A comparison of compounded-bioidentical hormone formulations versus FDA-approved hormone formulations in breast cancer progression

By

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Declaration of Originality

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Abstract

Introduction:

Oestrogen and oestrogen receptor-induced signalling plays an important role in breast cancer development and progression. Studies have shown that certain menopausal hormone therapies (MHTs) containing oestrogens and oestrogens in combination with progestogens, increase the risk of invasive breast cancer. Compounded-bioidentical hormone therapies (cBHTs), not FDA-approved or regulated by the Medicines Control Council of South Africa, have become a popular MHT and are advertised as safer efficient alternatives. Oestrogen alone and in combination with progestogens such as medroxyprogesterone acetate (MPA) and norethindrone (NET) enhance breast cell proliferation, migration and invasion. It is therefore important to determine the effects of compounded oestrogen formulations in the development and progression of breast cancer. This study aims to provide a comparative profile of the effects of traditional menopausal therapies (estrone + MPA and estrone + NETA), an FDA-approved bioidentical formulation (oestradiol + progesterone (bE_2+bP_4)) with the compounded bioidentical biest hormone formulation E_2 + estriol (bE_2+bE_3) on the progression of breast cancer.

Methods:

Human ER^+ mammary adenocarcinoma cells (MCF7) were used. Proliferation was assessed by determining the cell viability through water-soluble tetrazolium salt (WST-1) assays. The cell cycle was analysed with flow cytometry. Western blot analyses were performed to assess the proliferation marker MCM2, the PI3K/Akt signalling pathway and epithelial-to-mesenchymal transition (EMT) markers; E-cadherin, N-cadherin, Snail and β-catenin. Migration was measured through a wound healing assay.

Results and discussion:

All treatment combinations significantly increased cancer cell viability. The cell cycle analysis shows that FDA-approved estrone + MPA and estrone + NETA treatments induced the accumulation of MCF7 cells in the G_0/G_1 phase of the cell cycle. Western blot analysis revealed that all hormone treatments did not activate the PI3K/Akt pathway. Furthermore, treatment of BE_2 + BP_4 indicated mesenchymal characteristics of EMT. The wound closure assay showed

that the hormone treatments did not induce migration.

Conclusion:

According to our findings, there are both similarities and differences among the compounded biest combinations and FDA-approved hormone formulations. Concerningly, cBHT increases cell viability in a manner consistent with the FDA-approved formulations. Similar to FDA-approved therapies, they did not cause migration or activate the Akt pathway for cell proliferation. In contrast, when compared to their FDA-approved counterparts, cBHT formulations exhibited different effects on EMT and the cell cycle. All together these results demonstrate that cBHT treatments did not stimulate the pathways associated with breast cancer progression that was stimulated by the FDA-approved formulations. Future recommendations include investigating the effects of cBHT preparations on other pathways involved in breast cancer initiation and progression in comparison to the FDA-approved formulations.

Inleiding:

Estrogeen- en estrogeenreseptor-geïnduseerde seinoordrag speel 'n belangrike rol in borskanker ontwikkeling en bevordering. Studies het getoon dat sekere menopousale hormoonterapieë (MHT's) wat estrogeen en estrogeen in kombinasie met progestogene bevat, die risiko van indringende borskanker verhoog. Saamgestelde-bioidentiese hormoonterapieë (cBHTs), wat nie deur die FDA goedgekeur of gereguleer is deur die Medisynebeheerraad van Suid-Afrika nie, het 'n gewilde MHT geword, en word as veiliger doeltreffende alternatiewe geadverteer. Estrogeen alleen en in kombinasie met progestogene soos medroxyprogesteroon asetaat (MPA) en norethindrone (NET) verhoog bors sel proliferasie, migrasie en indringing. Dit is dus belangrik om die uitwerking van saamgestelde estrogeenformulerings in die ontwikkeling en vordering van borskanker te bepaal. Hierdie studie het ten doel om 'n vergelykende profiel te verskaf van die effekte van tradisionele menopousale terapieë, nl. (estrone + MPA en estrone + NETA), 'n FDA-goedgekeurde bioidentiese formulering (oestradiol + progesteroon (bE2+bP4)) met die van saamgestelde bioidentiese biest hormoonformulering E2 + estriol (bE2+bE3) op die vordering van borskanker.

Metodes:

Menslike ER+ borsadenokarsinoomselle (MCF7) is gebruik. Proliferasie is geassesseer deur die sellewensvatbaarheid deur wateroplosbare tetrazolium sout (WST-1) toetse te bepaal. Die selsiklus is met vloeisitometrie geanaliseer. Westelike klad ontledings is uitgevoer om die proliferasiemerker MCM2, die PI3K/Akt seinweg en epiteel-na-mesenkiemale oorgangsmerkers (EMT) te assesseer; E-cadherin, N-cadherin, Slac en b-catenin. Migrasie is gemeet deur 'n wondgenesingstoets.

Resultate en bespreking:

Alle behandelingskombinasies het die lewensvatbaarheid van kankerselle aansienlik verhoog. Die selsiklus analise toon dat FDA-goedgekeurde estrone + MPA en estrone + NETA behandelings die ophoping van MCF7 selle in die G0/G1 fase van die selsiklus geïnduseer het. Westelike klad-analise het aan die lig gebring dat alle hormoonbehandelings nie die PI3K/Akt-weg geaktiveer het nie. Verder het behandeling van BE2 + BP4 mesenkiemale kenmerke van EMT aangedui. Die wondgenesingstoets het getoon dat die hormoonbehandelings nie migrasie

veroorsaak het nie.

Gevolgtrekkings:

Volgens ons bevindinge is daar beide ooreenkomste en verskille tussen die saamgestelde biest-kombinasies en FDA-goedgekeurde hormoonformulerings. Wat betref, cBHT, verhoog die lewensvatbaarheid van selle op 'n wyse wat ooreenstem met die FDA-goedgekeurde formulerings. Soortgelyk aan FDA-goedgekeurde terapieë, het hulle nie migrasie veroorsaak of die Akt-weg vir selproliferasie geaktiveer nie. In teenstelling met hul FDA-goedgekeurde eweknieë, het cBHT-formulerings verskillende effekte op EMT en die selsiklus getoon. Saam demonstreer hierdie resultate dat cBHT-behandelings nie die weë gestimuleer het wat verband hou met borskankerprogressie wat deur die FDA-goedgekeurde formulerings gestimuleer is nie. Toekomstige aanbevelings sluit in die ondersoek na die uitwerking van cBHT-preparate op ander weë wat betrokke is by borskankerinisiasie en -bevordering ondersoek in vergelyking met die FDA-goedgekeurde formulerings.

Table of Contents

List of Abbreviations:

A

Akt	RAC-alpha serine/threonine-protein kinase (Protein kinase B)
AMH	Anti-müllerian hormone

B

BAD	BCL2-associated agonist of cell death
bE_1	Bioidentical estrone
bE_2	Bioidentical oestradiol
bE_3	Bioidentical estriol
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSL-2	Biosafety level-2

C

CAF	Central analytical facilities
cBHT	Compounded bioidentical hormone therapy
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEE	Conjugated equine oestrogen
CHD	Coronary heart disease
CK2	Casein kinase 2
CoA	Coenzyme A
COC	Combined oral contraceptive
CREB	cAMP-response element binding protein
c-Src	Cellular proto-oncogene tyrosine-protein kinase

D

DHEA	Dehydroepiandrosterone
DHEQ	17 alpha-dihydroequilin
DMEM	Dulbecco's Medium Eagle Modified

E

E_1	Estrone
E_2	Oestradiol
E_3	Estriol
E_4	Estetrol
ECM	Extracellular matrix remodelling
EDTA	Ethylenediamine tetraacetic acid
EE	Ethinylestradiol
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
EDFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPT	Oestrogen-progesterone therapy
ER	Oestrogen receptor
ERE	Oestrogen response element
ERK	Extracellular signal-regulated kinase
ER-α	Oestrogen receptor alpha
ER-β	Oestrogen receptor beta
E3N	Etude Epidémiologique auprès de femmes de la Mutuelle Générale de l'Education Nationale
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1

F

FBS	Fetal bovine serum
FDA	Food and drug administration
FGFR	Fibroblast growth factor-receptor
FMP	Final menstrual period

FoxO	Forkhead box O
FSH	Follicle stimulating hormone

G

GDF	Growth and differentiation factor
GPR-30/GPER1	G protein-coupled receptor-30
GSK-3	Glycogen synthase kinase 3
GSK-3β	Glycogen synthase kinase 3β
GSM	Genitourinary syndrome of menopause

H

HER2/ERBB	Human epidermal growth factor receptor
HGF	Hepatocyte growth factor
HR	Hormone receptor
HT	Hormone therapy

I

IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor

L

LEF	Lymphocyte enhancer factor 1
LH	Luteinising hormone
LNG-IUS	Levonorgestrel releasing-intrauterine system

M

MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MEK	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition
MHT	Menopausal hormone therapy
MMC	Mitomycin C

MPA	Medroxyprogesterone acetate
mPR	Progesterone membrane receptor
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2

N

NaF	Sodium fluoride
Na_3VO_4	Sodium orthovanadate
NCOR1	Nuclear receptor corepressor 1
NET	Norethindrone
NETA	Norethindrone acetate
NF-κB	Nuclear factor kappa B
nM	Nanomolar

P

P_5	Pregnenolone
P_4	Progesterone
PBS	Phosphate buffered saline
PDK-1	phosphoinositide-dependent kinase 1
PFA	Paraformaldehyde
PGMRC1	Progesterone receptor membrane component 1
PI3K	Phosphoinositide 3-kinase
PI3KCA	Phosphoinositidylinositol-4,5-bisphosphate3-kinase catalytic subunit alpha
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PMSF	Phenylmethylsulphonyl fluoride
POI	Premature ovarian insufficiency
PR	Progesterone receptor

PRE	Progesterone response element
PR-A	Progesterone receptor A
PR-B	Progesterone receptor B
PTEN	Phosphatase and tensin homolog
p70 S6K	p70 ribosomal S6 kinase

R

RANK	Receptor activator of NF-κB
RANKL	NF-κB ligand
RB1	Retinoblastoma
RIPA	Radioimmunoprecipitation
RT	Room temperature
RTK	Receptor tyrosine kinase

S

SAC	Spindle assembly checkpoint
SEM	Standard error of the mean
SERM	selective-oestrogen receptor modulator
Slug	SNAI2
SMAD	Suppressor of Mothers against Decapentaplegic
Snail	SNAI1
STAT	Signal transducer and activator of transcription
STRAW	Stages of Reproductive Aging Workshop
α-SMA	alpha-smooth muscle actin

T

TBS-T	Tris-buffered saline - Tween® 20
TCF	T-cell factor
TF	Transcription factor
TGF-β	Transforming growth factor-β
TNBC	Triple negative breast cancer
TSC	Tuberous sclerosis complex

TβRI	Transforming growth factor-β Type I receptor
TβRII	Transforming growth factor-β Type II receptor

V

VMS	Vasomotor symptoms

W

WST-1	Water-soluble tetrazolium salt
WHO	World health organization

X

XIAP	X-linked inhibitor of apoptosis

Z

ZEB1	Zinc finger E-box binding homeobox 1

List of Figures

List of Tables

CHAPTER 1: LITERATURE REVIEW

1.1. BREAST CANCER: INTRODUCTION

In 2020, 9.6 million deaths worldwide were attributed to cancer, with a peak incidence between the ages 45 and 65 (Ortega *et al.,* 2020; WHO, 2021). Cancer, which is an umbrella term for a large group of diseases that affect various organs and tissues, is the world's leading cause of death (Saha Roy & Vadlamudi, 2012; WHO, 2018). It is caused by various factors such as exposure to radiation, alcohol and tobacco use (Table 1.1). Breast cancer is the most frequently diagnosed cancer worldwide, with lung cancer being the leading cause of cancer-related mortality (WHO, 2021). In Africa, the most prevalent type of cancer in women is breast cancer (WHO Cancer Regional Profile, 2020). Recent statistics show a rapid increase in cancer incidence and mortality, both globally and in sub-Saharan Africa. The global cancer burden is expected to be 28.4 million cases in 2040, a 47 % increase from 2020 (Sung *et al.,* 2021; WHO, 2021). Additionally, sub-Saharan African mortality rates are currently among the highest in the world, which are largely attributable to late-stage presentation, where 77 % of all staged cases were stage III/IV at diagnosis (Sung *et al.,* 2021). Early detection of symptoms and appropriate treatment approaches are considered to have the potential to avert between 28 % to 37 % of breast cancer fatalities (Sung et al., 2021). Should more resources within health services not be implemented, a rise in cancer incidence will likely be accompanied by increases in mortality. Appropriate education and increased breast cancer awareness campaigns, as well as early identification and optimal treatment and management protocols should be implemented to combat the growing number of cancers cases.

Table 1.1.: Cancer risk factors

Cancer Risk Factors	
Physical inactivity	Hormonal changes
Alcohol and tobacco use	Reproductive factors
Personal or family history of cancer	Hormone therapy use
Genetics	Infections

Age	Exposure to radiation
Diet	Chemical exposure

As 50 % to 70 % of cancers are considered preventable, primary prevention remains an effective way to reduce cancer incidence and mortality (Sung et al., 2021; WHO, 2018). This can be accomplished by evading risk factors, implementing existing evidence-based prevention strategies and identifying novel prevention approaches. It is therefore crucial to conduct additional research into the molecular mechanisms of risk factors that may contribute to the development of cancer. For example, the report by Thomas Beatson in 1896 revealed that an oophorectomy (removal of one or both ovaries) resulted in the improvement of breast cancer patients, uncovering the stimulatory effects of oestrogen before the hormone was discovered (Love & Philips, 2002). Additionally, Charles Huggins discovered a drastic regression of metastatic prostate cancer after an orchiectomy (removal of one or both testicles) (Glina *et al.,* 2010). These discoveries laid the foundation for the modern use of hormone therapies such as tamoxifen, aromatase inhibitors and luteinizing hormone-releasing hormone analogues and inhibitors, which have greatly changed the way prostate and breast cancers are treated.

Although advances in our understanding of the molecular dynamics of breast cancer has greatly improved the development of preventative and treatment techniques, such as the design of selective-oestrogen receptor modulators (SERMs), many molecular mechanisms remain to be elucidated. There are currently, strong associations between hormonal risk factors like early menarche, late onset menopause, menopausal hormone therapy, and childbirth (parity) and the most prevalent breast cancer subtype, luminal A (Ellingjord-Dale *et al.,* 2017; Cenciarini & Proietti, 2019; American Cancer Society, 2020; Cotul *et al*., 2020). This warrants further investigation on the mechanisms associated with the hormonal and reproductive impact on the onset and progression of breast cancer.

1.1.1. Breast Cancer Classifications

Breast cancer is genetically and clinically heterogenous, thus, the ability to produce targeted treatments and predict their outcomes is limited (Malhotra *et al.,* 2010). The identification of

breast cancer molecular subtypes along with clinicopathological variables including tumour size, type and grade are used to determine patients which are most likely to respond to targeted therapies, and is utilised to predict prognosis (Malhotra *et al.*, 2010; Provenzano *et al*, 2018).

Cancer is categorised based on the tissues in which they originate. Adenocarcinoma is a type of cancer that originates in the glands that line the insides of organs. In breast cancer, adenocarcinomas originate in the ducts or lobules of the breast. According to the WHO classification of tumours of the breast, there are 19 different major histological subtypes (Harbeck *et al*., 2019; World Health Organization, 2019). The most common types are invasive ductal carcinoma (invasive carcinoma of no special type) and invasive lobular carcinoma (Waks & Winer, 2019). Other less common breast cancer types, include Paget's disease, medullary mucinous, ductal carcinoma *in situ* and tubular carcinoma (Sinn & Kreipe, 2013). There are many variables that contribute to the classification and categorization of breast tumours, all of which are critical in providing information on the prognosis and treatment of patients.

Four major intrinsic subtypes of breast cancer exist, namely luminal A-like, luminal B-like, human epidermal growth factor receptor positive (HER2+/ERBB) and triple negative breast cancer (TNBC) (Figure 1.1) (Bernhardt *et al*., 2016; Provenzano *et al*., 2018; Tong *et al*., 2018). Luminal breast cancers are oestrogen receptor (ER) positive, with luminal A being negative for HER2 and luminal B either being progesterone receptor (PR) negative or positive and/or HER2 positive (Johansson *et al.*, 2019; Provenzano *et al*., 2018). TNBC are typically synonymous with Basal-like cancers as they both lack hormone receptors (HR) and HER2, however not all TNBC's are basal-like.

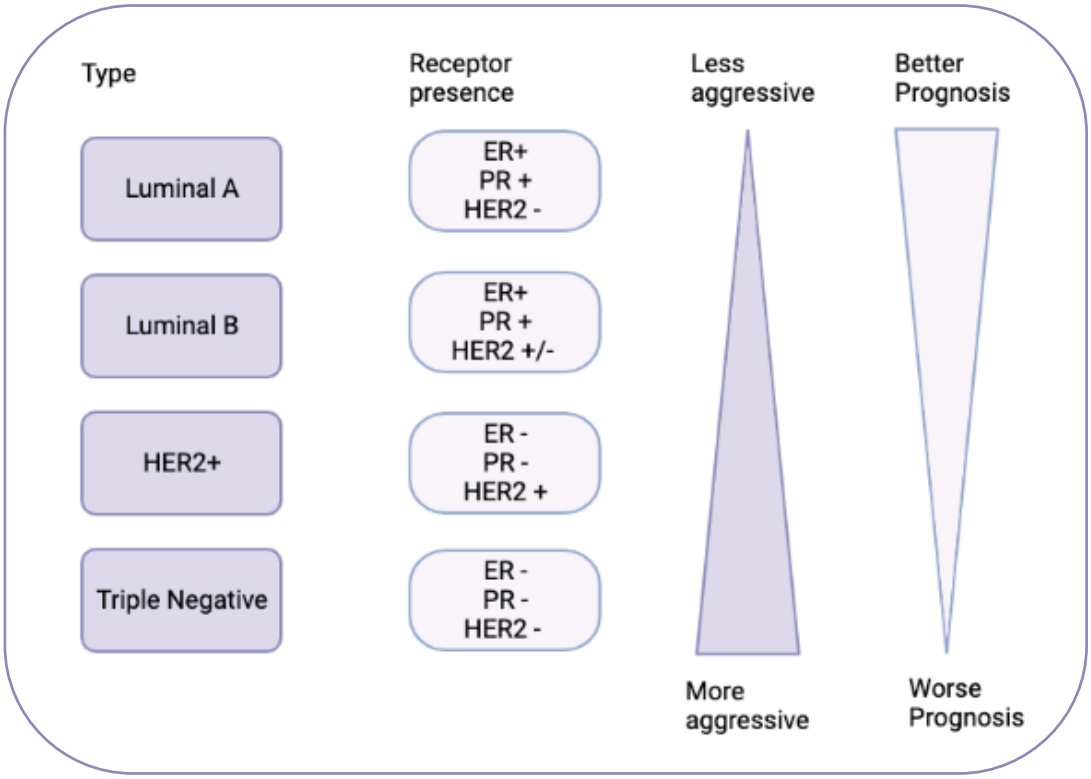


Figure 1.1.: Breast cancer subtypes; Abbreviations: ER – oestrogen receptor, HER – human epidermal growth factor receptor, PR – progesterone receptor. (Adapted from Provenzano *et al.,* 2018) (Created with BioRender.com)

Each of the molecular subtypes present with different risk factors for their incidence, therapeutic response, disease progression, and site of metastasis. Luminal A tumours, which account for 73 % of breast cancer cases, are characterised as slow growing and less aggressive (American Cancer Society, 2020). Moreover, the diagnosis is associated with a favourable prognosis in part because luminal A tumours are more responsive to hormone therapy (American Cancer Society, 2020; Provenzano *et al.*, 2018; Tong *et al.*, 2018). Similarly, the widespread use of targeted therapies for HER2+ tumours has significantly improved patient outcomes for prognosis and overall survival rate. In contrast to Luminal A, Luminal B tumours are higher grade and are associated with a poorer prognosis, presenting with a high expression of the proliferation marker Ki67 and a lower expression of oestrogen and progesterone receptors (Bernhardt *et al.,* 2016). Basal-like or TNBC's have a poorer prognosis than the other molecular subtypes and are more aggressive and difficult to treat, lacking targeted therapies. Interestingly, there is a high prevalence of TNBC diagnosis among African and African American

women, and a diagnosis is also more common in premenopausal women (American Cancer Society, 2020; Harbeck *et al.*, 2019; Waks & Winer, 2019).

1.1.2. Hallmarks of Cancer

Cellular activities that are crucial to the maintenance of multicellular organisms, such as growth, differentiation, apoptosis, and tissue integrity, are often dysregulated in cancer cells (Mareel & Leroy, 2003). As cells transition from normal growth and development to neoplastic growth and progression, they acquire a set of functional capabilities. These are recognized as the hallmarks of cancer and are central to the development of malignant tumours (Fouad & Aanei, 2017; Hanahan, 2022). There are eight hallmark capabilities and two enabling characteristics, with more recent literature proposing emerging hallmarks and enabling characteristics (Figure 1.2) (Hanahan, 2022). For the scope of this thesis, two hallmarks will be reviewed, namely the sustained proliferative signalling and activating invasion and metastasis.

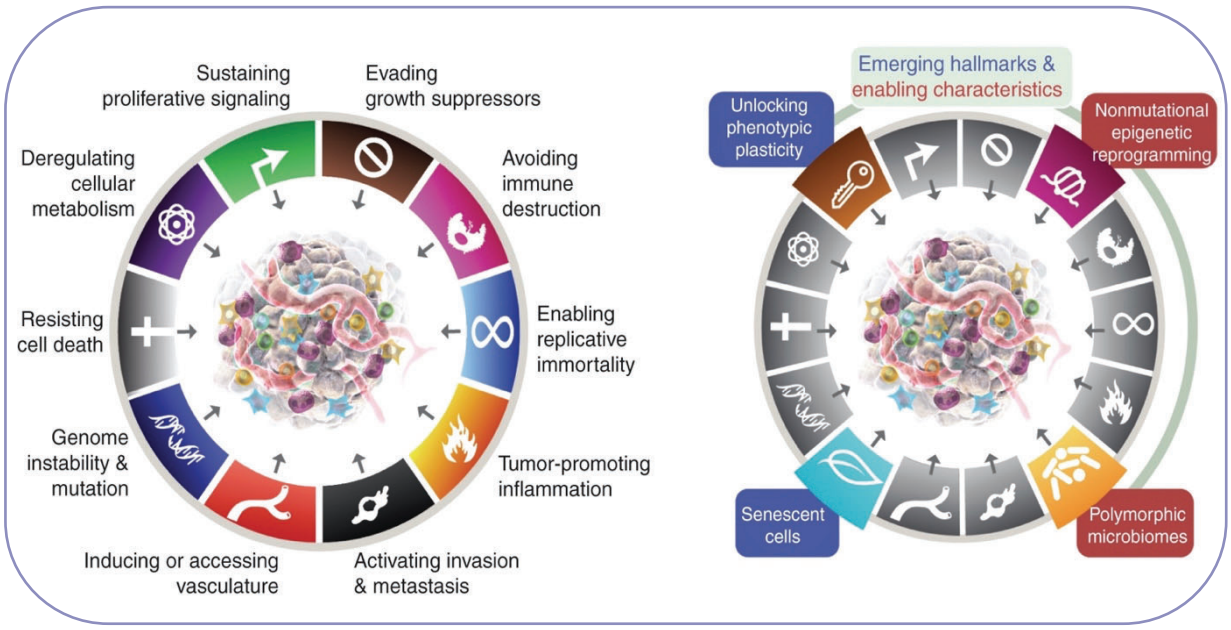


Figure 1.2.: Hallmarks of Cancer. Left: Currently there are eight hallmark capabilities and two enabling characteristics; tumour-promoting inflammation and inducing or accessing vasculature. Right: Current proposed emerging hallmarks and enabling characteristics. (Hanahan, 2022)

1.1.2.1. Sustaining Proliferative signalling

Modifications to intracellular signalling pathways promote tumour proliferation, secondary site invasion and metastasis as well as tumour cell survival. These modifications result from mutations in oncogenes that overexpress certain proteins, mutated proteins that present uncontrolled activity, or inactivation of tumour suppressor genes that support these processes (Ortega *et al*., 2020). Various alterations in the phosphoinositide 3-kinase/RAC-alpha serine/threonine-protein kinase/ mammalian target of rapamycin complex 2 (PI3K/Akt/mTOR) pathway have been found in up to 60 % of all human tumours, including breast cancer. Its dysregulation is associated with the development of several cancer hallmarks, including sustained proliferation, genomic instability, metabolic reprogramming, evasion of apoptosis and metastasis (Wang *et al*., 2018; Ortega *et al*., 2020).

