumour
Authors: Kimberly Thando Peta, Chrisna Durandt, Marlene B VanHeerden, Michael Sean Pepper, Melvin Anyasi Ambele *
Received: 23 May 2023
E-mails: u10432885@tuks.co.za, chrisna.durandt@up.ac.za, marlene.vanheerden@up.ac.za, michael.pepper@up.ac.za, melvin.ambele@up.ac.za
Submitted to section: Gene and Cell Therapy,
https://www.mdpi.com/journal/pharmaceutics/sections/Gene_Cell_Therapy
The Roles of Mesenchymal Stromal or Stem Cells in Cancer Therapy
https://www.mdpi.com/journal/pharmaceutics/special_issues/ZRP6992R4W

Appendix F: Chapter 3 manuscript submission

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Melvin Ambele <melvin.ambele@up.ac.za>

Cell Biochemistry & Function - Manuscript number CBF-23-0192

Editorial Office Cell Biochemistry & Function <onbehalfof@manuscriptcentral.com> 10 May 2023 at 06:40

Reply-To: cbfeo@wiley.com
To: kimberlypeta23@gmail.com
Cc: kimberlypeta23@gmail.com, chrisna.durandt@up.ac.za, marlene.vanheerden@up.ac.za, annie.joubert@up.ac.za, michael.pepper@up.ac.za, melvin.ambele@up.ac.za

10-May-2023

Dear Dr. Peta,

Your manuscript entitled "Effect of 2-methoxyestradiol treatment on early- and late-stage breast cancer progression in a mouse model" has been successfully submitted online and is presently being given full consideration for publication in Cell Biochemistry & Function.

Your manuscript number is CBF-23-0192. Please mention this number in all future correspondence regarding this submission.

If you submitted this manuscript through our Research Exchange site, you can view the status of your manuscript by logging into the submission site at wiley.atyponrex.com/journal/cbf.

If you submitted this manuscript through ScholarOne, you can view the status of your manuscript by checking your Author Center after logging in to https://mc.manuscriptcentral.com/cbf.

This journal offers a number of license options for published papers; information about this is available here: https://authorservices.wiley.com/author-resources/Journal-Authors/licensing/index.html. The submitting author has confirmed that all co-authors have the necessary rights to grant in the submission, including in light of each co-author's funder policies. If any author's funder has a policy that restricts which kinds of license they can sign, for example if the funder is a member of Coalition S, please make sure the submitting author is aware.

Thank you for submitting your manuscript to Cell Biochemistry & Function.

Sincerely,

Cell Biochemistry & Function Editorial Office

Appendix G: Chapter 4 manuscript submission

FEBS Open Bio - Manuscript ID FEBSOPEN-23-0334 Inbox ×

openbio@febs.org FEBS Open Bio Editorial Office <onbehalfof@manuscriptcentral.com> 01:16 (7 minutes ago)
to me, chrisna.durandt, marlene.vanheerden, annie.joubert, michael.pepper, melvin.ambele

30-May-2023

Dear Miss Peta,

Thank you for submitting your manuscript entitled "Effect of 2-methoxyestradiol on mammary tumour initiation and progression" by Peta, Kimberly; Durandt, Chrisna; van Heerden, Marlene; Joubert, Anna; Pepper, Michael; Ambele, Melvin to FEBS Open Bio. The manuscript is presently being given full consideration for publication in FEBS Open Bio.

Co-authors: Please contact the Editorial Office as soon as possible if you disagree with being listed as a co-author for this manuscript. If you have an ORCID iD, we encourage you to link it to your ScholarOne account. To do this, please log in to ScholarOne at https://mc.manuscriptcentral.com/febsopen. Please then click on your name on the right-hand side of the black banner at the top of the page, and select 'Email/Name' in the dropdown menu that appears. Please then select 'Associate your existing ORCID iD' and follow the on-screen instructions. There is also the option to create an ORCID iD on this page if you do not have one already.

Your manuscript ID is FEBSOPEN-23-0334.

Please mention the above manuscript ID in all future correspondence or when calling the office. If there are any changes in your street address or e-mail address, please log in to ScholarOne Manuscripts at https://mc.manuscriptcentral.com/febsopen and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Center after logging in to https://mc.manuscriptcentral.com/febsopen.

Thank you for submitting your manuscript to FEBS Open Bio.

Sincerely,

FEBS Open Bio Editorial Office
openbio@febs.org