Sustained proliferative signalling: PI3K/Akt signalling pathway activation

Given its association with cancer, the family of lipid kinases known as PI3K's are of interest. They regulate key cellular processes required for homeostasis. The protein kinase B (PKB) also known as Akt, is a downstream target in the PI3K signalling pathway. The activation of the PI3K signalling pathway is dependent on the binding of hormones, growth factors, or other extracellular stimuli, to their respective cell surface receptors, receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPR-30/GPER1). The regulatory subunit (p85) and the catalytic subunit (p110) make up the PI3K heterodimer. The activation of p110, which reacts to various stimuli, is controlled by p85. Upon activation, PI3K phosphorylates phosphatidylinositol 4,5 bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), indicated in Figure 1.3. Akt is subsequently phosphorylated at Thr308 and Ser473 by its activators, PDK-1 and mTORC2, respectively, triggering an intracellular signalling cascade (Nicholson & Anderson, 2002; Finley & Thompson, 2014; Miricescu *et al*., 2021). Akt phosphorylates various target proteins resulting in the stimulation of cell survival, growth, and proliferation. Forkhead box O transcription factors (FoxO), the BCL2-associated agonist of cell death (BAD), and glycogen synthase kinase 3 (GSK-3) are some of the downstream substrates that Akt activates to promote cell cycle entry and cell survival. Additionally, Akt activates mTORC1, which stimulates protein translation and protects against apoptosis (Wang, 2021).

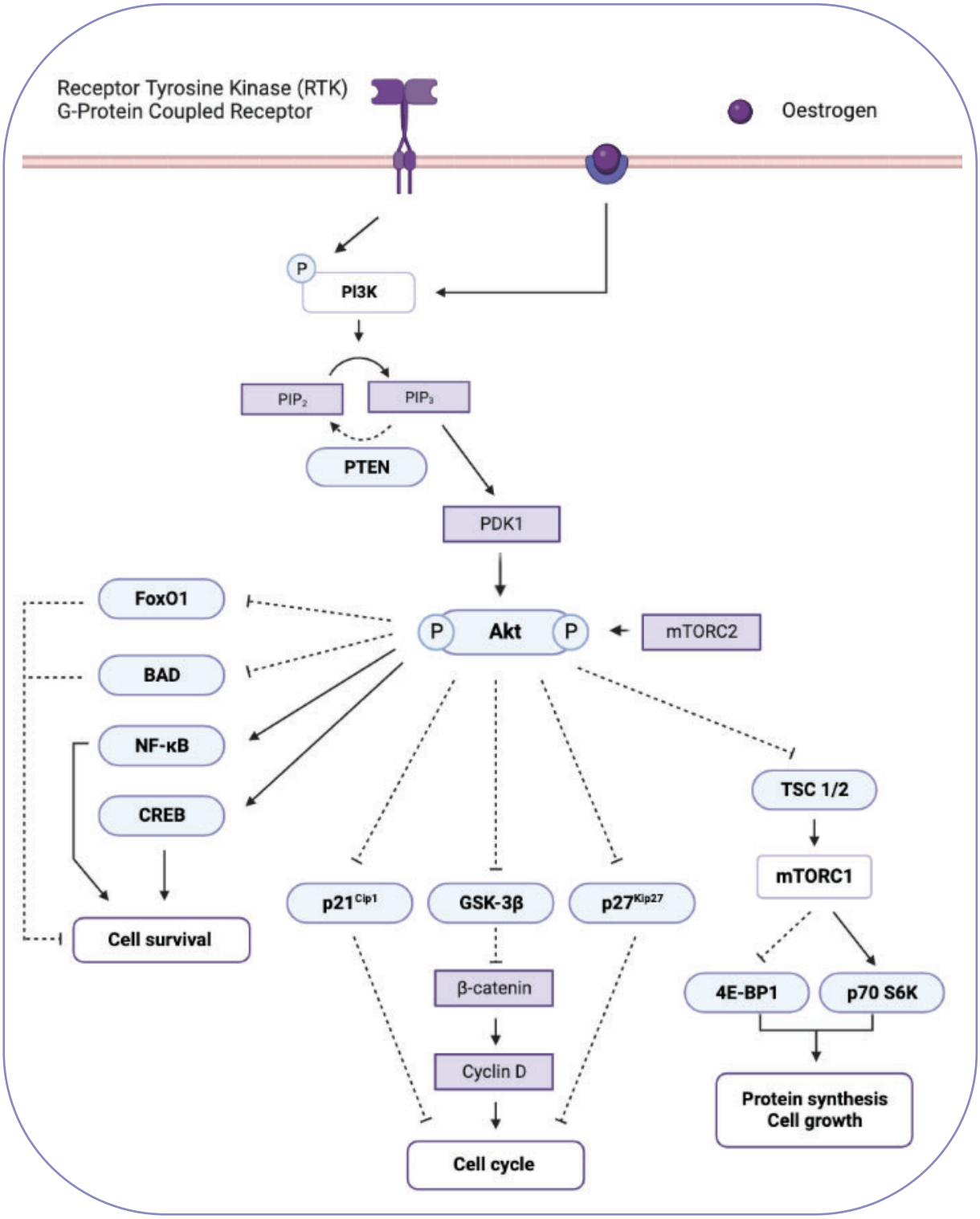


Figure 1.3.: The PI3K/Akt signalling pathway activation and its role in tumourigenesis; Abbreviations: Akt – RAC-alpha serine/threonine-protein kinase, BAD – BCL2-associated agonist of cell death, CREB – cAMP-response element binding protein, FoxO1 – forkhead box O transcription factor 1, GSK-3β – glycogen synthase kinase 3 beta, mTORC – mammalian target of rapamycin complex, NF-κB – Nuclear factor kappa B, PDK – phosphoinositide-dependent kinase 1, PI3K – phosphoinositide 3-kinase, PIP2 – phosphatidylinositol 4,5 bisphosphate, PIP3 – phosphatidylinositol 3,4,5-trisphosphate, p70 S6K – p70 ribosomal S6 kinase, TSC – Tuberous sclerosis complex, 4E-BP1 – eukaryotic translation initiation factor 4E-binding protein 1. (Adapted from Miricescu et al., 2021; Ortega et al., 2020) (Created with BioRender.com)

The PI3K pathway is tightly regulated by a number of factors to ensure homeostasis, specifically RTKs that are tightly controlled by the availability of growth factors, and lipid phosphatase and tensin homolog (PTEN). PTEN is an important tumour suppressor which inhibits cell proliferation by dephosphorylating PIP_3 to PIP_2, dampening the PI3K pathway through a negative feedback loop (Finley & Thompson, 2014; Paplomata & O'regan, 2014; Ortega *et al*., 2020). In many malignant tumours, the PTEN gene has undergone alterations/mutations, resulting in aberrant PTEN that is unable to exert its inhibitory effect on the PI3K/Akt/mTOR pathway, allowing for sustained signalling.

Activation of the PI3K/Akt signalling pathway has been associated with promoting cell survival as well as the cell cycle progression. Akt activation induces proliferation through the inhibition of the downstream molecule GSK-3β (Xie *et al*., 2019). GSK-3β has multiple roles ranging from glucose homeostasis to the key role it plays in the Wnt signalling pathway (Nicholson & Anderson, 2002; Vadlakonda *et al*., 2013). GSK-3β inactivation is directly related to cell metabolism reprogramming, such as the uptake and use of glycogen, which supports the Warburg effect (cancer cells' preference for anaerobic glucose metabolism over aerobic glucose metabolism). GSK-3β has been shown to increase cyclin D degradation, by increasing β-catenin degradation through the ubiquitin-proteasome pathway (Nicholson & Anderson, 2002; Wang *et al*., 2021). As such, the inhibition of GSK-3β through Akt activation stimulates the cell cycle by increasing cyclin D1 expression.

Cyclin D1 is an important regulator of the cell cycle, and its upregulation leads to entry of cells from Gap phase 0 (G_0) to Gap phase 1 (G_1) increasing cell cycle progression (Wang, 2021). Furthermore, Akt directly stimulates the cell cycle progression by phosphorylating p21 and p27 (Ortega *et al*., 2020). Inhibition of p27 by Akt phosphorylation, localizes p27 to the cytoplasm. This inhibits p27 binding to nuclear cyclin-dependent kinase 2 (CDK2) resulting in the progression of the cell cycle. Following p21 phosphorylation is DNA synthesis and activation of various CDKs which also results in cellular proliferation. The relevance of the Akt pathway in cancer is demonstrated by its role in cellular proliferation, metabolism, and protein synthesis.

1.1.2.2. Sustained proliferative signalling: Cell cycle progression

Cancer is characterised by unchecked proliferation, which is dependent on the cell cycle. The cell cycle is an ordered sequence of events that cells undergo to complete DNA replication to produce two genetically identical daughter cells (Bower *et al.*, 2017). There are four phases of the cell cycle, G_1, synthesis (S), Gap phase 2 (G_2) and mitosis (M) (Figure 1.4) (Bower *et al.*, 2017; Fouad & Aanei, 2017). DNA is replicated during the S phase, while the M phase is characterized by cell division into two daughter cells. The gap phases correspond to intervals between the M and S phases. After mitosis cells can exit the cell cycle and differentiate or go into a quiescent stage in G_0 (Pucci *et al.*, 2000; Bower *et al.*, 2017) .

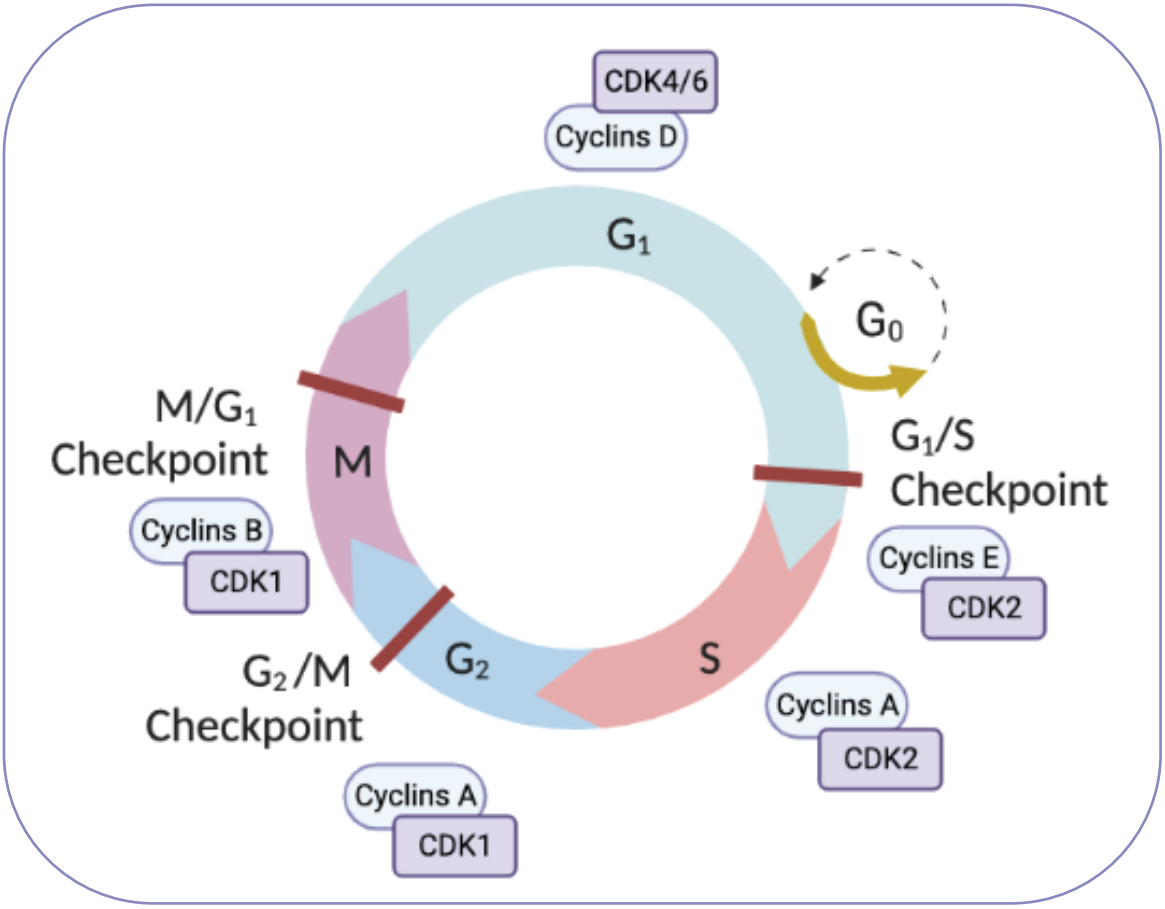


Figure 1.4.: Cell cycle regulation; Abbreviations: CDK – cyclin-dependent kinase. (Adapted from Wang, 2021) (Created with BioRender)

The cell cycle is a tightly regulated process responsible for the controlled proliferation of cells, maintenance of genomic stability and prevention of carcinogenesis (Pucci *et al.*, 2000;

Hengst & Nigg, 2004). It is modulated by four distinct cell cycle checkpoints, several types of cyclin and CDKs, and external factors such as growth factors. When the M/G_1, G_2/M or G_1/S checkpoints are activated, it leads to the arrest of the cell cycle for repair of the damaged DNA or activation of apoptotic signalling cascades (Pucci *et al*., 2000). Cell cycle control is based on a combination of regulated protein synthesis, phosphorylation and proteolysis, as well as transcriptional and translation regulators (Hengst & Nigg, 2004). Transitions between the different phases of the cell cycle are governed by changes in the kinase activity of CDKs (Ali *et al*., 2020). The CDKs belong to a well conserved family of serine/threonine protein kinases (Pucci *et al*, 2000). Cyclins are the regulatory units that control the kinase activity of CDKs. In each phase of the cell cycle, specific cyclins bind to CDKs to form CDK/cyclin heterodimers through phosphorylation (Ali *et al*., 2020). As many cyclins are expressed at different stages of the cell cycle, they are classified according to those stages. During the G_1 phase, cyclin D associates with CDK4 or CDK6, known as the G_1 phase cyclin, to form the cyclin/CDK complex (Pucci *et al*., 2000; Ali *et al*., 2020) (Figure 1.4). This allows for the progression of the dividing cells into the next phase of the cell cycle following proliferative signalling (Hengst & Nigg, 2004).

The decision to proceed with cell division or exit from the cell cycle is made during the G_1 phase. After a specific point in G_1, called the restriction point (R-point), cells that enter S phase no longer respond to growth factors or differentiation signals, but commit themselves to divide (Pucci *et al*, 2000; Ali *et al*., 2020). At least four cell cycle checkpoints, including the restriction point (G_0/G_1), the G_1 and G_2 checkpoints, and the mitosis-associated spindle assembly checkpoint (SAC), may be dysregulated in cancer cells (Bower *et al*., 2017). The dysregulation of the cell cycle checkpoints enables cancer cells to remain in a continuous cycle of division, evading apoptosis and DNA repair. It has been demonstrated that PTEN inhibition or PI3K activation of Akt are essential for growth factor-induced cell cycle progression in cancer cells as a mechanism of sustained proliferation.

The upregulation of the Akt pathway in cancer cells can promote cell cycle progression by multiple mechanisms including (Nicholson & Anderson, 2002; Wang, 2021):

1. Phosphorylating CDK inhibitory proteins $p21^{CIP1}$ and $p27^{kip1}$ causing their cytoplasmic accumulation.

2. Decreasing $p27^{kip1}$ transcription by phosphorylating and negatively regulating the forkhead family transcription factor.

3. Increasing cyclin D transcription by stabilising β-catenin through the inhibition of GSK-3 or by activating CREB transcription factor.

4. Increasing cyclin D mRNA translation.

In addition, the activation of the PI3K/Akt signalling pathway is also central in the regulation of epithelial-mesenchymal transition (EMT), which facilitates metastasis of cancer cells from the primary tumour to secondary sites.

1.1.2.3 Activating Invasion and Metastasis

Metastasis is the migration of cancer cells from the primary tumour site, through blood vessels and the lymphatic system, to secondary sites in different tissues and organs. More than 90 % of all cancer-related deaths have metastatic disease as their primary cause, making metastasis one of the major factors in cancer therapy failure and mortality (Fares *et al*., 2020). Cancer cells can invade surrounding tissues and spread through the basement membrane during the multi-step process of metastasis (Wang *et al.,* 2021). This process entails the local invasion of cells, cellular migration known as intravasation, extravasation and colonization in organs and tissues (Figure 1.5). Interestingly, cancer cell migration is site-specific, a process known as organotropism (Wang *et al*., 2021). This mechanism is reliant on EMT, which is described by epigenetic and phenotypic alterations in cancer cells (Kokkinos *et al*., 2007). As such, metastasis is a hallmark of cancer and an important characteristic of tumourigenesis.

EMT is characterized by changes in cell polarity and shape, transdifferentiating from an epithelial phenotype into fibroblastic migratory cells acquiring a mesenchymal phenotype (Kokkinos *et al*., 2007). Epithelial cells maintain a cobble-stone appearance, with distinct contact between cells, while mesenchymal cells present an elongated appearance with reduced cell-cell contact (Kokkinos *et al*., 2007). There are three types of EMT, type I refers to the migration or movement of epithelial-derived cells throughout common biological processes such as embryogenesis and organ development (Kokkinos *et al.*, 2007; Kalluri & Weinberg, 2009). Type II is associated with tissue regeneration, and type III is a

pathophysiologic adaptation of the process. Type III EMT is linked to the development of neoplasia in cells that have undergone specific epigenetic and genetic alterations. Epithelial cells undergo a variety of metabolic alterations during type III EMT that encourage migratory, invasive, stress-resilient, and antiapoptotic characteristics (Saha Roy & Vadlamudi, 2012; Fares *et al*., 2020; Wang *et al*., 2020). The transition of one state to another is governed by a number of growth factors and signalling pathways (Fares *et al*., 2020). It is suggested that signals originating from the tumour-associated stroma, such as epidermal growth factor (EGF), platelet-derived growth factor, hepatocyte growth factor (HGF), and transforming growth factor-β (TGF-β) result in type III EMT (Kalluri & Weinberg, 2009). Furthermore, TGF-β signalling can stimulate metastatic dissemination, for instance during metastasis of breast and prostate tumour cells to bone and lung.

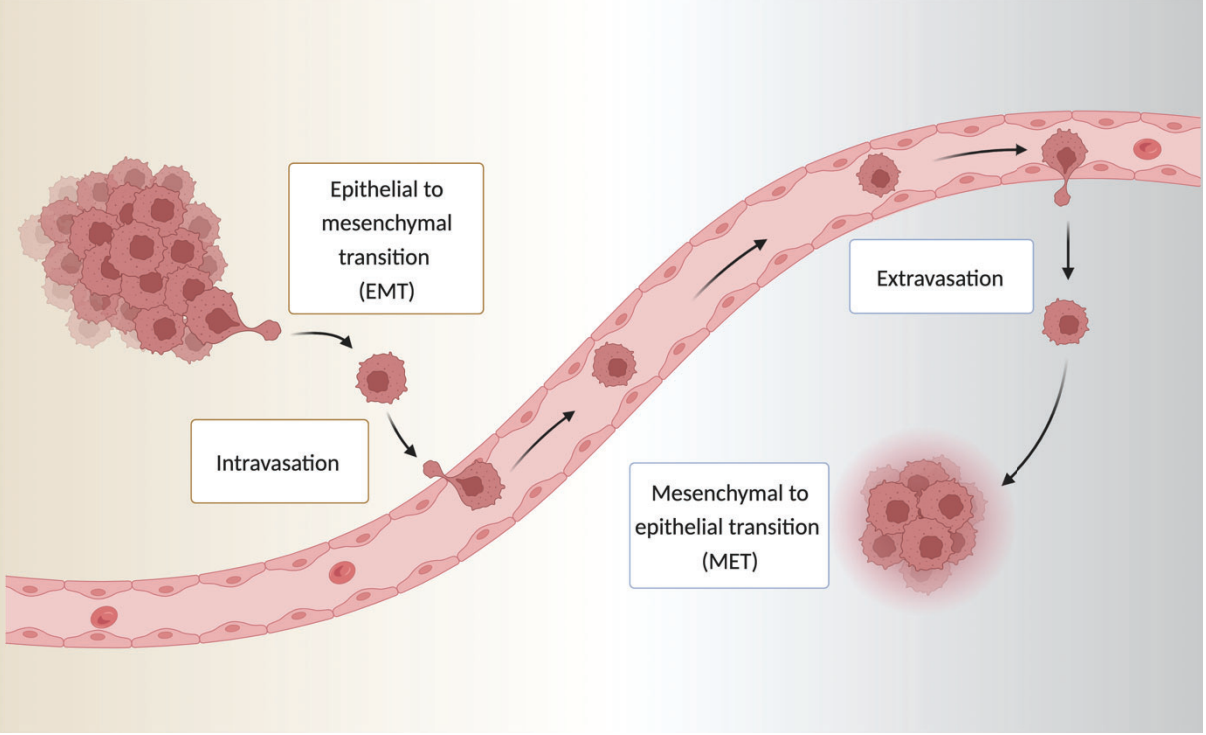


Figure 1.5.: Epithelial to mesenchymal transition; Abbreviations: EMT – epithelial-mesenchymal transition, MET – mesenchymal-epithelial transition. (Adapted from Fares *et al.,* 2020) (Created with BioRender.com)

TGF-β is a key regulator of EMT acting as a tumour suppressor during the early phases of cancer progression and as a tumour promoter in the later stages (Xie *et al.*, 2017). TGF-β is part of a superfamily of cytokines consisting of bone morphogenetic proteins (BMPs), activins, inhibins, nodal, growth and differentiation factors (GDFs) (Xie *et al.,* 2017). Each family member plays crucial roles in many cellular processes, including immune-suppression, growth inhibition, EMT, cell migration, invasion, and extracellular matrix (ECM) remodelling. TGF-β signalling can promote EMT through downstream activation of transcription factors that are programmed to repress epithelial genes and activate mesenchymal genes (Kalluri & Weinberg, 2009; Xie *et al.,* 2017). TGF-β signalling can occur through the regulation of suppressor of Mothers against Decapentaplegic (SMAD) signalling as well as through non-SMAD signalling, such as through crosstalk with other signalling pathways, including PI3K/Akt/mTOR, the mitogen activated protein kinases (MAPK), ERK, p38 and JNK and Rho-like GTPase signalling (Xie *et al*., 2017; Loh *et al*., 2019). TGF-β cytokines signal through a transmembrane receptor complex that comprises of the Type I and Type II receptor serine-threonine kinases. TGF-β binds to the constitutively active Type II receptor (TβRII), initiating the recruitment of the TGF-β Type I receptor (TβRI) (Figure 1.6) (Xie *et al.,* 2017; Loh *et al.,* 2019). TRβI activation leads to the phosphorylation of SMAD2/3 and their interaction with SMAD4, which then translocates to the nucleus to regulate the expression of the target genes. Additionally, both TβRII and TβRI appear to be directly involved in the activation of the PI3K/Akt pathway by interacting with the p85 subunit of PI3K leading to the activation of Akt downstream targets (Xie *et al.,* 2017).

Transcription factors such as SNAI1 (snail), SNAI2 (slug), zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and FOX families are activated directly or indirectly by TGF-β signalling. These transcription factors are responsible for the loss of epithelial cell adhesion proteins such as E-cadherin, EpCAM, cytokeratin, and claudin-1, and induce an increase in mesenchymal proteins including N-cadherin, vimentin, alpha-smooth muscle actin (α-SMA), and fibronectin (Kalluri & Weinberg, 2009; Wang *et al.,* 2021; Park *et al*., 2022). TGF-β directly stimulates snail and slug by binding SMAD3 on their promoter region (Loh *et al.,* 2019). Snail is a prominent inducer of EMT and represses E-cadherin expression. Expression of snail positively correlates with tumour grade, recurrence, metastasis and poor prognosis in various cancers (Wang *et al*., 2014). Additionally, TGF-β indirectly increases N-cadherin by

activating MAPKs and controlling WNT-7A production through the β-catenin/T-Cell Factor (TCF) pathway.

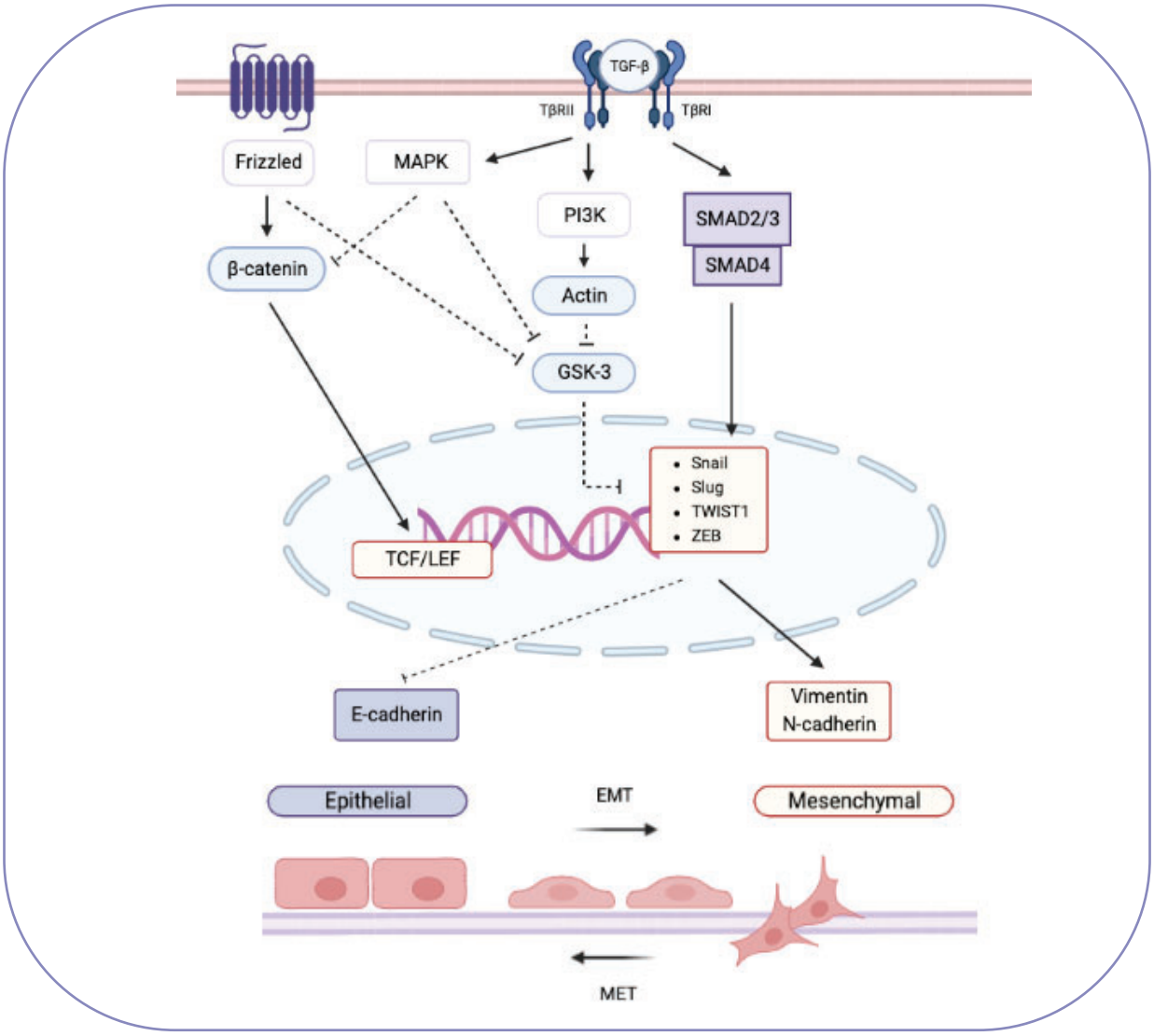


Figure 1.6.: Signalling pathways involved in epithelial-mesenchymal transition in cancer; Abbreviations: GSK-3 – glycogen synthase kinase 3, MAPK – mitogen activated protein kinases PI3K – phosphoinositide 3-kinase, Snail – SNAI1, Slug – SNAI2, SMAD – suppressor of Mothers against Decapentaplegic, TGF-β - transforming growth factor beta, ZEB – zinc finger E-box binding homeobox 1. (Adapted from Loh *et al.,* 2019; Wang *et al.,* 2007; Xie *et al.,* 2018) (Created with BioRender.com)

Localized at cell-cell contacts, E-cadherin helps maintain apical-basal polarity and stable epithelial morphology by interacting with catenins and the cytoskeleton (Kokkinos e*t al*., 2007; Zhou *et al*., 2021). A loss in E-cadherin is seen as a result of transcription factors' binding to

the E-cadherin promoter region. Decreased E-cadherin expression results in the subsequent loss of cell-cell adhesion and epithelial morphology (Kokkinos e*t al*., 2007). To preserve epithelial features in cancer cells, E-cadherin sequesters β-catenin at the membrane, when E-cadherin expression is inhibited, the movement of β-catenin to the nucleus forms part of a TCF/Lymphocyte enhancer factor 1 (LEF) complex, increasing N-cadherin and Vimentin expression and the subsequent acquisition of the mesenchymal phenotype (Kokkinos *et al.,* 2007; Kalluri & Weinberg, 2009; Loh *et al.,* 2019; Park *et al*., 2022). Vimentin is involved in cellular motility, shape maintenance and directional migration, while N-cadherin facilitates cell migration, augmentation of fibroblast growth factor-receptor (FGFR) signalling, and modulation of the Wnt signalling pathway (Mrozik *et al*., 2018; Zhou *et al.*, 2021).

Additionally, epigenetic and posttranslational modulators also play a vital role in regulating EMT. Moreso, the integrin-mediated adhesion and debonding interactions with matrix components is critical for regional migration (Wang e*t al.,* 2021). Once EMT has taken place and cancer cells have metastasized to their specific organ/tissue site, mesenchymal-epithelial transition (MET) is needed for metastatic progression. After extravasation, during MET, there is an increase in the expression of E-cadherin (Kokkinos *et al*., 2007). MET is the reverse process of EMT, where cells are converted from a mesenchymal phenotype to an epithelial phenotype with cell-cell adhesions.

Metastatic cancer encompasses a diverse collection of cells that possess different phenotypic characteristics and genetic profiles (Fares *et al*., 2020). The most predominant changes in genes and proteins during metastasis include tumour protein p53 (*TP53*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), PTEN, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), and retinoblastoma (RB1) (Wang *et al*., 2020). The dysregulation of these genes and proteins can promote a pro-metastatic tumour microenvironment.

It has been well established that breast cancer cell lines that express oestrogen receptors are susceptible to changes in gene expression and resulting changes in intracellular signalling. The activation of these pathways may promote proliferation and metastasis in the presence of oestrogen, such as when patients receive hormone replacement therapies.

1.2. THE ROLE HORMONES PLAY IN BREAST CANCER DEVELOPMENT

Hormones are chemical messengers that regulate a variety of processes in multicellular organisms, such as metabolism, growth and development, sexual development, mood, sleep patterns, and stress (Davidge-Pitts & Solorzano, 2022). The main reproductive hormones, oestrogen, testosterone and progesterone, are instrumental in maintaining male and female secondary sex characteristics, reproduction, bone density, the development and function of sperm, breast tissue and sexual organs, brain function, cholesterol mobilization, and immune system regulation (Valadez-Cosmes *et al*., 2016; Fuentes & Silveyra, 2019). When hormones are balanced, they help maintain homeostasis by protecting against conditions like osteoporosis and cardiovascular disease. However, when dysregulated, could lead to serious health problems, including increased susceptibility to autoimmune conditions, infections and the development of cancer (Valadez-Cosmes *et al*., 2016).

Hormone-mediated signalling can be divided into genomic and non-genomic events. Genomic events are those involving the binding and migration of receptor complexes to directly bind to DNA, whereas non-genomic effects involve the indirect regulation of gene expression by activating signalling transduction mechanisms, second messengers, kinases, and ion channels (Valadez-Cosmes *et al*., 2016; Cenciarini & Proietti, 2019). Non-genomic signalling occurs in the membrane or cytoplasm by generating short-term or rapid effects interacting with intracellular proteins, such as MAPKs, nuclear factor κB (NF-κB) and PI3K to induce protein transcription (Nilsson *et al*., 2001; Valadez-Cosmes *et al.,* 2016; Cenciarini & Proietti, 2019; Committee on the Clinical Utility of Treating Patients with Compounded Bioidentical Hormone Replacement Therapy, 2020).

Both oestrogen and progesterone have been identified as potent breast mitogens and act as major risk factors for invasive breast cancer (Wang & Lee, 2016; Africander & Storbeck, 2018; Cenciarini & Proietti, 2019). Fundamental, descriptive, quantitative and experimental data all show that oestradiol's important cellular action is to promote growth and proliferation. In contrast, despite circumstantial proliferative effects, progesterone's dominant actions in cells are to inhibit proliferation, to enhance differentiation and promote maturation (Wang & Lee, 2016; Africander & Storbeck, 2018; Cenciarini & Proietti, 2019; Prior, 2020). It is well-established that oestrogen has a dose-related risk on breast cancer (Fuentes & Silveyra, 2019).

Among the factors that contribute to the risk of oestrogen-induced breast cancer, are the ovulatory cycles women encounter throughout the course of their lives, which are influenced by a variety of situations, such as early menarche and late onset menopause.

There are four main occurring oestrogens, estrone (E_1), oestradiol (E_2), estriol (E_3) and estetrol (E_4) (Atwood & Ekstein, 2019; Fuentes & Silveyra, 2019). Oestradiol is the most prevalent oestrogen in the body during the reproductive years. It is mainly responsible for the development of female sexual characteristics and reproduction. After menopause, estrone becomes the dominant oestrogen, with estriol being present in small amounts in premenopausal women (Atwood & Ekstein, 2019; Fuentes & Silveyra, 2019). These oestrogens elicit their biological effects by binding to membrane-bound GPR-30/GPER1 and oestrogen receptors (ER-α) and -β (ER-β), which are found in the cytoplasm (Figure 1.7). The binding affinities of oestrogens vary depending on the receptor types they bind to, with oestrogens all having a higher affinity for ER-α than ER-β (Perkins *et al.*, 2017). Furthermore, E_2 has the strongest binding affinity for ER-α. It's also important to note that ER-α and ER-β have opposing effects, wherein ER-α drives cell growth and ER-β inhibits ER-α mediated proliferation (Perkins *et al.*, 2017).

By triggering multiple kinase cascades, such as the Src kinase, MAPK, cAMP, PI3K, and protein kinase C pathways, ER-α extranuclear signalling induces its rapid cellular effects (Saha Roy & Vadlamudi, 2012; Yu & Hongyan, 2022). Kinases, such as ERK and Akt are implicated in breast cancer metastasis. As such, ER-α extranuclear signalling has the potential to promote breast cancer cell migration and metastasis. Although different from those of ER-α, ER-β plays a comparable role to ER-α as a transcription factor by influencing diverse physiological responses to oestrogen signalling. ER-β acts as a suppressive player of proliferation processes, stimulating the differentiation of cells (Montanari *et al.*, 2017). For instance, it has been demonstrated that ER-β expression is lower in invasive breast cancer than it is in less aggressive, proliferative tumours (Saha Roy & Vadlamudi, 2012). Furthermore, ER-β downregulation has been associated with EMT in prostate cancer cells.

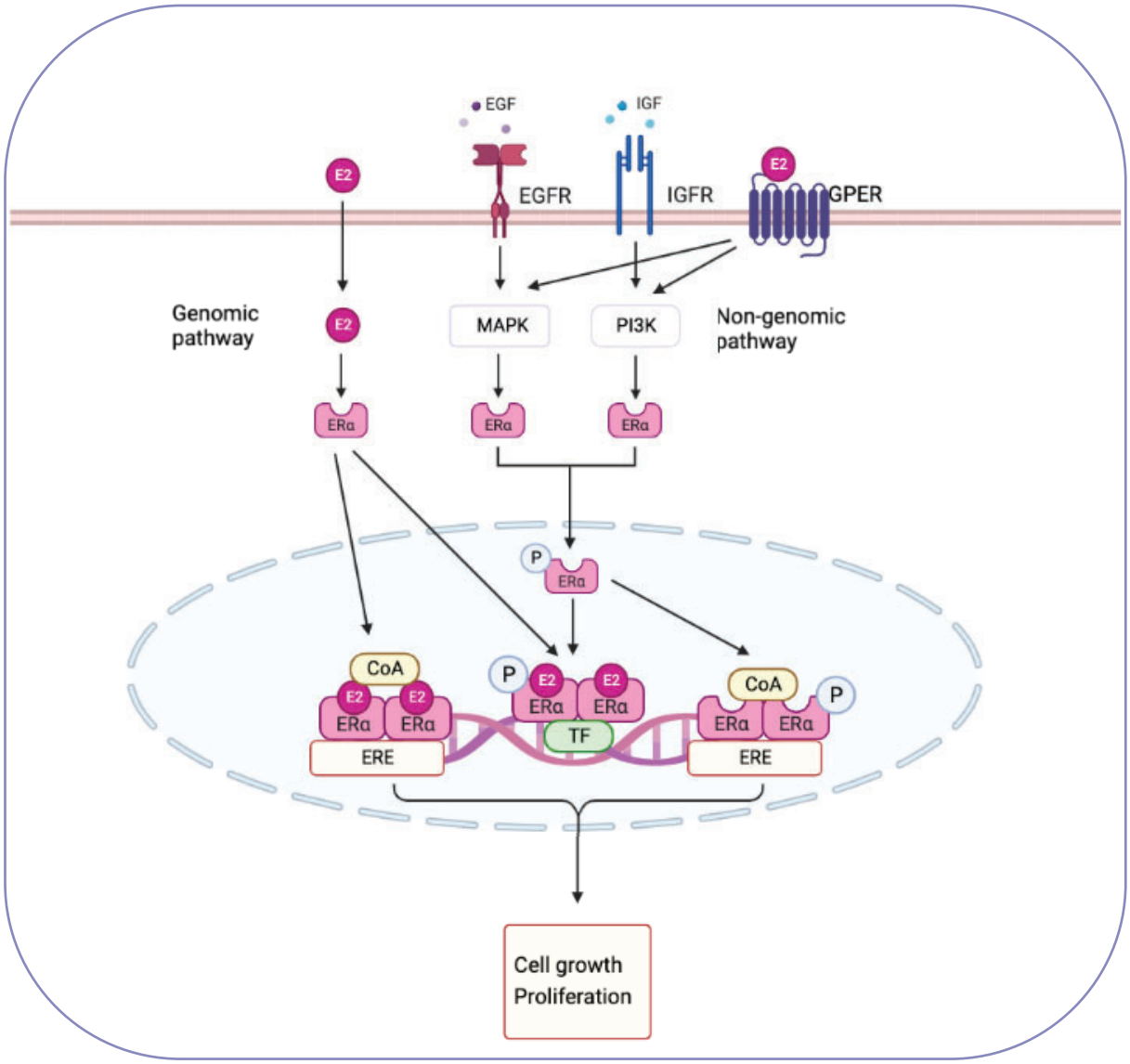


Figure 1.7.: Oestradiol signalling in breast cancer; Abbreviations: CoA – coenzyme A, EGF – epidermal growth factor, EGFR – epidermal growth factor receptor, ER – oestradiol receptor, ERE – oestradiol response element, E_2 – oestradiol, GPER – G-protein coupled receptor, IGF – insulin-like growth factor, IGF – insulin-like growth factor receptor, MAPK – mitogen activated protein kinase , PI3K – phosphoinositide 3-kinase, TF – transcription factor. (Adapted from Ramírez-de-Arellano et al., 2022; Yu and Hongyan, 2022) (Created with BioRender.com)

Although the effects and mechanisms of the hormones are often studied in isolation, under normal physiological conditions, progesterone receptors (PRs) and ERs are usually co-expressed and their functions are mutually dependent on their expression and activity (Cenciarini & Proietti, 2019). Most epithelial cells that express PRs also express ER-α in the adult mammary gland. PR is an upregulated target gene of ER and oestrogen is required to maintain high expression of PR (Obr & Edwards, 2012). It's interesting to note that ER-α-mediated transcriptional processes contribute to the transcription of PR (Trabert *et al.*, 2020).

Furthermore, oestrogen acts as a key mediator of PR's extranuclear signalling effects (Trabert *et al*., 2020).

Progesterone directly exerts its effects by binding to progesterone receptors (PRs), then binding to progesterone response elements (PREs) or other DNA-binding transcription factors to modify target gene expression (Figure 1.8) (Yu & Hongyan, 2022). In non-genomic pathways, progesterone indirectly regulates gene transcription by activating second messenger cascades through PRs, progesterone membrane receptors (mPRs) and progesterone receptor membrane component 1 (PGRMC1) (Yu & Hongyan, 2022).

Progesterone (P_4) binds to two predominant PR isoforms, PR-A and PR-B, which have different transcriptional and functional activities (Trabert *et al*., 2020). The ratio of PR-A to PR-B in target cells likely predict the overall cellular response to progesterone. Under normal physiologic conditions, a 1:1 ratio of PR-A and PR-B exists in human breast and in benign breast lesions (Obr & Edwards, 2012; Cenciarini & Proietti, 2019). In breast cancer, this ratio is altered with a higher PR-A:PR-B, and is associated with a more aggressive tumour phenotype, with resistance to endocrine therapies (Obr & Edwards, 2012; Trabert *et al.*, 2020; Yu & Hongyan, 2022). This alteration is specifically seen with the use of progestins, synthetic progesterone's, which may potentially be a mechanism by which breast cancer risk is increased (Holtorf, 2009).

PR-B is more crucial for the proliferative responses to progesterone in the mammary epithelium, while ovarian and uterine development and function rely primarily on PR-A (Obr & Edwards, 2012). In contrast PR-A does not efficiently mediate rapid activation of the protein kinase signalling pathway. PR-B undergoes phosphorylation after ligand binding or due to the action of growth factors (Cenciarini & Proietti, 2019). Particularly, phosphorylation of Ser294 is coupled to rapid ubiquitin-dependent turnover of the receptor and is associated with high transcriptional activity. PR-Ser294 phosphorylation is widely observed in breast tumours and is primarily found in premalignant regions (Cenciarini & Proietti, 2019).

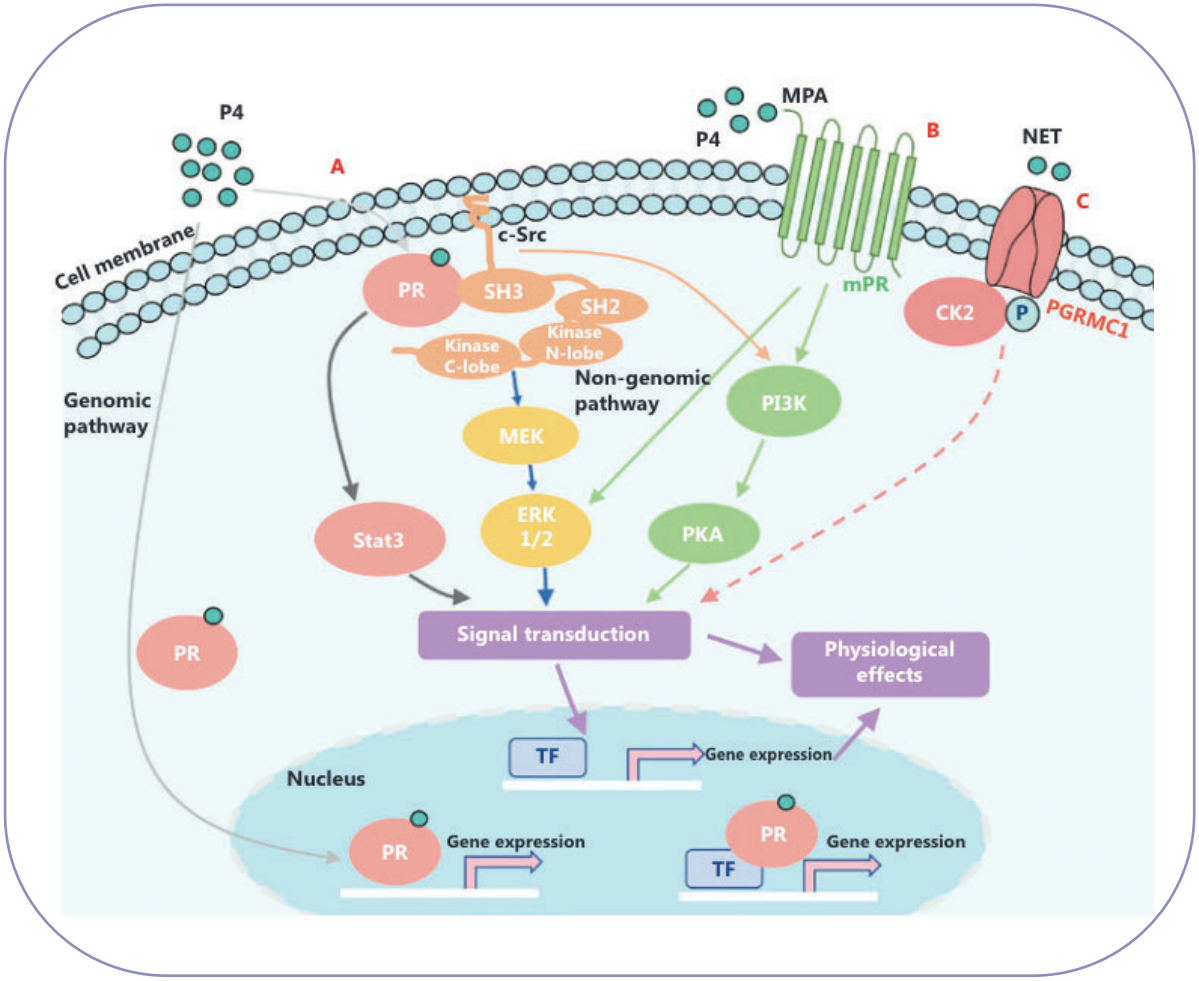


Figure 1.8.: Progestogen signalling in breast cancer; Abbreviations: CK2 – casein kinase 2, c-Src – cellular proto-oncogene tyrosine-protein kinase, ERK – extracellular signal-regulated kinase , MEK – mitogen-activated protein kinase, MPA – medroxyprogesterone acetate, mPR – progesterone membrane receptors, NET – norethisterone, PGRMC1 – progesterone receptor membrane component 1, PI3K – Phosphoinositide 3-kinase , PKA – protein kinase A, PR – progesterone receptor, P_4 – progesterone, TF – transcription factor. (Yu & Hongyan, 2022)

Progestins are a class of synthetic compounds structurally distinct but functionally similar to progesterone with differing potency and pharmacokinetics (Figure 1.9) (Africander *et al.*, 2011; Asi *et al.,* 2016). Progesterone taken orally has few biological effects since it is poorly absorbed, even in micronized form, and is substantially metabolized during the hepatic first pass (Trabert *et al.*, 2020). The biochemistry, metabolism, as well as beneficial and harmful effects of the various synthetic progestins differ widely between native progesterone's and between each other (Asi *et al.*, 2016). Progestins mimic some of the effects of progesterone but may have different actions on progesterone receptors (Asi *et al.*, 2016). In addition to binding PR, these compounds may also have an affinity for androgen, glucocorticoid and

mineralocorticoid receptors (Asi *et al.,* 2016). Such cases have been reported when exogenous progestins were administered with oestrogen as menopausal hormone therapy or as contraceptives (Louw-du Toit *et al.,* 2017; Busund *et al.,* 2018).

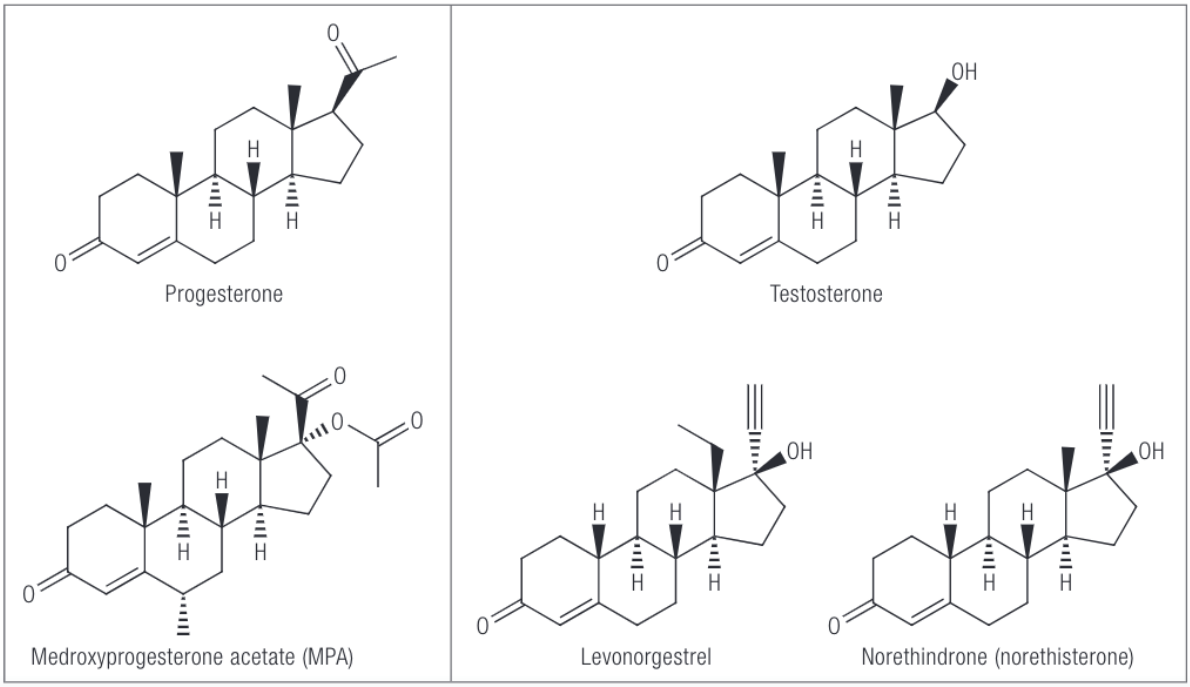


Figure 1.9.: Chemical structure of progesterone and medroxyprogesterone acetate (MPA), containing 21 carbons compared to testosterone, levonorgestrel and norethindrone, containing 19 carbons. (Trabert *et al.,* 2020)

There has been evidence that an association between the use of progestins and breast cancer risk exists. Studies have reported that combined oestrogen and progesterone menopausal hormone therapy (MHT) increased human mammary proliferation above oestrogen alone treatment (Miricescu *et al.,* 2021). It has been suggested that progestin stimulation of second messenger pathways, including, PI3K/Akt, MAPK/ERK, and NF-κB ligand/receptor activator of NF-κB (RANKL/RANK), leads to breast cell proliferation (Yu & Hongyan, 2022). Researchers also hypothesise that progesterone promotes pre-neoplastic proliferation by stimulating cyclical proliferation of the mammary stem cell pools or occult tumour initiating cells, as seen in models of hormone-dependent mammary tumours (Trabert *et al*., 2020). The proliferative effects of progesterone in the adult breast occur mainly through

paracrine actions between progesterone receptor positive (PR+) and negative (PR-) breast cells (Prior, 2020; Trabert *et al*., 2020). Further evidence points to progesterone/PR signalling and a shift from paracrine to autocrine regulation of proliferation as causes of cancer progression (Sivaraman e*t al.,* 2001; Obr & Edwards, 2012). There are two waves of progesterone-induced proliferation in the mammary gland. In the first 24 hours after ovariectomized mice, it is primarily the PR-positive cells that proliferate in a cyclin D1-dependent manner (Cenciarini & Proietti, 2019).

While there is limited epidemiologic evidence to establish a link between circulating levels of progesterone and risk of breast cancer, mechanistic investigations have linked progesterone to the development of the disease (Khan, 2020). The possibility that progesterone exposure is associated with breast cancer is an understudied topic. In premenopausal women, research has been hampered by the cyclical variation of serum progesterone levels, so that even when studied, no clear trends emerge (Khan, 2020). Recent research that investigated serum progesterone levels, including the Nurses' Health Study and the Breast and Bone Follow-up to the Fracture Intervention Trial (Missmer *et al*., 2004; Trabert *et al*., 2020). It was discovered that women with high progesterone and low oestradiol levels have a lower risk of developing breast cancer: the relative risk in the Nurses' Health Study population was 0.5 (95 % CI, 0.2 to 1.3), meaning it is more likely to be beneficial than harmful. Similarly, the hazard ratio, which is how often women with high progesterone and low oestradiol levels developed breast cancer, in the Breast and Bone Follow-up to the Fracture Intervention Trial report was 0.38, which indicates low association (Trabert *et al*., 2020). These findings support the context-specific action of progesterone, which seems to require a minimal oestrogen concentration to initiate the biological effects, such as sustained proliferative signalling, that favour the development of cancer. Contrary to popular belief, MHT with oestrogen and progesterone does not cause the *de novo* onset of breast cancer; rather, it encourages the faster growth of occult breast tumours that are too small to be seen on mammography (Song *et al*., 2013). Moreover, it should be emphasized once more that while hormones function synergistically in balance, they can also advance disease when they are dysregulated.

1.3. MENOPAUSE

As women age, they experience the loss of ovarian follicles resulting in a natural decline of the reproductive hormones (Atwood & Ekstein, 2019; Trabert *et al*., 2020). This can occur between 40 and 60 years of age, and results in the cessation of the menstrual cycle, a phenomenon known as menopause. Reproductive hormones play important roles throughout the course of an individual's development. In women, steroid hormones are mainly responsible for reproduction and contribute to the development of female characteristics, cognitive health, bone health as well as cardiovascular health (Fuentes & Silveyra, 2019; Harbeck *et al*., 2019). During the menstrual cycle, fluctuations in oestrogen and progesterone together with follicle stimulating hormone (FSH) and luteinising hormone (LH), drive the ovulation process and direct the mammary gland epithelium to undergo sequential waves of proliferation, differentiation and apoptosis (Weiss *et al*., 2004; Bernhardt *et al*., 2016). During menopause women experience a drastic fluctuation in hormones, accentuated by a decline in oestrogen levels (Figure 1.10). As the hormone dynamics change, women begin to experience vasomotor symptoms (VMS), most commonly 'hot flashes/flushes', night sweats, genitourinary syndrome of menopause (GSM), sexual dysfunction as well as mood and sleep disturbances (Figure 1.11) (Al-Safi & Santoro, 2014; O'Neill & Eden, 2020; Armeni *et al*., 2021).

Ovarian follicle loss is accelerated as women enter perimenopause. Perimenopause, also referred to as menopausal transition, describes the variable time where hormonal changes and clinical symptoms occur a few years before and after menopause or climacteric (O'Neill & Eden, 2020; Lobo, 2022). It is characterized by increased menstrual cycle irregularity, lengthy periods of anovulation, and fluctuation in hormone levels (Trabert *et al.*, 2020). A woman is declared menopausal once amenorrhea occurs for more than 12 consecutive months (Files & Kling, 2020; O'Neill & Eden, 2020). Early menopause is defined as the permanent cessation of menstruation before the age of 45 (Armeni *et al.*, 2021; O'Neill & Eden, 2020). Premature ovarian syndrome (POI), as its name implies describes the premature loss of ovarian activity before the age of 40 (Armeni *et al.,* 2021). POI is commonly iatrogenic, following surgery, chemotherapy and radiotherapy, and it can occur spontaneously (O'Neill & Eden, 2020).

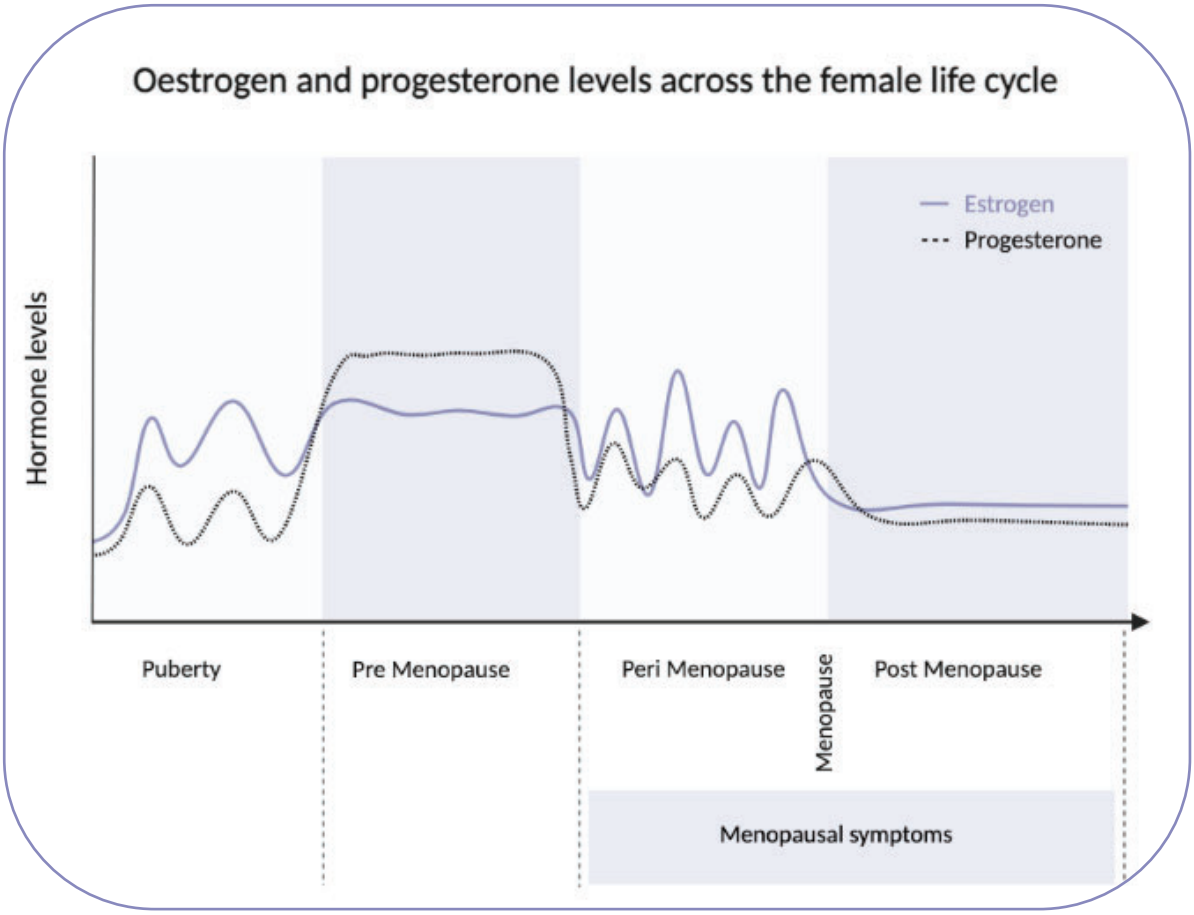
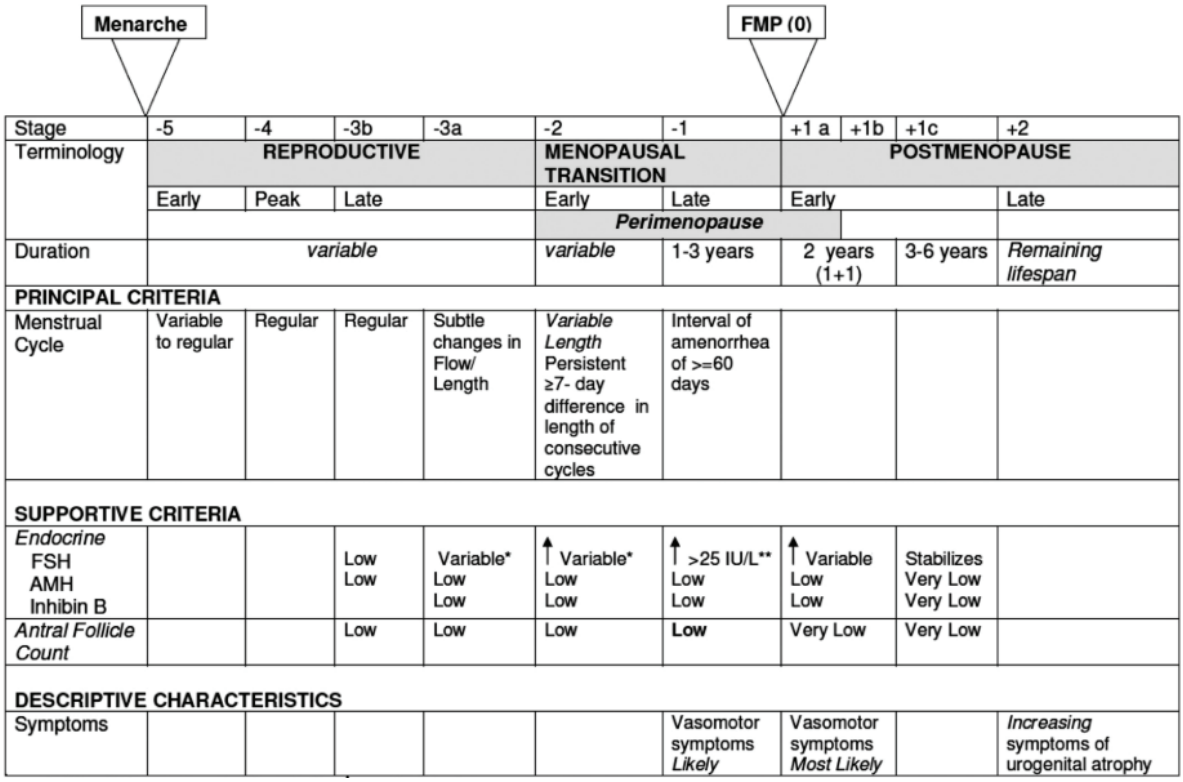


Figure 1.10.: Oestrogen and progesterone levels during the female life cycle. (Adapted from Harrington, 2020) (Created with BioRender.com)

These symptoms occur during perimenopause with some individuals experiencing symptoms post menopause. The long-term consequences of menopause, which include osteoporosis and cardiovascular disease, may result in morbidity and mortality (Armeni *et al.*, 2021; O'Neill & Eden, 2020). The severity and intensity of symptoms, which vary according to ethnicity, health and body composition, may impact a woman's quality of life, resulting in fatigue, irritability and a depressed mood (Al-Safi & Santoro, 2014; O'Neill & Eden, 2020). To address these symptoms and to provide preventative measures for chronic diseases, menopausal hormone therapy or the use of non-hormonal medications can be implemented (Al-Safi & Santoro, 2014).



Stage	-5	-4	-3b	-3a	-2	-1	+1 a	+1b	+1c	+2
Terminology	REPRODUCTIVE				MENOPAUSAL TRANSITION		POSTMENOPAUSE			
	Early	Peak	Late		Early	Late	Early			Late
					Perimenopause					
Duration	*variable*				*variable*	1-3 years	2 years (1+1)		3-6 years	*Remaining lifespan*
PRINCIPAL CRITERIA										
Menstrual Cycle	Variable to regular	Regular	Regular	Subtle changes in Flow/ Length	*Variable Length* Persistent ≥7- day difference in length of consecutive cycles	Interval of amenorrhea of >=60 days				
SUPPORTIVE CRITERIA										
Endocrine FSH AMH Inhibin B			Low Low	Variable* Low Low	↑ Variable* Low Low	↑ >25 IU/L** Low Low	↑ Variable Low Low		Stabilizes Very Low Very Low	
Antral Follicle Count			Low	Low	Low	**Low**	Very Low		Very Low	
DESCRIPTIVE CHARACTERISTICS										
Symptoms						Vasomotor symptoms *Likely*	Vasomotor symptoms *Most Likely*			*Increasing* symptoms of urogenital atrophy

Figure 1.11.: The stages of Reproductive Aging Workshop + 10 (STRAW+10) Staging System; Abbreviations: AMH – anti-müllerian hormone, FMP – final menstrual period, FSH – follicle stimulating hormone. (Harlow *et al.*, 2012)

1.4. MENOPAUSAL HORMONE TREATMENT

To alleviate menopausal symptoms, the most effective treatment is MHT. The decision to use hormone therapy should be part of a comprehensive health assessment to allow for personalized treatment. Women should be assessed in the context of their medical history, considering the presence of osteoporosis and cardiovascular disease, cultural norms, needs and preferences (Lee *et al.*, 2020; O'Neill & Eden, 2020; Armeni *et al.*, 2021). It's recommended that hormone therapy be conducted based on the frequency and severity of symptoms as well as lifestyle adjustments, as the long-term effects on cardiovascular disease and breast cancer are unclear (Lee *et al.*, 2020). MHT is suggested for perimenopausal women and postmenopausal women with risk factors for osteoporosis including women with POI, where it employs the lowest possible effective dose to control and inhibit bone loss (Table 1.2) (Armeni *et al.*, 2021).

Table 1.2.: Benefits and risks of using menopausal hormone treatments

Benefits	Risks
Relieves menopausal symptoms, such as:	Breast cancer
• Vulvovaginal atrophy symptoms	Stroke
• Osteoporosis	Dementia
• Diabetes	Venous thromboembolism
	Cardiovascular disease

Oestrogen with or without progesterone is the most effective in treating VMS and GSM (Al-Safi & Santoro, 2014; Files & Kling, 2020). Oestrogens are administered continuously to substitute the deficiency of reproductive hormones and to control menopausal symptoms (Stute *et al*., 2018; Archer *et al*., 2019). Oestrogen therapy increases endometrial hyperplasia in women with an intact uterus, increasing the risk of endometrial cancer, thus progestogens are supplemented to act as an oestrogen antagonist, providing endometrial protection (Santoro *et al*., 2016; Goyette *et al*., 2017). Other forms of menopausal treatment used for symptom relief include androgens, non-hormonal products, bisphosphonates and SERM's (Al-Safi & Santoro, 2014; Lee *et al.*, 2020). Currently, levonorgestrel releasing-intrauterine system (LNG-IUS) with oral or percutaneous oestrogen, low-dose combined oral contraceptives (COCs), and oestrogen-progesterone therapy (EPT) are generally recommended (Lee *et al.*, 2020; Yu & Hongyang, 2022). Oestrogens are classified into natural and synthetic oestrogens. Oestradiol valerate, E_2, and conjugated equine oestrogen (CEE) are the main natural oestrogens, while nylestriol and ethinylestradiol (EE) are synthetic oestrogens (Yu & Hongyan, 2022).

Progestogen is a term used to describe both synthetic (progestin) and natural progesterone (Armeni *et al.*, 2021). Natural progestogen is represented by P_4, whereas progestins mainly include dydrogesterone, medroxyprogesterone acetate (MPA), norethindrone (NET), and drospirenone (Yu & Hongyang, 2022). Progestogen regimens are either sequential or continuously administered (Al-Safi & Santoro, 2014). Sequential regimens entail progestogen intake for 12-14 days per cycle, which results in monthly withdrawal

bleeding. It is usually prescribed for patients experiencing POI, early menopause and perimenopause (Armeni *et al.*, 2021). Notably, cyclic regimens were theoretically intended to mitigate long-term risks associated with prolonged progestogen use, such as invasive breast cancer (Al-Safi & Santoro, 2014; Goyette *et al.*, 2017). Continuous regimens, on the other hand, are usually recommended for postmenopausal women and result in endometrial atrophy and amenorrhea (Armeni *et al.*, 2021).

Hormone therapy can also result in side effects such as headaches, bleeding, bloating, mood changes and nausea (Rinker-Schaeffer *et al.*, 2007). Oestrogen treatment is associated with increased risk of serious diseases such as venous thromboembolism and stroke (Al-Safi & Santoro, 2014). When combined with progestins, a further risk of coronary events and breast cancer is observed, as well as breast tenderness and increased density (Al-Safi & Santoro, 2014; Stute *et al.*, 2018). In addition to the risk MHT carries, the various hormonal formulations, preparations, routes, and modes of administration, further contribute to breast cancer risk. The oestrogen and oestrogen-progestogen combination preparations are available for oral ingestion, transdermal use, vaginal application and parenteral application (Lobo, 2022). For example, oral oestrogen administration is associated with hepatic-first pass, which results in the metabolization of oestrogen and a consequent reduction in the systemic bioavailability of oestrogen (Armeni *et al.*, 2021). Furthermore, this causes an increase in HDL-cholesterol, triglycerides, coagulations factors and sex hormone-binding globulin. On the other hand, in transdermal therapy, oestrogens are directly delivered into the systemic circulation, thus are associated with a lower risk of venous thrombosis. This emphasizes how crucial the need for caution in selecting treatment and administration methods.

1.4.1. Menopausal hormone therapy and breast cancer risk

The Women's Health Initiative (WHI) trial tested commonly combined hormone preparations in postmenopausal women, which brought to light the increased risk of breast cancer incidence through MHT. The study aimed to define the risks and benefits of the hormone treatments on the important aspects of health which were defined as coronary heart disease (CHD), invasive breast cancer, stroke, pulmonary embolism, endometrial cancer, hip fracture and death

(Writing Group for the Women's Health Initiative Investigators, 2002). This study demonstrated that the oestrogen-progestogen combination; CEE + MPA, increased the risk of breast cancer when compared to oestrogen therapy alone (Writing Group for the Women's Health Initiative Investigators, 2002). These results indicated a 15 % increased risk for oestrogen-progestogen use for less than 5 years, and a 53 % increased risk for five years or more (Writing Group for the Women's Health Initiative Investigators, 2002).

Due to the results of the 2001 WHI trial, there was a misconception that all MHT increases the risk of developing breast cancer, which resulted in a subsequent decline in the use of MHT and a shift toward the use of alternative therapies. However, contrary to the WHI results which demonstrate a decreased risk with oral oestrogen (CEE) use, the E3N (Etude Epidémiologique auprès de femmes de la Mutuelle Générale de l'Education Nationale) cohort (evaluated over 80 000 postmenopausal women taking different menopausal hormone regimens) showed that oestrogen only treatment increased breast cancer risk by 29 % (Clavel-Chapelon, 2015). It is important to note that the oestrogen only treatment was mostly administered transdermally. In the Million Women Study, breast cancer risk was increased two-fold (95 % CI 1.88 – 2.12) for current users of combined therapy, compared to a factor of 1.3 (95 % CI 1.2 – 1.4) for oestrogen only therapy (Million Women Study Collaborators, 2003). Although, the risk of breast cancer was not significant until 5 years of MHT use, it was reduced after discontinuation (Krämer *et al.,* 2005; Trabert *et al*., 2020). In contrast, new insights indicate that the risk associated with MHT use may continue longer than what was previously thought (Rymer *et al*., 2019; Vinogradova *et al*., 2020; Thomas *et al*., 2022).

The E3N cohort reported that oestrogen combined with dydrogesterone or progesterone may be the least harmful contributor to breast cancer risk (Clavel-Chapelon, 2015). Similarly, Stute *et al.* (2018), found that oestrogen combined with MPA or NET, increased the risk of breast cancer but not dydrogesterone or micronized progesterone. Furthermore, the cohort found that the risk of oestrogen only treatment did not differ significantly from the combination of oestrogen and P_4/dydrogesterone but differed significantly compared to the other progestogen combinations. The other progestogens combined with oestrogen in the cohort include medrogestone, chlormadinone acetate, promegestone, nomegestrol acetate, NET acetate (NETA) and MPA. Collectively, these studies

indicate that breast cancer risk varies between regimens, hormone combinations and progestins (Asi *et al*., 2016). Throughout this thesis, the term conventional therapy will be used to refer to FDA-approved and commonly used menopausal hormone preparations.

A recent study that evaluated protein biomarkers with proteomics discovered a link between MHT use and a higher risk of breast cancer in women (Thomas *et al*., 2022). While characterizing the circulating proteins, researchers found that lower levels of proteins involved in cell adhesion and immunoregulation were associated with the cluster of females on MHT. Furthermore, this cluster was also associated with higher levels of proteins involved with cell fate, DNA integrity, the female reproductive system and metabolism. These findings support that MHT may mediate the effects of risk factors, such as alcohol and tobacco use, obesity, and parity, or contribute to the emergence of breast cancer (Thomas *et al*., 2022). Additionally, this study discovered that the proteomic profile persisted years after MHT was discontinued, in contrast to earlier studies that suggested a complete reversal of MHT risk on breast cancer (Thomas *et al*., 2022). The study's limitations include the lack of specific information regarding the MHT regimens employed, the length of the course of treatment, dosage, and specifics regarding tumour features. Furthermore, it is difficult to ascertain how well alterations in circulating protein concentrations can reflect the physiological activities and changes in protein expression of the breast tissue (Thomas *et al.,* 2022). Overall, the results of this investigation call for additional research to confirm the long-lasting effects of MHT and its impact on proteins associated to tumour development and progression.

The use of conventional hormone therapy has decreased as a result of the controversy, despite the fact that there are other factors that raise the risk of breast cancer, such as the duration of hormone therapy (HT), the type of HT used, breast density, prior exposure to HT, and the overall risk that HT poses with obesity, inactivity, and alcohol and tobacco use (Kerlikowske *et al*., 2010; Guidozzi *et al*., 2014; Beral *et al*., 2019; Rymer *et al*., 2019; Lambrinoudaki, 2021). Numerous studies have demonstrated that natural oestrogen and/or progesterone combinations have less side effects than their synthetic counterparts, leading to an increase in their use (Fournier *et al.,* 2008; Holtorf, 2009; Liu *et al*., 2020; Martins *et al*., 2020). As such, there has been an increase in what is described as bioidentical hormones for the treatment of menopausal symptoms (Holtorf, 2009; Perkins *et al*., 2017).

1.4.2. Compounded-bioidentical Hormone therapy

In the advancement of precision medicine, compounding pharmacies custom make compounded bioidentical formulations to produce tablets, lozenges and creams in combination doses, or preparations that are not routinely available (Boothby *et al.,* 2004; Committee on Obstetric Practice, 2012). Bioidentical hormones have the same chemical structure as endogenous hormones, mimicking the activities of their endogenous counterparts (Holtorf, 2009; Committee on the Clinical Utility of Treating Patients with Compounded Bioidentical Hormone Replacement Therapy, 2020). Unlike FDA-approved bioidentical formulations, compounded bioidentical hormones are not subject to the same tests of efficacy, safety, or dosing consistency (Committee on Obstetric Practice, 2012; Newson & Rymer, 2019).

The rationale behind compounding is that compounded bioidentical hormone products are superior to FDA-approved treatments because they are safer and offer individualised preparations, as well as a wider range of doses and dosage forms (Committee on the Clinical Utility of Treating Patients with Compounded Bioidentical Hormone Replacement Therapy, 2020). The hormones commonly found in compounded bioidentical preparations include E_1, E_2, E_3, pregnenolone (P_5), P_4, testosterone, and dehydroepiandrosterone (DHEA). The Committee on the Clinical Utility of treating Patients with Compounded Bioidentical Hormone Replacement (2020), found 741 compounding formulations, of which 289 included more than one active pharmaceutical ingredient. Although a combination of multiple hormones in a single formulation is attractive or convenient to patients, the combination of active pharmaceutical ingredients requires careful consideration of drug-drug interactions, the compatibility of all the ingredients and whether the quantity of hormones is sufficient to elicit the desired outcomes.

Each compounding pharmacy has its own process for formulating a compounded prescription. Compounder-specific factors that can influence prescriptions include ingredient testing, ingredient choices, quality systems, compounding skills, available facilities, equipment and environmental skills (Committee on the Clinical Utility of Treating Patients with Compounded Bioidentical Hormone Replacement Therapy, 2020). For example, different compounders will have different processes to compound an identical prescription, which will likely result in varying preparations even though it is an identical prescription with the same label. Previous research has shown that compounded bioidentical therapies can cause patient

injury and even death, as was the case with glucocorticoid formulations contaminated with fungus (Santoro *et al*., 2016).

1.4.2.1. Implications of Compounded-bioidentical formulations in cancer

Popular formulations that are recommended for MHT include the biest and triest preparations, which combine bioidentical E_2 (bE_2) and bE_3 in a 20:80 ratio, respectively, and a bE_2, bE_3 and bE_1 formulation with a ratio of 10:80:10 (Newson & Rymer, 2019). According to the Committee on Obstetric Practice (2012), the ratios are based on the milligram quantity of the agents added together and not on each agent's potency. This may lead to overdosage and underdosage because of the variable bioavailability and bioactivity of agents, as well as variations in purity and human error across pharmacies, possibly increasing the risk of endometrial cancer and thromboembolism (Committee on Obstetric Practice, 2012; Perkins *et al.,* 2019; Newson & Rymer, 2019).

A comparison between two FDA-approved capsules and compounded progesterone formulations shows the varying strengths of compounded progesterone formulations (Figure 1.12). Of concern are the low progesterone preparations, as low dose progesterone is not effective in protecting the endometrium from unopposed oestrogen (Committee on the Clinical Utility of Treating Patients with Compounded Bioidentical Hormone Replacement Therapy, 2020). Thus, low dose progesterone for menopausal hormone therapy is ineffective as it fails to reduce the risk of developing endometrial cancer. Additionally, higher concentrations of progesterone have not yet been tested for safety and efficacy in FDA-approved drug products, raising a different set of safety concerns.

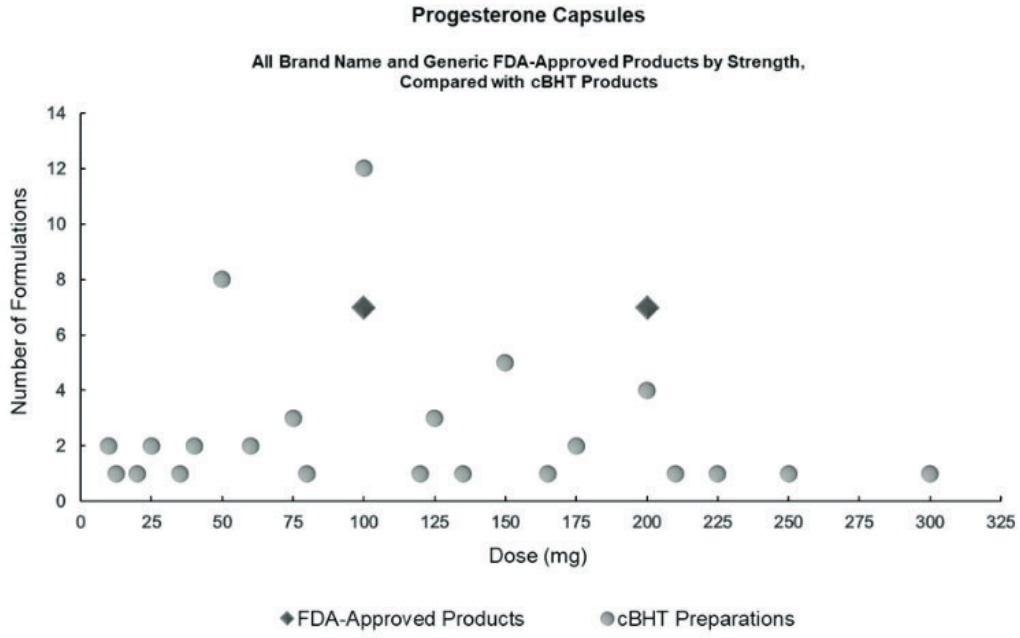


Figure 1.12.: A comparison of various FDA-approved progesterone products by strength with compounded-bioidentical progesterone products; Abbreviations: cBHT – compounded bioidentical hormone therapy, FDA – Food and Drug Administration. (Committee on the Clinical Utility of Treating Patients with Compounded Bioidentical Hormone Replacement Therapy, 2020).

A comparison of the different doses of compounded biest oestrogen cream with oral progesterone to a conventional oestradiol patch and Prometrium showed that the compounded bioidentical formulations yielded less oestrogen levels. This randomized clinical trial tested three doses of the compounded cream 2.0 mg, 2.5 mg, and 3 mg, with 100 mg of oral progesterone, while the patch was tested at 0.05 mg, with 100 mg of Prometrium (Sood *et al.*, 2013). As compounded bioidentical hormones come in various doses compared to conventional hormone preparations, it is important to investigate the effectivity and risks of these doses.

Additionally, the hormone combinations also require further study as the biest and triest formulations are based on the premise that E_3 and E_1 are weaker, safer oestrogens than E_2, in terms of the transactivation and transrepression of gene expression (Boothby *et al.*, 2004; Holtorf, 2009; Perkins *et al.*, 2018). Compared to the other oestrogens, E_3 has a higher binding affinity to ER-β, which inhibits proliferation and prevents breast cancer development through G_2 cell cycle arrest (Holtorf, 2009), whereas estrone selectively binds to ER-α at a ratio of 5:1

and oestradiol at 1:1. As oestrogen receptor-α is known to promote breast cell proliferation, the addition of E_3 is hypothesized to antagonize the negative effects of E_2 and E_1, while aiding in the alleviation of menopausal symptoms.

Looking more closely at E_3, it was reported that when combined with E_2, it inhibits transcription. It functions as an antioestrogen, competitively inhibiting oestradiol binding as well as activated receptor binding to the oestrogen response element (ERE) (Holtorf, 2009). Contrary to the theories about E_3's weakness and ability to antagonize E_2, Boothby *et al.* (2004) displayed that E_3 does not inhibit E_2 binding nor block the development of endometrial hyperplasia associated with E_2 and E_1. More recently, Perkins *et al.* (2018) demonstrated that bE_3 and bE_1 are not weaker compared to oestrogen in terms of transactivation and transrepression of gene expression. Furthermore, they showed E_3 did not act as an antagonist to E_2-induced breast cancer proliferation and anchorage dependent growth using the MCF-7 BUS breast cancer cell line (Perkins *et al.,* 2018). To note, there are no FDA-approved E_3-containing products. Although E_3 is termed as a "weaker" oestrogen, studies have shown that in the presence of oestrogen, it functions as an antioestrogen (Holtorf, 2009). The research is inconclusive as some studies find that estriol acts as an oestradiol antagonist, others find it makes no difference, and still others find it has a similar ability to the other oestrogens.

A recent study that aimed to characterize oestrogens found that bioidentical oestrogens frequently mimicked synthetic EE and mimicked the activity of their standard equivalents when compared to the corresponding commercially available standards (Perkins *et al.*, 2017). Furthermore, they showed that bE_3 and bE_1 are full oestrogen receptor agonists and are not weaker oestrogens compared to bE_2, suggesting the re-evaluation of biest and triest custom compounded formulations. Results from this study suggested that compounded bioidentical hormone therapy (cBHT) may not be a safer alternative to conventional hormone therapy.

Although clinical studies have shown the effectiveness of bioidentical hormone use, with less side effects, there is much that is still unknown about the effects of compounded bioidentical hormones (Conaway, 2011; Martins *et al.*, 2020). There are no large, long-term, randomized, double-blinded, placebo-controlled studies that have determined the effectiveness, safety, or adverse effects of custom-compounded bioidentical hormones

(Santoro *et al.,* 2016). More specifically, in the context of breast cancer risk, it is essential to investigate the effects of the compounded biest and triest formulations in breast cancer.

Classical examples of reprogrammed activities in cancer cells either support cell survival under stressful conditions or allow cells to grow and proliferate at pathologically elevated levels. In order to compare and understand the metabolic roles that hormone therapies play in the progression of cancer, the effects of the treatments on cell viability, proliferation, and migration will be investigated.

1.5. PROBLEM STATEMENT

There are a variety of treatment options available to alleviate menopausal symptoms, however the great majority are ineffective, and some may even be harmful (Guidozzi *et al*., 2014). Although some bioidentical hormone preparations have been FDA-approved, the South African Menopause Society have not updated their consensus on menopausal hormone therapy, which recommends against the use of bioidentical hormone and compounding products. Furthermore, the Medicines Control Council in South Africa requires the registration and mandatory regulation of conventional hormone therapy drugs (Guidozzi *et al*., 2014). These hormone products are subjected to regular testing for purity, potency, efficacy and safety, whereas compounded bioidentical hormone products are not subject to such tests. Therefore, claims of superior efficacy and safety of compounded bioidentical hormones need to be investigated.

The relationship between the conventional hormones and breast cancer risk have been established and studies have shown that CEE+MPA and CEE+NETA treatments promote proliferation and metastasis in cancer cells. The question then remains, whether the use of compounded bioidentical hormones will elicit similar effects in these cancer-specific processes, and what effect does that have on increasing breast cancer risk for women currently on cBHT.

1.6. AIMS

This research aims to elucidate the relationship between cBHT and breast cancer progression. Furthermore, to compare the effects of FDA-approved treatment and cBHT on proliferation, metastasis and the cell cycle in breast cancer cells.

1.7. RESEARCH QUESTIONS

1. Does the biest compounded-bioidentical hormone formulation increase breast cancer proliferation in the MCF7 breast cancer cell line?

2. Does the biest compounded-bioidentical hormone formulation contribute to invasion and metastasis in comparison with the FDA-approved hormone formulations in cancer cells
3. How do the menopausal hormones treatments in question contribute to the sustained proliferative signalling in cancer?

1.8. OBJECTIVES

I. To determine whether MCF7 and MCF-12A cell lines have oestrogen receptors present with western blot analysis.

II. To identify the effects on cell viability of the hormones that combine to form the menopausal hormone treatments in MCF7 breast cancer cells with a WST-1 assay.

III. To identify the effects on cell viability of compounded-bioidentical hormone therapy treatment vs conventional hormone therapy treatment in MCF7 breast cancer cells with a WST-1 assay.

IV. To determine the effects of compounded-bioidentical hormone therapy treatment vs conventional hormone therapy treatment on the proliferation of MCF7 breast cancer cells with cell cycle and western blot analyses.

V. To analyse the effects of compounded-bioidentical hormone therapy treatment vs conventional hormone therapy treatment on the metastasis of MCF7 breast cancer cells with western blot analyses and migration assays.

VI. To investigate the effects of compounded-bioidentical hormone therapy treatment vs conventional hormone therapy treatment on the PI3K/Akt signalling pathway in MCF7 breast cancer cells with western blot analyses.

CHAPTER 2: METHODS AND MATERIALS

2.1. MATERIALS AND STUDY DESIGN

2.1.1. Hormone treatments

Bioidentical hormones share the same molecular structure as endogenous hormones, however compounding pharmacies, where bioidentical hormones are produced, are not regulated by a governing body. Thus, for quality purposes a commercial standard is required. Therefore, all cells were treated with commercial standard hormones, bioidentical hormones, and/or conventional hormones, listed in Table 2.1. For oestrogen-only therapy as well as oestrogen-progestogen therapy, conjugated equine oestrogen (CEE) is commonly prescribed. In this study estrone 3-Sulfate salt will be represented by CEE as it is the primary active ingredient and constitutes 70% of the total content.

Table 2.1.: A table listing the conventional, bioidentical, and commercially available hormones being investigated.

Commercial Standard Hormones	Bioidentical Hormones	Conventional Hormones
E_2	bE_2	CEE
E_3	bE_3	MPA
P_4	bP_4	NETA

All hormones were diluted in 100 % ethanol and stored as stock solutions at -20 $^{\circ}$C. Prior to each experimental treatment, stock solutions were diluted further in growth media to a working solution, where the final concentration of ethanol is 0.1 % per treatment group. Cells were given a working solution of 0.1 % ethanol in growth media in order to control for any ethanol-related effects.

Cells were exposed to 0.1 nM, 1 nM, 10 nM, and 100 nM doses for each hormone to determine the optimal concentrations to utilize for the combination treatments. Concentration ranges are based on available literature (Goyette et al., 2017; Hasan et al., 2020;

Perkins et al., 2017; Seeger, Rakov & Mueck, 2005; Seeger, Wallwiener & Mueck, 2003). Oestrogen to progesterone ratios in clinical settings are typically 1:100 and, in some situations, 1:10; the ratio for this investigation was determined to be 1:100. The biest ratio commonly recommended is 2:8. See Table 2.2 for the working solution concentrations.

Table 2.2.: Optimised concentrations for the commercial standard hormone treatments, compounded-bioidentical hormone treatments and FDA-approved hormone formulations for the duration of the study.

Treatment	Formulation	Ratio	Concentration (nM)
Commercial standard hormone	$E_2 + P_4$	1:100	1 nM + 100 nM
	$E_2 + E_3$	2:8	1 nM + 4 nM
Compounded-bioidentical hormone formulation	$bE_2 + bE_3$	2:8	1 nM + 4 nM
FDA-approved formulations	CEE + MPA	1:100	1 nM + 100 nM
	CEE + NETA	1:100	1 nM + 100 nM
	$bE_2 + bP_4$	1:100	1 nM + 100 nM

2.2. Cell culture

The human breast adenocarcinoma cell line, MCF7, was used in this study. MCF7 cells were maintained in Dulbecco's Medium Eagle Modified (DMEM) (Gibco®, ThermoFisher Scientific, MA, USA) supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin-Streptomycin and stored at 4 °C. To study the effects of the steroid hormones without the confounding effects of endogenous hormones in serum, cells were cultured in 5 % charcoal-stripped FBS and phenol-red free media for 48 hours prior to the start of the experiments.

Cells were incubated under a humidified atmosphere containing 5 % CO_2 at 37 °C. Cells were sub-cultured using 0.4 % trypsin once a confluency of approximately 70-80 % was reached. Cells were not sub-cultured more than 20 times. Strict aseptic control was always applied according to biosafety level-2 (BSL-2) regulatory conditions.

2.3. Treatment protocol

MCF7 cells were incubated for 48-hours in the appropriate phenol-red free media prior to treatment. Cells were treated with the appropriate hormone combinations for 72-hours as listed in Table 2.2. Additionally, experimental endpoints of five, 10, and 15 minutes after treatment at 48 hours were observed in order to analyse protein expression on the rapid non-genomic effects of the PI3K/Akt pathway. For each experimental technique, cells were seeded as displayed in Table 2.3. All experiments were conducted in triplicate and when mentioned, in quadruplet.

Table 2.3.: Seeding densities for each experimental technique

Technique	Culture Vessel	Surface area (cm^2)	Seeding Density ($cells/cm^2$)
WST-1	96-well plate	0.32	1.0×10^5
	48-well plate	1.1	2.5×10^5
Flow cytometry	T-25 flask	25	8.0×10^5
Migration assay	48-well plate	1.1	2.5×10^5
Western blot	T-25 flask	25	8.0×10^5

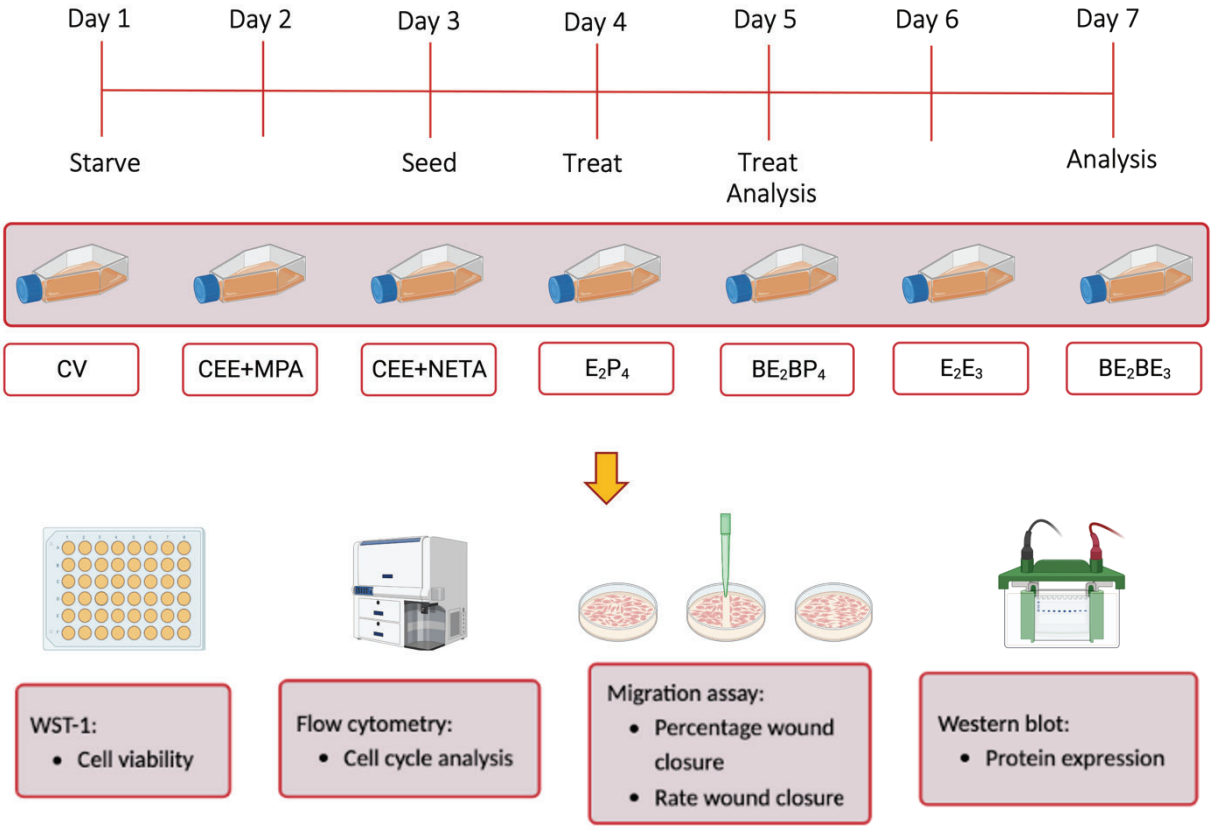


Figure 2.1.: Study design; Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

2.4. WST-1

The WST-1 Cell Proliferation Assay (Roche, Sigma-Aldrich) was used to examine cell viability. This assay measures the viability of cells through its metabolic activity, by quantifying mitochondrial reductive capacity. Briefly, this colorimetric assay measures the cleavage of tetrazolium salt, MTS, by mitochondrial dehydrogenases to form formazan in viable cells. The levels of formazan can then be used to quantify cell proliferation or cytotoxicity. After the 72-hour incubation with hormone treatments, cells were incubated with a 1:10 ratio of WST-1 to volume per well, for 90 minutes at 37 °C. Colorimetric readings were measured at a wavelength of 450 nm using a EL800 Universal Microplate Reader (BioTek Instruments Inc., VT, USA) and the KC Junior software.

This assay was firstly used to determine the optimal concentrations of each hormone. In this instance cells were seeded in 96-well plates and treated accordingly. Secondly, this assay was utilized to assess the hormone combination effects on cell viability in 48-well plates.

2.5. Western Blot Analysis

1.5.1 Protein harvest from cells

Following the 72-hour, and 48-hour hormone treatment period, the cell culture flasks were placed on ice. The cell culture media was aspirated from the flasks, and the cell monolayers was subsequently washed twice with ice cold phosphate buffered saline (PBS). Modified radioimmunoprecipitation (RIPA) buffer consisting of 65 mM Tris, 154 mM sodium chloride (NaCl), 1 % NP-40, 1 % Na-deoxycholate, 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA) and 0.1 % Sodium dodecyl sulfate (SDS) at a pH of 7.4 was used for cell lysis. The following was added to the RIPA buffer immediately before use; protease inhibitor cocktail (Sigma), phenylmethylsulphonyl fluoride (PMSF), sodium fluoride (NaF) and sodium orthovanadate (Na_3VO_4). The cell monolayer was incubated in 150 µl RIPA buffer for 2-3 minutes on ice. The cell monolayer was then scraped using a sterile cell scraper, which was washed in 100% ethanol between treatment groups. The cell lysates were then transferred from the flask to a pre-cooled microcentrifuge tube, incubated for an hour over ice, briefly vortexed, and centrifuged at 16.3 *g* for 2 minutes at 4 ^{o}C. Following the Bradford assay and sample preparation, cell lysates were stored at -80 °C.

1.5.2 Bradford assay and sample preparation

The Bradford assay was used to measure the total concentration of extracted proteins present in the cell lysates. A 5X Bradford stock solution was prepared by dissolving 500 mg Coomassie Brilliant Blue G-250 in 250 ml 95 % ethanol and 500 ml phosphoric acid, this was then made up to a final volume of 1 L with distilled H_2O. The 5X stock solution was then filtered overnight until it appeared brown. A 1X working solution was prepared by diluting the stock with distilled H_2O and filtering until brown. Bovine serum albumin (BSA, Roche) was used to prepare a

standard curve for each assay. In order to produce the standard curve, 0 µg (Blank), 2 µg, 4 µg, 8 µg, 12 mg, 16 µg, and 20 µg BSA were prepared in a final volume of 100 µl H_2O with the addition of 900 µl of Bradford working solution (in duplicate). A volume of 5 µl for each sample (in duplicate) was diluted in 95 µl distilled H_2O and 900 µl Bradford working solution. Absorbencies were measured on the Cecil CE 2021 spectrophotometer (Cecil Instruments) set to a wavelength of 595 nm. The spectrophotometer was zeroed using the blank (0 µg) sample containing 100 µl distilled H_2O and 900 µl Bradford working solution. The readings obtained for the Bradford assay were used to determine the volume of each sample to load 50 µg of total protein. For sample preparation, a 3X Laemmli's sample buffer was used to prepare samples for Western blots. A ratio of 2:1 (protein: sample buffer) was prepared with final concentrations 62.5 mM Tris, 4 % SDS, 10 % glycerol, 0.03 % bromophenol blue and 5 % β-mercaptoethanol (for a 1X solution). Samples were then stored at -80^{o}C until use.

1.5.3 SDS-PAGE

Western blotting samples were allowed to thaw on ice. The samples were then briefly vortexed, heated for five minutes at 95 ^{o}C to ensure that the proteins were denatured, then briefly centrifuged for 6-8 seconds. The BLUeye Prestained Protein Ladder (Sigma-Aldrich) was loaded on each gel as a molecular weight marker. The Tris/Glycine/SDS running buffer (Bio-Rad, CA, USA) was used for gel electrophoresis. Protein was loaded onto a 12 % TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, CA, USA). Proteins were separated at 100 V until samples entered the resolving gel, (approx.10 minutes) and thereafter at 120 V until the blue dye reaches the bottom of the gel (approx. 1 hour). Proteins were transferred onto PVDF membranes with the Trans- Blot® Turbo™ RTA Mini PVDF Transfer kit (Bio- Rad, CA, USA) and the Trans-Blot® Turbo Transfer System (Bio-rad, CA, USA). Following protein transfer, the membranes were washed three times for five minutes in 1X Tris-buffered saline with 0.1 % Tween® 20 detergent (TBS-T), and total protein images were obtained on the ChemiDoc™ MP (Bio-rad, CA, USA) system to confirm protein transfer. Membranes were blocked with either 5 % milk and TBS-T, or 5 % BSA (for phosphorylated proteins) for an hour with gentle shaking, to prevent non-specific binding of the primary antibodies. Membranes were then washed three times for five minutes in TBS-T, followed by an overnight incubation on a roller at 4 °C with the

appropriate primary antibodies. All antibodies were prepared in 5 ml TBS-T in 50 ml canonical tubes as shown in Table 2.4. The following day, membranes were washed three times for five minutes in TBS-T and then incubated in IgG horseradish peroxidase conjugated secondary antibodies antibody on the roller for one hour at room temperature (RT). After incubation in the secondary antibody, membranes were washed three times for five minutes in TBS-T and subsequently imaged on the ChemiDoc™ MP (Bio-rad, CA, USA) system with Clarity™ ECL Substrate (Bio-Rad, CA, USA).

Table 2.4.: Primary and secondary antibody concentrations used for western blot analysis

Primary Antibody	Antibody concentration	Molecular weight (kDa)	Secondary Antibody	Antibody concentration
PI3K/Akt Signalling				
Total Akt	1:1 000	60	Anti-rabbit-HRP	1: 10 000
Phosphorylated Akt (ser473)	1:1 000	60	Anti-rabbit-HRP	1: 10 000
Phosphorylated GSK-3β (ser9)	1:1 000	46	Anti-rabbit-HRP	1: 10 000
Phosphorylated PTEN (ser380)	1:1 000	54	Anti-rabbit-HRP	1:10 000
Phosphorylated PDK-1	1:1 000	58 -68	Anti-rabbit-HRP	1:10 000
Proliferation marker				
MCM2	1:1 000	125	Anti-rabbit-HRP	1:10 000
EMT: Epithelial-mesenchymal transition markers				
E-cadherin	1:1 000	135	Anti-rabbit-HRP	1:10 000
Slug (SNAIl2)	1:1 000	30	Anti-rabbit-HRP	1:10 000
N-cadherin	1:1 000	140	Anti-rabbit-HRP	1:10 000
β-catenin	1:1 000	92	Anti-rabbit-HRP	1:10 000
Oestrogen Receptors				

ER-α	1:1 000	65	Anti-rabbit-HRP	1:10 000
ER-β	1:1 000	55	Anti-rabbit-HRP	1:10 000

2.6. FLOW CYTOMETRY

To assess the cell cycle, DNA content was quantified with flow cytometry, as cells in different phases of the cell cycle have different amounts of DNA present. Following the appropriate treatment protocols, cells were harvested with 0.4 % trypsin and centrifuged at 1750 RPM for four minutes. Thereafter, cells were resuspended in warm PBS and counted with the Countess™ 3 and centrifuged at 1750 RPM for four minutes. Cells were fixed by mixing the pellet with ice-cold 70 % ethanol with gentle vortexing, then incubated on ice for one hour. After an hour, cells underwent a five-minute centrifugation at 3000 RPM followed by a PBS wash step. The pellet was then resuspended in a mixture of 100 μg/ml RNase A and 50 μg/ml propidium iodide (PI). Cells were incubated at room temperature for 30 minutes and covered with foil. Analysis was done using a BD FACS Melody cell sorter at the Central Analytical Facility (CAF).

2.7. WOUND-HEALING ASSAY

1.7.1 Mitomycin C (MMC)

In order to exclude the effects of cellular proliferation from the wound-healing assay, cells are treated Mitomycin C (MMC). MMC is a strong DNA crosslinker that inhibits the synthesis of DNA. MMC (Sigma-Aldrich) was prepared at a concentration of 0.4 mg/ml in sterile PBS. As MMC is light sensitive, the experimental protocol was performed in the dark. Dose response experiments were performed to determine the optimal concentration for MMC for the MCF7 cell line over 48 hours. The cells were left to adhere for 24 hours, after which they were treated with MMC. It was previously shown that a dose of 2 μg/ml successfully suppressed growth after 24 hours (Table 2.6) since MMC was not stable for 48 hours as seen in Table 2.5. To determine the optimal concentration of MMC, cells were seeded onto sterile coverslips in 6-

well plates, a 0-hour control coverslip was fixed with 4 % paraformaldehyde (PFA) at the time of MMC treatment. The remaining coverslips were fixed with 4 % PFA following 48 hours. The coverslips were fixed in 1:1 ratio of 4 % PFA and media for 5 minutes, followed by fixation of 4 % PFA only for five minutes. The coverslips were then washed three times with PBS. Nuclear staining was performed with Hoechst for 10 minutes, after which they were washed three times. The coverslips were then mounted onto microscope slides with DAKO Fluorescent Mounting Medium (DAKO) and were left to dry at RT for 1 hour. A total of 9 images at random fields of view were acquired on a Nikon Eclipse E400 microscope equipped with a DS-F12 colour digital camera (Nikon, Japan) with a DAPI barrier filter (excitation 340-380 nm, emission 435-483 nm). Nuclear counts were performed to assess cellular proliferation using the ImageJ software, and 0-hour control groups were compared to 48 hour treated groups.

Table 2.5.: Nuclear count of cells during mitomycin c optimisation for 48 hours

Group (time point)	Control (0 hours)	Control (48 hours)	2 μg/ml (48 hours)	4 μg/ml (48 hours)
Average count (n=9)	517.33	1464.89	285.67	187.44
Total count	4309	13184	2571	1687

Table 2.6.: Nuclear count of cells during mitomycin c optimisation for 24 hours (du Plessis, 2022)

Group (time point)	Control (0 hours)	Control (24 hours)	1 μg/ml (24 hours)	2 μg/ml (24 hours)
Average count (n=9)	359.22	800	386.78	314.22
Total count	3233	7200	3481	2828

1.7.2 Migration Assay Procedure

Cellular migration was evaluated using a wound healing assay, also referred to as a scratch assay. The experiment is based on the capacity of confluent monolayered cells to fill a

gap over time. Cells were treated with a cytostatic drug, Mitomycin C, to eliminate the effects of cellular proliferation. This ensures that the closure of the gap is due to migration and not proliferation. Following the appropriate treatment protocols, a wound or scratch was made in each well, with a scratcher. Control images were taken at a 0-hour time-point, acquired with the 4x objective on a Zeiss Olympus® CKX41 inverted microscope (Olympus®, GMBH Japan) using the Zeiss Laboscope software (Carl Zeiss, Germany). For each well, a distinguishable marker was made on the lid of the culture plate, which indicated the area of the scratch to be imaged. The wells were then refreshed with media supplemented with appropriate treatments and MMC, then incubated at 37 °C covered with foil. Analyses of the images were performed with ImageJ analysis software and the Wound Healing Size Tool plugin was used to determine the area of the wounds. The area of wounds was calculated, in μm^2, by the software by demarcating the wounded area along the migration front on scaled images.

The following formula was used to calculate the percentage of wound closure:

$$\frac{wound\ area\ at\ (0\ hr) - wound\ area\ (x\ hr)}{wound\ area\ (0\ hr)} \times 100 = \%\ wound\ closure\ at\ x\ hr$$

The rate of wound closure was calculated with the following equation:

$$\frac{\%\ wound\ closure\ at\ (x\ hr)}{x} = rate\ of\ wound\ closure\ at\ x\ hr\ (\%.hr^{-1})$$

1.8 STATISTICAL ANALYSIS

Data was represented visually and statistically using GraphPad Prism® Version 9 for Mac OS (GraphPad Software, San Diego, CA). All values were represented as a percentage of the control and mean ± standard error of the mean (SEM). Data was tested for normality using the Shapiro-Wilk test. One-way ANOVAs and two-way ANOVAs were used where applicable, using Dunnet's test, Tukey, or Fisher's LSD post-hoc tests with a p-value < 0.05 considered as statistically significant.

Table 2.7.: Statistical analysis performed for each method

Method	Statistics
WST-1	One-way ANOVA, Dunnets tests
Flow cytometry	Two-way ANOVA, Tukey test
Western blot	One-way ANOVA, uncorrected Fisher's LSD
	One-way ANOVA, uncorrected Fisher's LSD
Migration assay	Two-way ANOVA, uncorrected Fisher's LSD

CHAPTER 3: RESULTS

3.1. OESTROGEN RECEPTOR PRESENCE

Western blot analysis was utilized to assess whether oestrogen receptors (ERs) -α and -β were present on the MCF7 and MCF12-A cell lines. The MCF7 cell line displayed the presence of ER-α but not ER-β (Figure 3.1). Neither ER-α nor ER-β were present on the MCF12-A cells. Based on these results, we conducted the rest of the experiments on the MCF7 cell line.

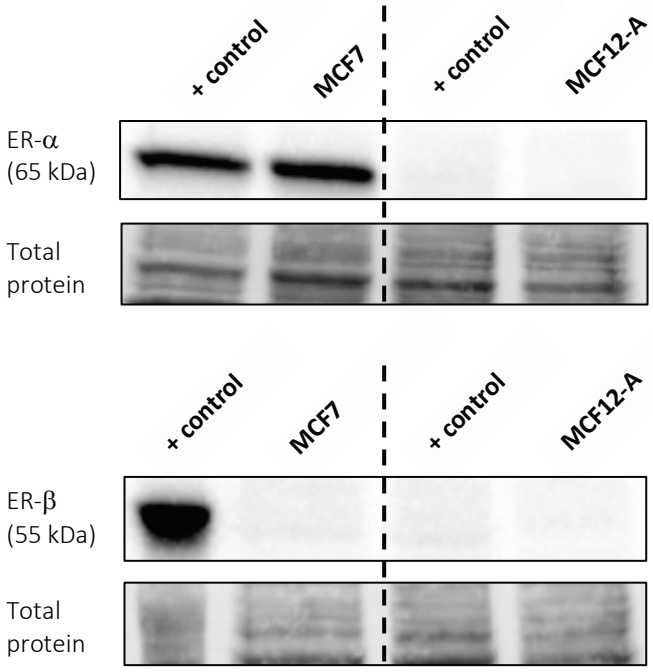


Figure 3.1.: Protein expression of ER-α and ER-β of MCF7 and MCF12A cells; Abbreviations: ER – oestrogen receptor.

3.2. CONCENTRATION-RESPONSE CURVES

The WST-1 assay was employed to assess the individual effects of the hormone treatments on breast cancer cell viability. The tumourigenic MCF7 cell line was exposed to various concentrations of steroid hormones; oestradiol (E_2), estrone 3-Sulfate salt (CEE), estriol (E_3), bioidentical E_2 (BE_2), BE_3, progesterone (P_4), medroxyprogesterone acetate (MPA), norethindrone acetate (NETA), for 72 hours. Based on these results and literature, we chose

the optimal concentrations of each hormone treatment for the combination experiments.

Treatment of CEE did not significantly alter cell viability of MCF7 cells (Figure 3.2.1.A). However, a concentration of 1 nM was chosen to be used in combination with MPA and NETA, as the cell viability was at the highest peak at 1 nM.

Treatment of E_2 increased cell viability when treated at 1 nM compared to the control ($p<0.05$). Although not significant, we also observed a decrease in MCF7 cell viability at the 10 – 100 nM range (Figure 3.2.1.B). As the concentration of 1 nM elicited a significant increase in cell viability, it was chosen as the concentration to be used in combination with E_3 and P_4, throughout this study.

Treatment of BE_2 significantly increased cell viability at all concentrations (Figure 3.2.1.C). Exposure to BE_2 increased cell viability significantly at 1 nM, followed by lowered viability from 10 – 100 nM. A concentration of 1 nM was chosen to be used in combination with BE_3 throughout this study.

The 72-hour treatment of E_3 increased cell viability significantly from the at 1 nM and 10 nM (Figure 3.2.1.B). The greatest increase in cell viability activity occurred at 1 nM ($p<0.001$). A concentration of 4 nM was chosen to be used in combination with E_2, in accordance with the ratio for combined treatments.

BE_3 induced an increase in cell viability at 1 nM ($p<0.01$) and 10 nM ($p<0.05$) (Figure 3.2.1.E). Similar to E_3, BE_3 was chosen to be combined with BE_2 at a dose of 4 nM throughout the duration of this investigation.

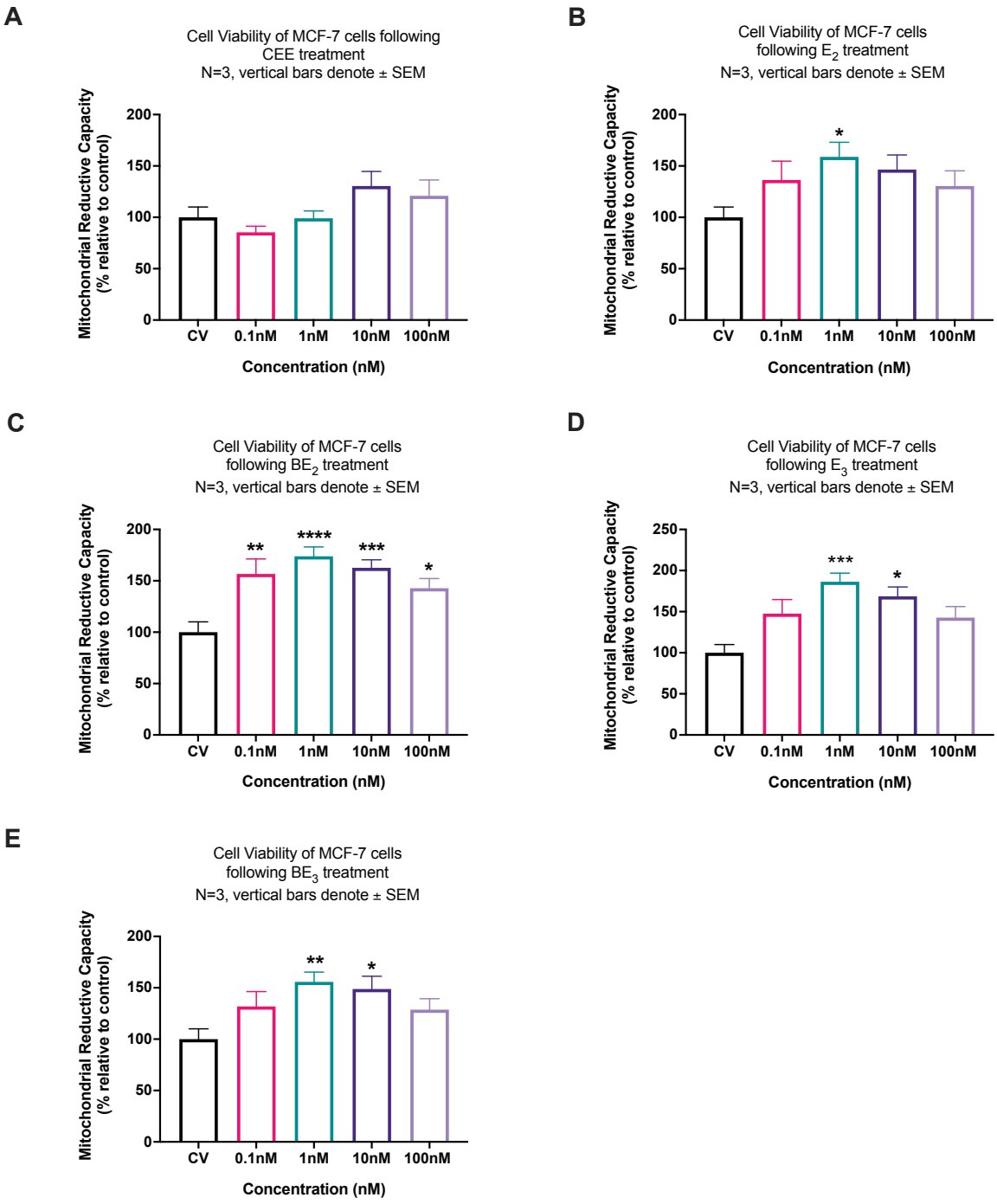


Figure 3.2.1. Cell viability of MCF7 cells following oestrogen treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol; * $p<0.05$ vs control vehicle; ** $p<0.01$ vs control vehicle; *** $p<0.001$ vs control vehicle; **** $p<0.0001$ vs control vehicle.

The 72-hour exposure of MCF7 cells to MPA exhibited an increase in cell viability at all concentrations (Figure 3.2.2.A). Particularly, the greatest increase was observed at 100 nM ($p<0.0001$) and 1 nM ($p<0.0001$). A concentration of 100 nM was chosen to be used in combination with CEE throughout this study.

Treatment of NETA on MCF7 cells seemed to increase cell viability in a concentration-dependent manner, with reduced viability at 100 nM ($p=0.05$) (Figure 3.2.2.B). Activity peaked at 1 nM ($p<0.01$) and 10 nM ($p<0.01$). Similar to P_4 a concentration of 100 nM was chosen in combination with CEE for the duration of the study, even though a significant response was not elicited.

Treatment of P_4 for 72 hours significantly increased cell viability at 0.1 nM ($p<0.05$) and 1 nM ($p<0.05$) (Figure 3.2.2.C). Although a significant increase in activity was not observed at 10 nM ($p=0.05$) or 100 nM ($p=0.9802$), a concentration of 100 nM was chosen to be used in the combination with E_2, in accordance with the accepted ratio of hormone combinations.

Treatment of BP_4 for 72 hours significantly increased cell viability at 1 nM ($p<0.0001$) and 10 nM ($p<0.0001$) (Figure 3.2.2.D). Although 0.1 nM and 100 nM did not influence cell viability, a concentration of 100 nM was chosen to be used in the combination with BE_2, in accordance with the accepted ratio of hormone combinations.

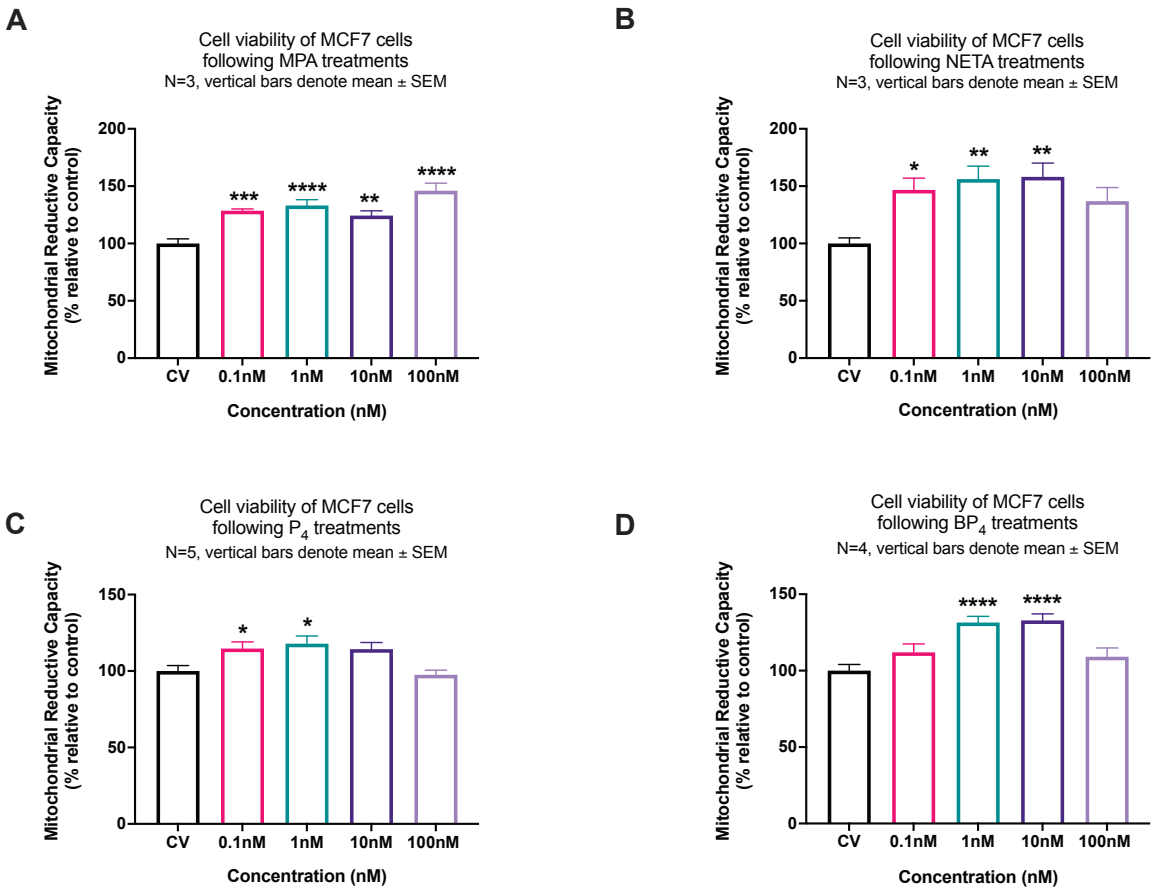


Figure 3.2.2. Cell viability of MCF7 cells following progestogen treatments for 72 hours. Values expressed as a percentage of the vehicle control. Vertical bars denote mean ± SEM. The results are representative of three independent experiments unless otherwise stated. Abbreviations: BP_4 – bioidentical progesterone, CV – control vehicle, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate; * $p<0.05$ vs control vehicle; ** $p<0.01$ vs control vehicle; *** $p<0.001$ vs control vehicle; **** $p<0.0001$ vs control vehicle.

3.3. COMBINED HORMONE TREATMENTS INCREASE THE CELL VIABILITY OF MCF7 CELLS

The exposure of all hormone combination treatments on MCF7 cells for 72 hours increased cell viability significantly when compared to the control vehicle ($p<0.0001$) (Figure 3.2).

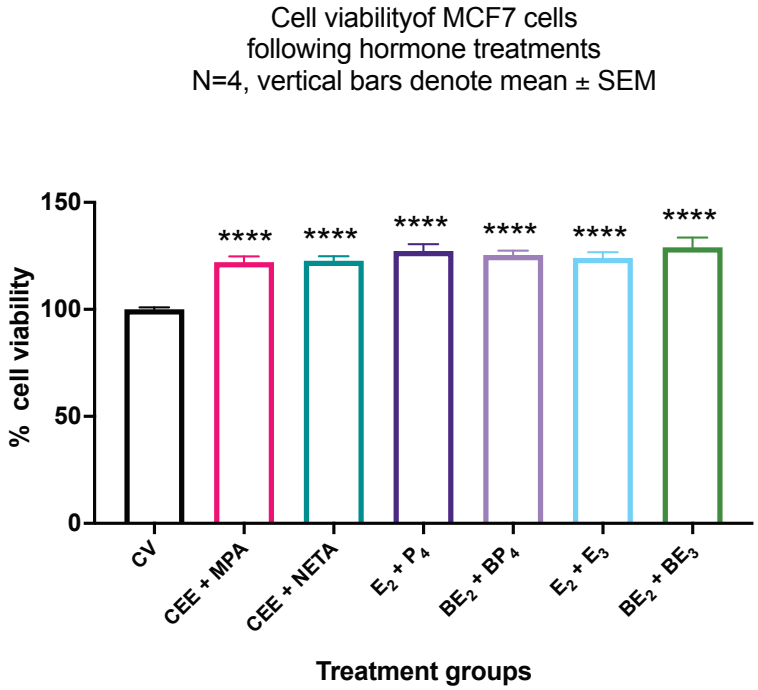


Figure 3.2.3.: Cell viability of MCF7 cells following combined hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of four independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate; **** $p<0.0001$ vs control vehicle.

3.4. THE CEE + MPA AND CEE + NETA TREATMENTS INDUCED THE ACCUMULATION OF MCF7 CELLS IN THE G0/G1 PHASE OF THE CELL CYCLE

Using the fluorescent dye, propidium iodide, DNA content in cells was determined in the different stages of the cell cycle. Cells preparing for cell division contain increasing amounts of DNA and display proportionally increased fluorescence. The DNA content distribution is represented as cell count versus linear fluorescence in a histogram. The proportion of cells increased significantly in the G_0/G_1 phase upon treatment of CEE+MPA ($p<0.0001$) and CEE+NETA when compared to the control vehicle ($p<0.05$) (Figure 3.3). Furthermore, CEE+MPA had a greater proportion of cells in the G_0/G_1 phase when compared to the other treatment combinations. No significant differences of cell percentage in the S and G_2/M phase were observed when comparing the treatment groups to the control or each other.

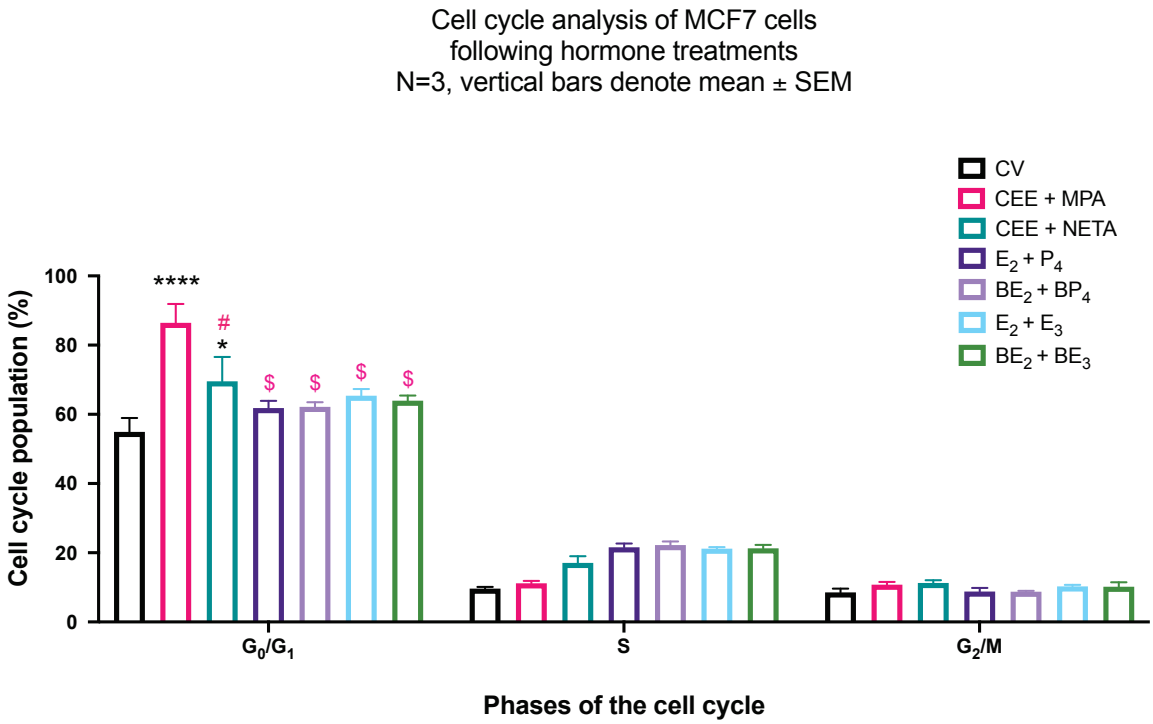


Figure 3.3.: Cell cycle analysis of MCF7 cells following hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle (CV). Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate. * $p<0.05$ vs control vehicle; **** $p<0.001$ vs control vehicle; # $p<0.001$ vs CEE + MPA; $ $p<0.0001$ vs CEE + MPA.

3.5. THE COMBINATION HORMONE TREATMENTS DID NOT INDUCE INCREASED MCM2 EXPRESSION

MCM2 plays a key role in DNA replication. To evaluate whether the increases in cell viability following hormone combination treatment is a result of increased proliferative capacity, the protein expression of MCM2 was assessed. Exposure to hormone treatments did not significantly increase proliferation compared to the control vehicle (Figure 3.4).

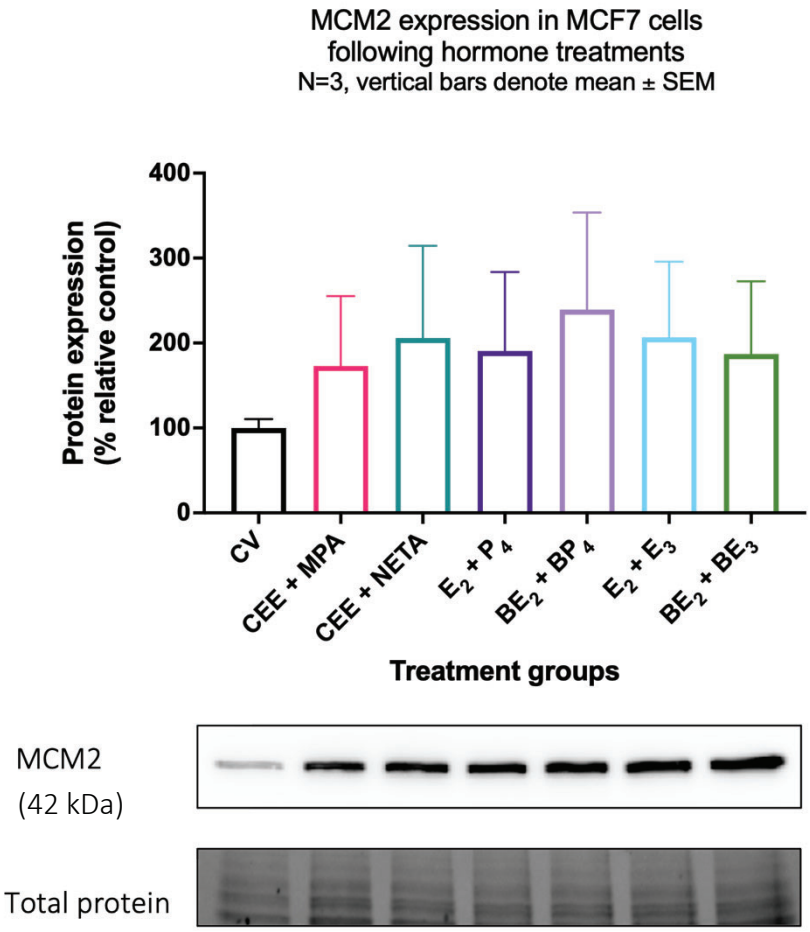


Figure 3.4.: MCM2 protein expression of MCF7 cells following hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

3.6. THE COMBINATION HORMONE TREATMENTS DID NOT ACTIVATE THE PI3K/AKT SIGNALLING PATHWAY

Activation of the PI3K signalling pathway induces a rapid activation of transcription factors and downstream proteins that promote cancer cell proliferation. Thus, the effects of hormone exposure on MCF7 cells were investigated after treatment exposure at five, 10 and 15 minutes, respectively. Western blot analysis was used to evaluate the protein expression of the various components of the PI3K signalling pathway.

The PI3K pathway is regulated by PTEN, which when activated, converts PIP_3 back to PIP_2, dampening Akt activation. Hormone treatments did not elicit any significant changes in PTEN phosphorylation after five-minute treatment exposure compared to the control vehicle (Figure 3.5). Exposure to hormone treatments for 10 minutes did not seem to change PTEN phosphorylation compared to five-minute exposure, or between treatment groups. Furthermore, neither of the treatment combinations elicited changes in PTEN phosphorylation after 15-minutes compared to the control vehicle.

PDK-1 phosphorylation on Thr308 and Ser473 phosphorylation by MTORC1 are both necessary for full Akt activation. When compared to the control vehicle, none of the treatment groups showed increased PDK-1 phosphorylation after hormone exposure (Figure 3.6). Although no significant differences were observed, PDK-1 phosphorylation trended upwards following 10-minute treatment exposure of $E_2 + P_4$ ($p=0.099$), $BE_2 + BP_4$ ($p=0.083$), $E_2 + E_3$ ($p=0.094$) and $BE_2 + BE_3$ ($P=0.082$) when compared to the control vehicle.

Similar to PDK-1 phosphorylation, exposure to hormone treatments at five, 10 and 15 minutes did not to induce changes in Akt phosphorylation compared to the vehicle control and in all treatment groups (Figure 3.7). Moreover, no differences can be seen between treatment groups at any of the time points.

Phosphorylation of GSK-3β, a downstream target of Akt activation, leads to the inhibition of GSK-3β activity. Exposure to all hormone treatments did not significantly increase GSK-3β phosphorylation after five- and 10-minutes of exposure (Figure 3.8) compared to the control vehicle. After 15 minutes, treatment exposure to of $E_2 + E_3$ ($p<0.05$) significantly elevated GSK-3β's phosphorylation in comparison to the control vehicle.

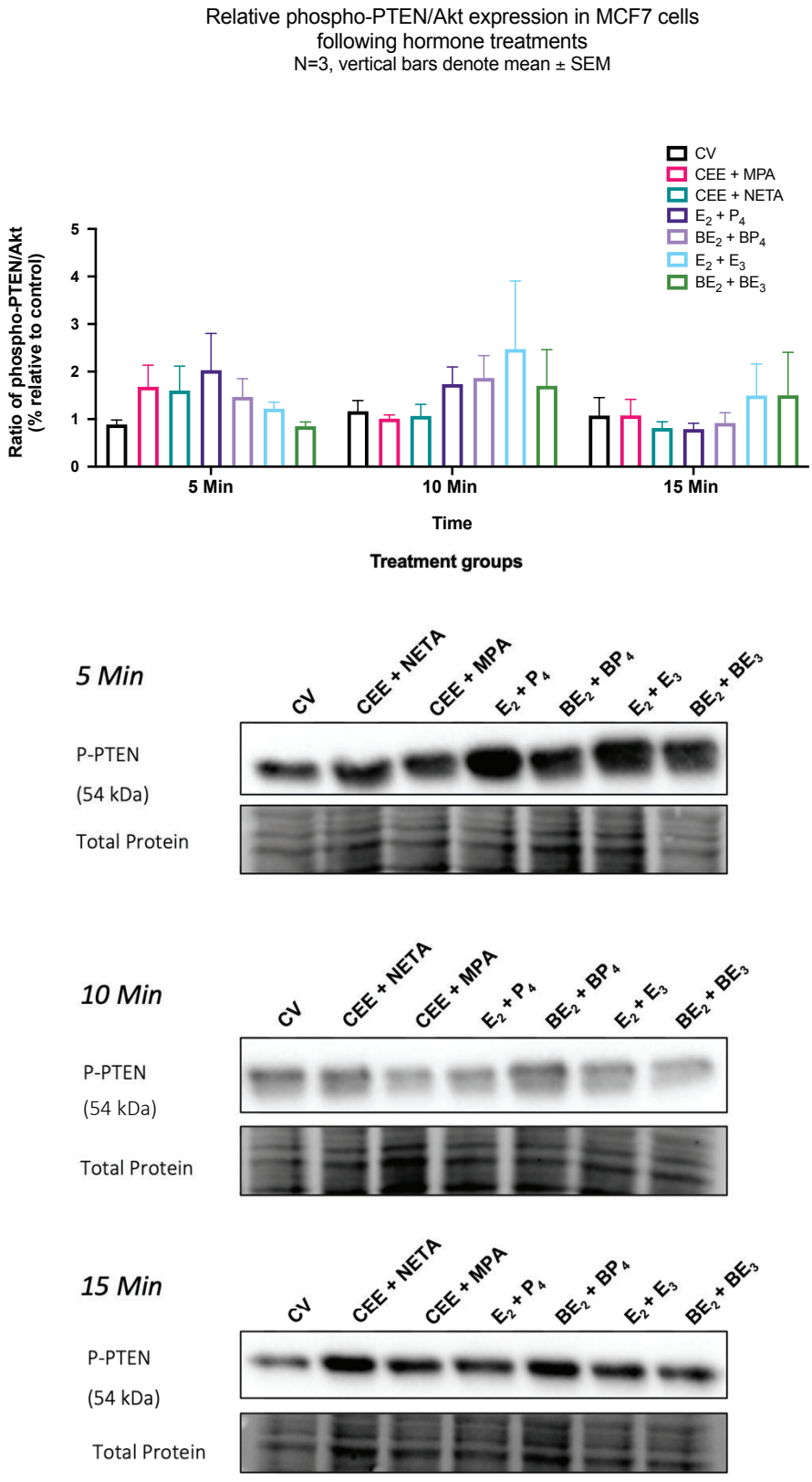


Figure 3.5.: Relative protein expression of phosphorylated P-PTEN/Akt protein expression of MCF7 cells at five, 10 and 15 minutes after 48-hour hormone treatment. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

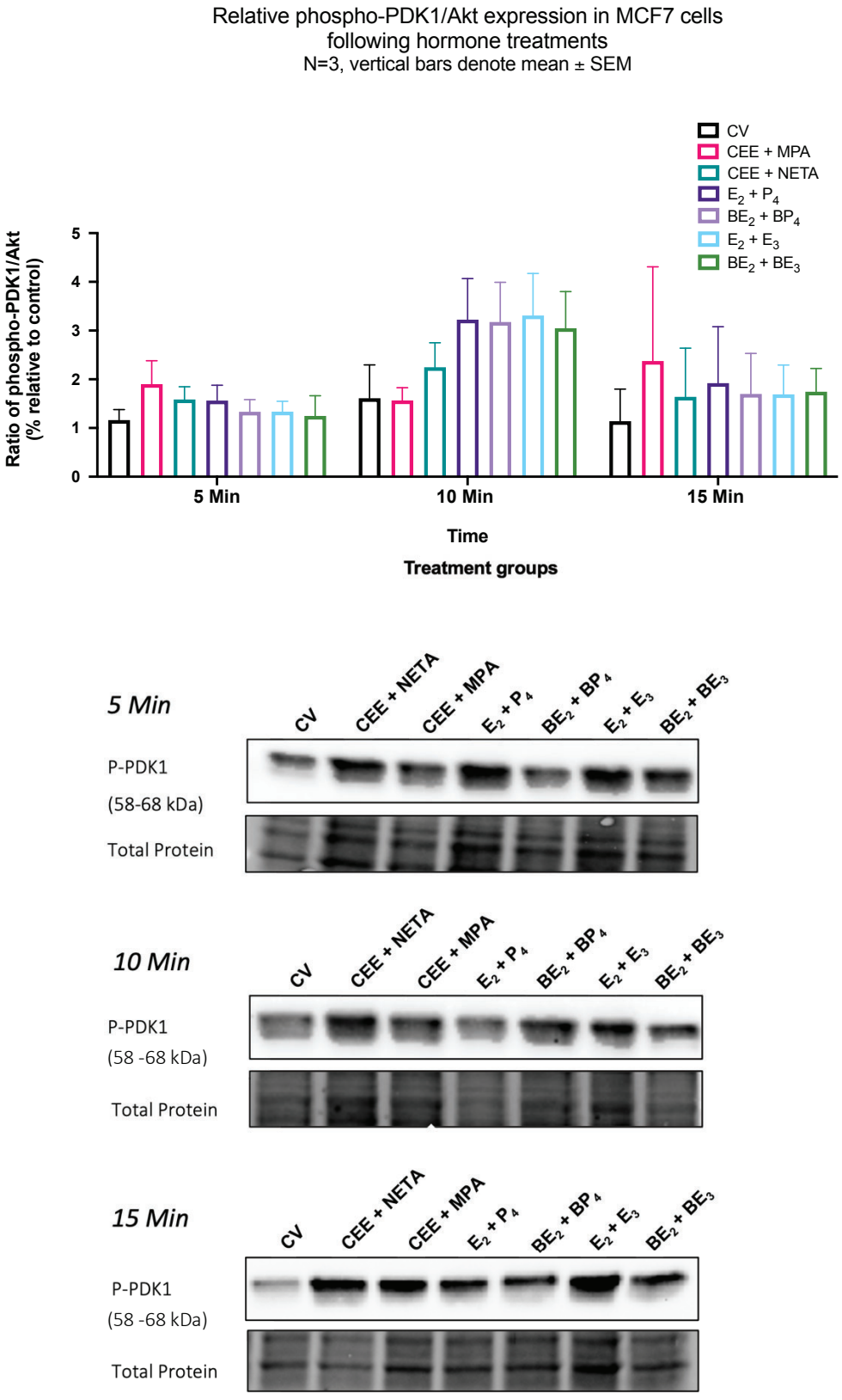


Figure 3.6.: Relative protein expression of phosphorylated PDK1/Akt protein expression of MCF7 cells at five, 10 and 15 minutes after 48-hour hormone treatment. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

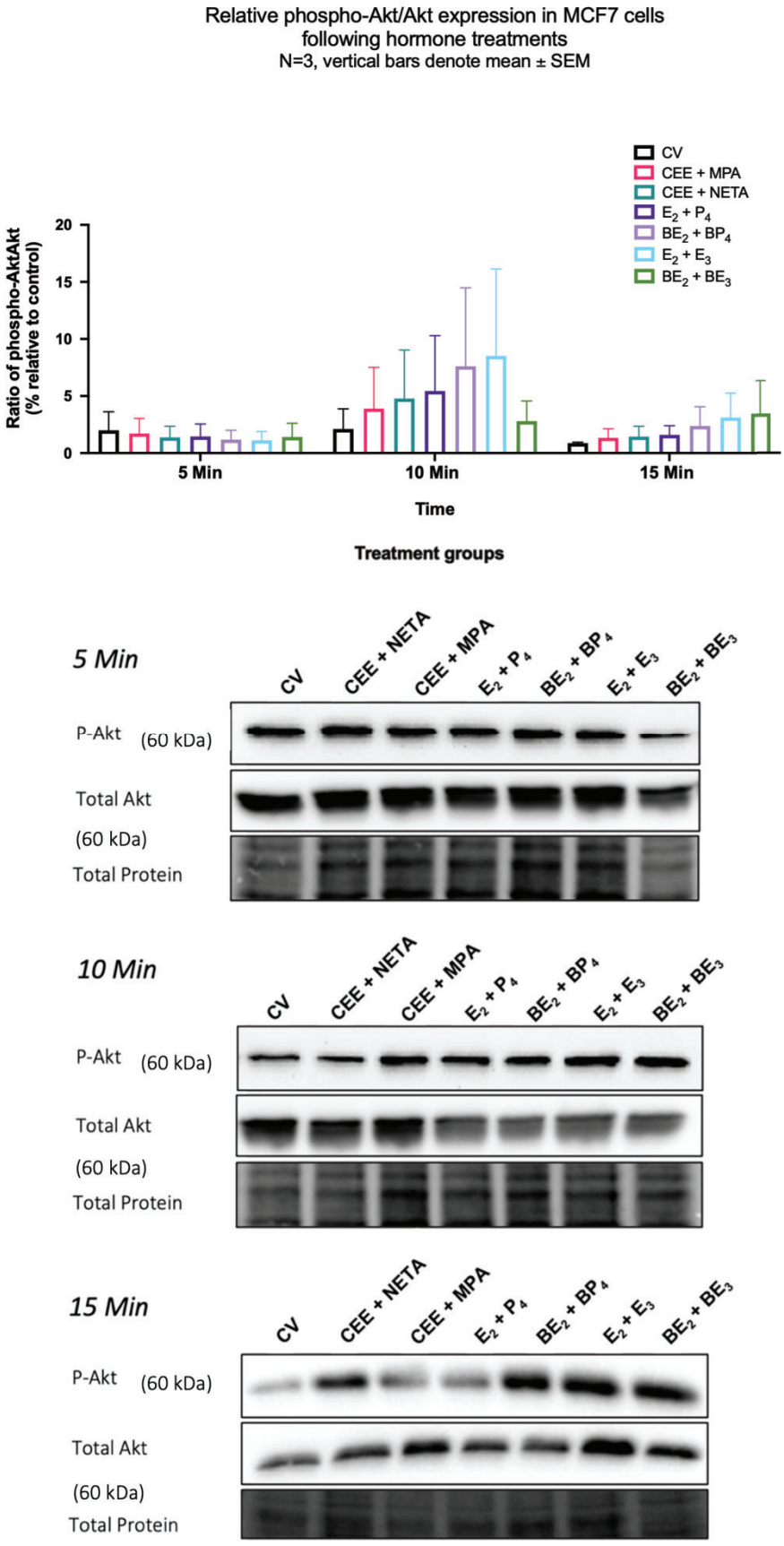


Figure 3.7.: Relative protein expression of phosphorylated Akt/Akt protein expression of MCF7 cells at five, 10 and 15 minutes after 48-hour hormone treatment. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments.

Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

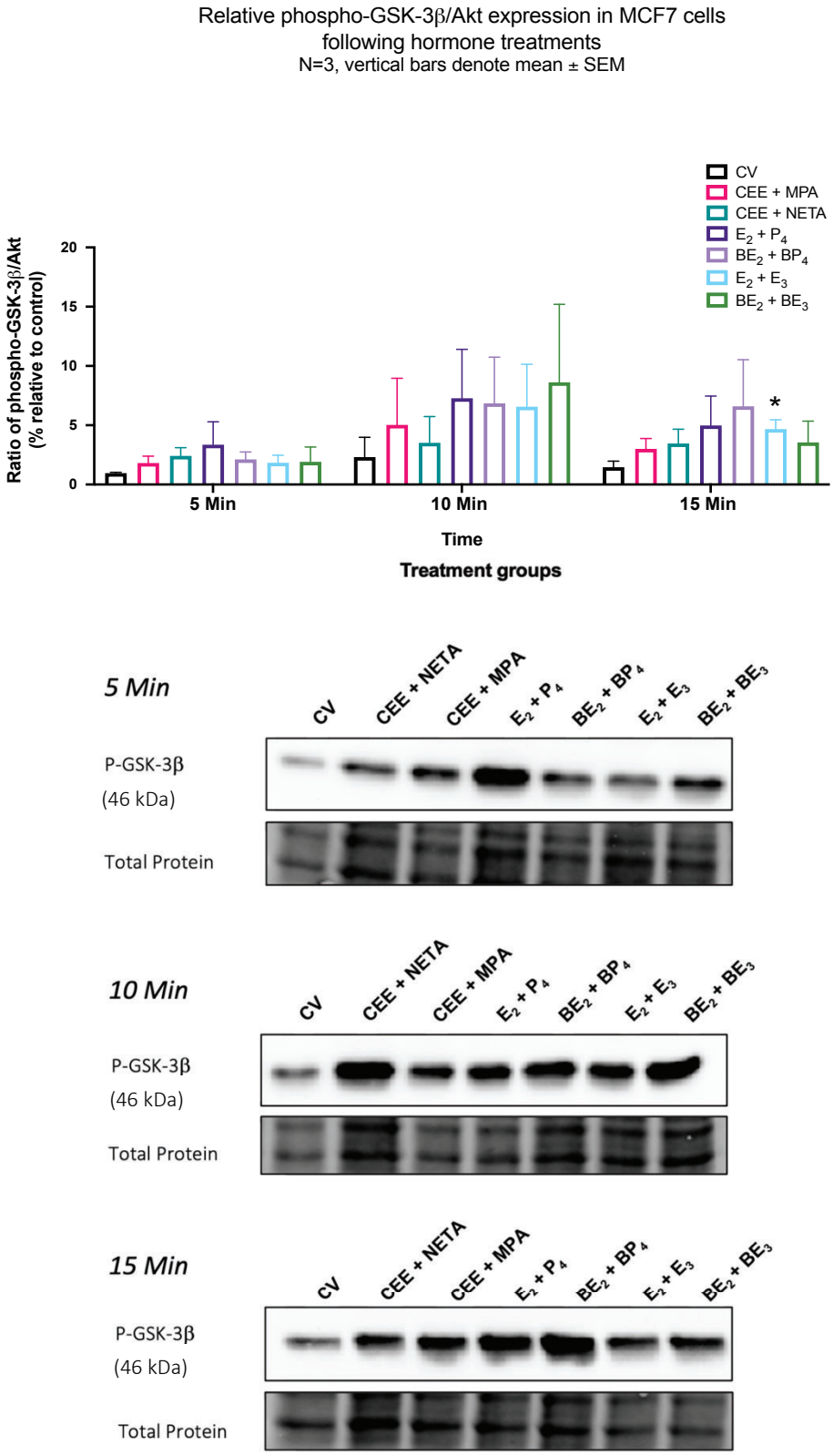


Figure 3.8.: Relative protein expression of phosphorylated GSK-3β/Akt protein expression of MCF7

cells at five, 10 and 15 minutes after 48-hour hormone treatment. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate; * $p<0.05$ vs control vehicle.

3.7. METASTASIS

To study the effects of the hormone treatments on cell migration, we performed western blots to investigate the effects of the hormone treatments on the protein expression of markers instrumental in the epithelial-to-mesenchymal transition. Additionally, we performed a migration assay to quantify the effects on migration and the rate of migration.

3.7.1. The effect of hormone combination treatments on epithelial-to-mesenchymal transition (EMT)

Hormone exposure significantly increased N-cadherin expression ($p<0.05$) of $E_2 + P_4$, $BE_2 + BP_4$ and $E_2 + E_3$ compared to the control vehicle (Figure 3.9.A). Exposure of $BE_2 + BP_4$ for 72 hours induced a significant decrease ($p<0.05$) in the protein expression of the mesenchymal marker E-cadherin compared to the control vehicle (Figure 3.9.B). Additionally, $E_2 + E_3$ treatment significantly increased ($p<0.05$) E-cadherin expression compared to $BE_2 + BP_4$. Furthermore, $BE_2 + BE_3$ treatment significantly increased ($p<0.05$) E-cadherin expression compared to CEE + MPA, $E_2 + P_4$ and $BE_2 + BP_4$. β-catenin, an activator of Slug, did not show any significant differences or changes in protein expression when compared to control vehicle (Figure 3.10.A). Furthermore, hormone exposure did not induce changes in protein expression of the transcription factor, slug, between groups (Figure 3.10.B).

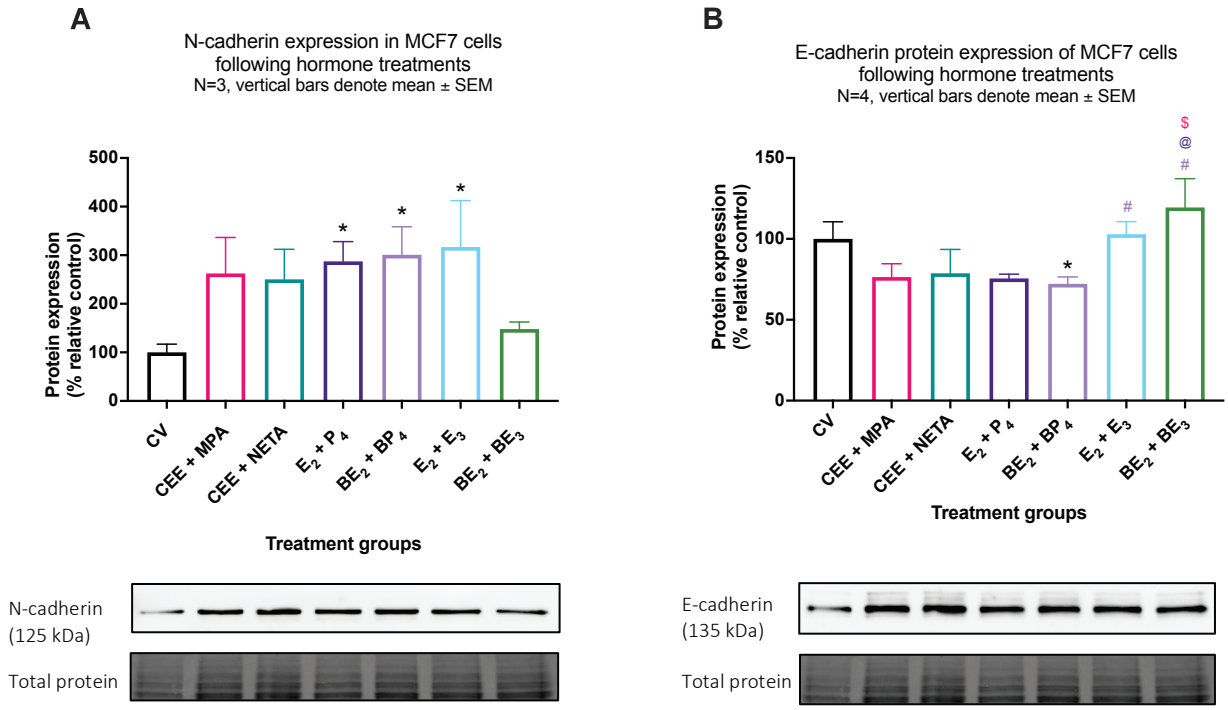


Figure 3.9.: Protein expression of epithelial to mesenchymal transition markers. A. N-cadherin protein expression of MCF7 cells following hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. B. E-cadherin protein expression of MCF7 cells following hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of four independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate; * $p<0.05$ vs control vehicle; # $p<0.05$ vs $BE_2 + BP_4$; @ $p<0.05$ vs $E_2 + P_4$; $ $p<0.05$ vs CEE + MPA.

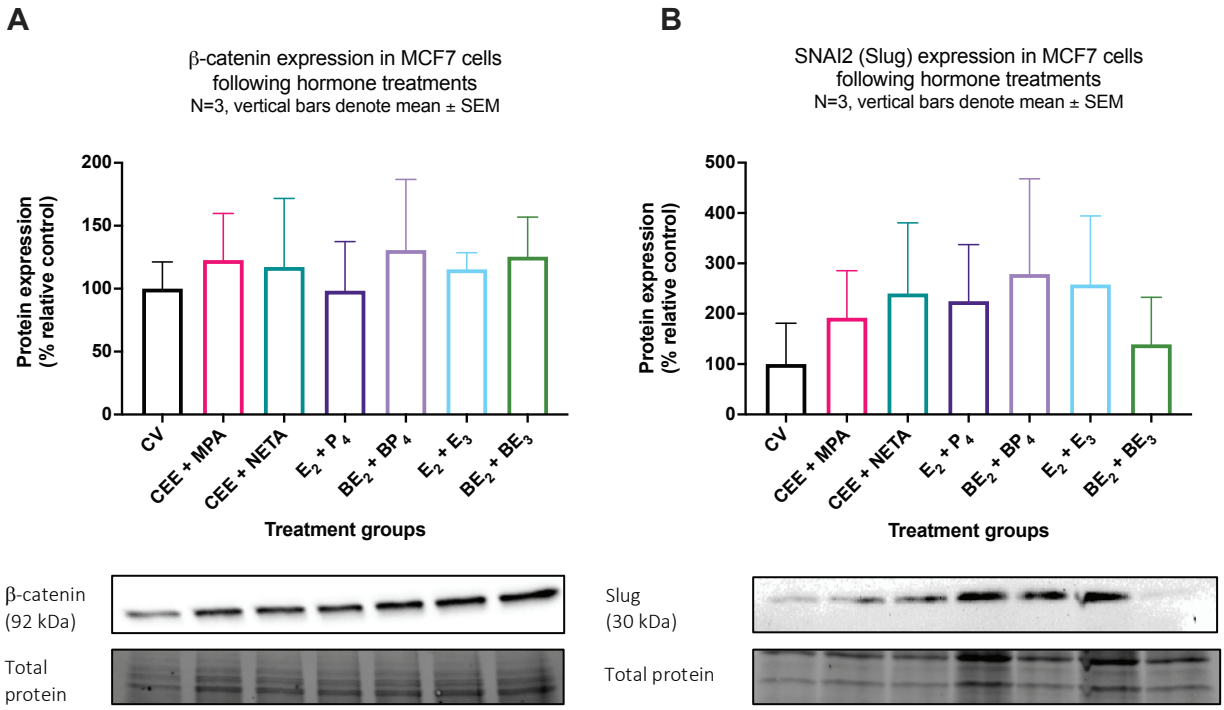


Figure 3.10.: Protein expression of epithelial to mesenchymal transition markers. A. b-catenin protein expression of MCF7 cells following hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. B. Slug (SNAIl2) protein expression of MCF7 cells following hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

3.7.2. Migratory capacity

A scratch or wound was made in the cell monolayer, cell proliferation was inhibited and the migration, as well as rate at which the wound closed, was subsequently measured. A significant difference in percentage wound closure was observed at 6 hours, when comparing $E_2 + P_4$ to the control vehicle ($p<0.05$) (Figure 3.). No significant changes were observed for percentage wound closure as well as rate of wound closure between other treatment groups over 24 hours (Figure 3.).

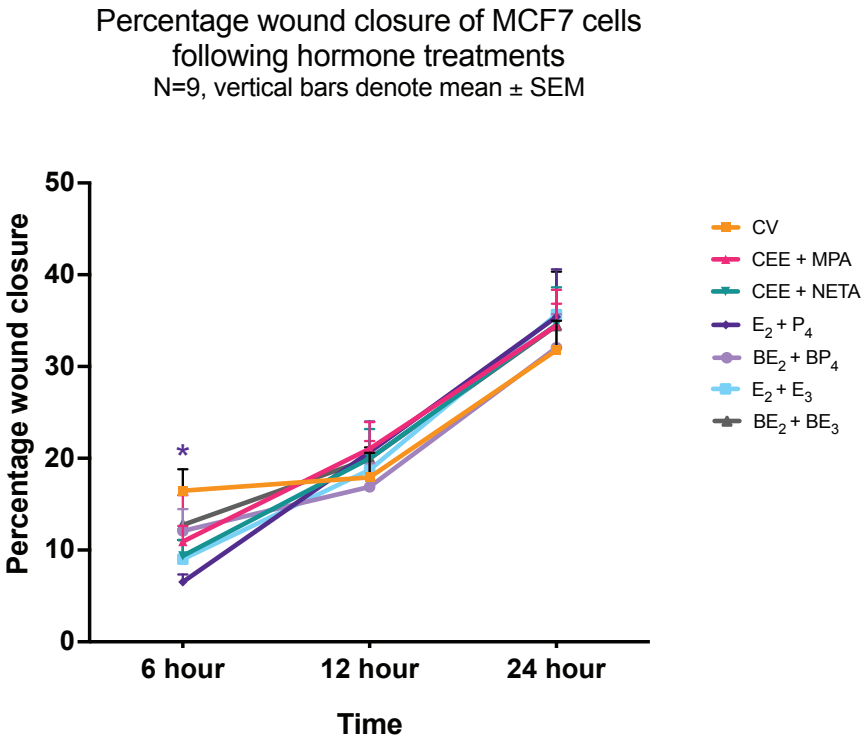


Figure 3.11.: Percentage wound closure of MCF7 cells following hormone treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate; * $p<0.05$ vs vehicle control

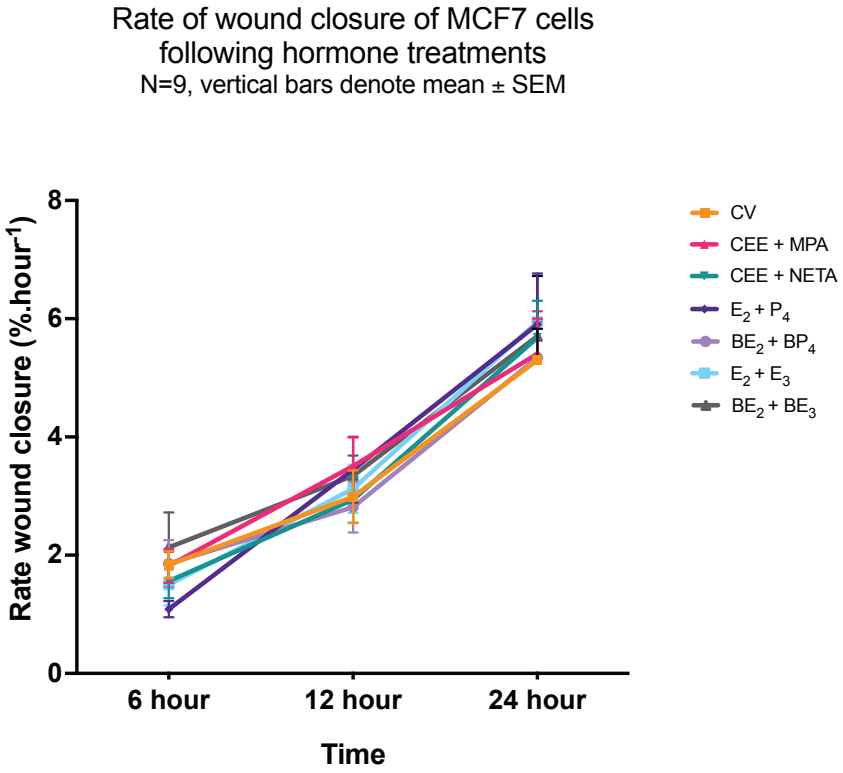


Figure 3.12.: Rate wound closure of MCF7 cells following hormone treatments for 72 hours. Values

expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

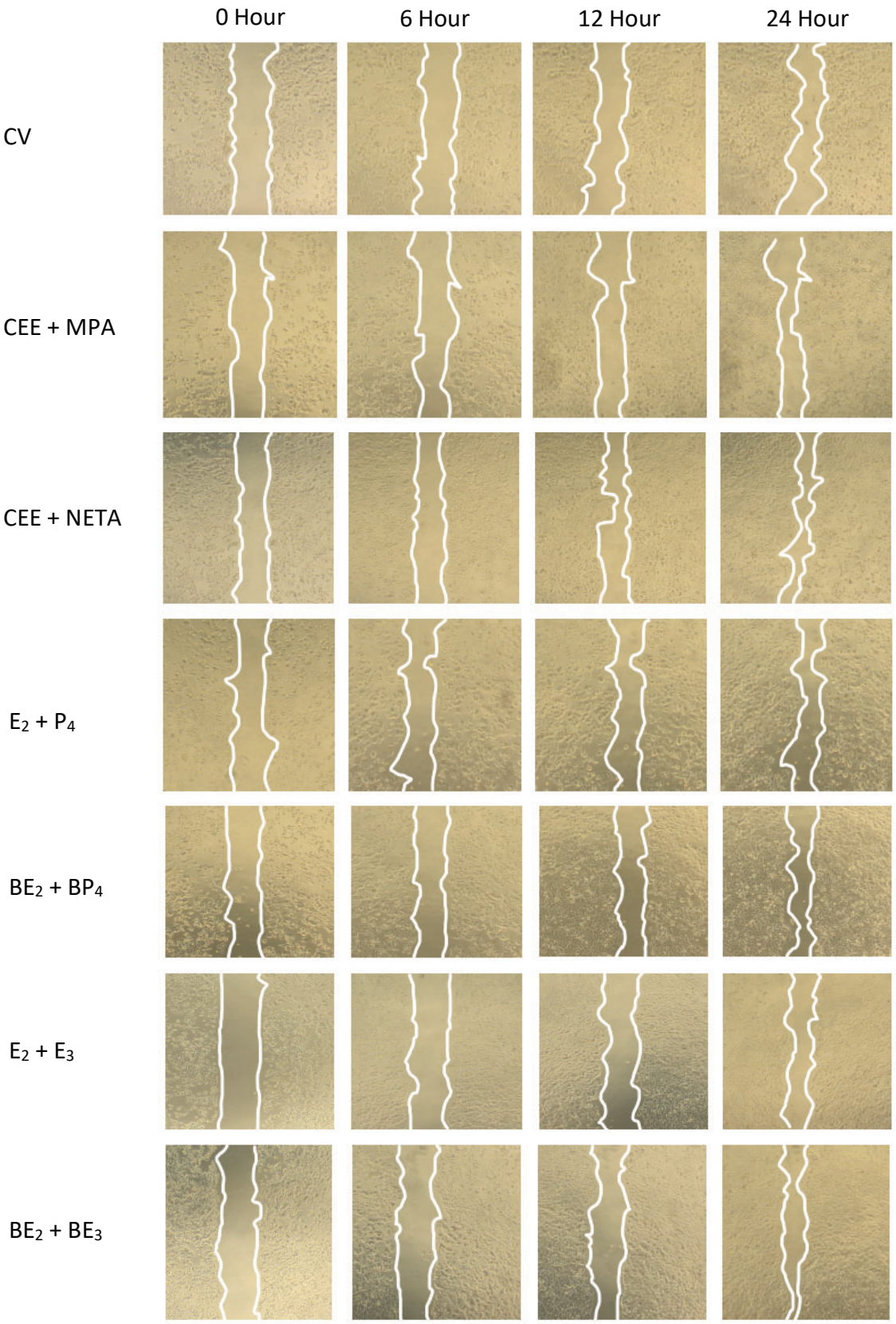


Figure 3.11. Representative images of the wound closure of MCF7 cells following hormone treatments for 72 hours. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, BP_4 – bioidentical progesterone, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate.

CHAPTER 4: DISCUSSION

Menopausal hormone therapy (MHT) has been widely used for the clinical treatment of symptoms associated with menopause in women. There are several different types of menopausal treatments available, with oestrogen monotherapy and combined oestrogen and progesterone therapy being the most common. Conjugated equine oestrogen (CEE) is a well-known therapy and is present in high levels in serum following absorption (Boothby *et al.,* 2004). Due to their longer half-lives than natural progesterone, synthetic progesterone's are also recommended. Despite MHT being the most effective treatment for menopausal symptoms, the majority of published epidemiological studies - but not all - indicate an elevated risk of invasive breast cancer in patients using long-term combination MHT with a progestin (Gompel & Plu-Bureau, 2018). In response to safety concerns, women have increasingly turned to phytoestrogens and other "natural" products as safer alternatives to prescription dose forms of oestrogens and/or progestogens.

Regarding the use of conventional hormone therapies, a major concern is the role progestins play in breast cancer risk. Estriol (E_3) and estrone (E_1) have been proposed as an alternate antagonist to oestrogen-induced proliferation of the endometrium since progesterone is thought to be the major contributor to increased risk. Compounding pharmacies commonly prescribe bioidentical oestradiol (E_2) monotherapy, $E_2 + E_3$ (biest) and $E_1 + E_2 + E_3$ (triest) oestrogen combinations. These formulations are based on the theory that E_1 and E_2 are safer and have the capacity to counteract the potent oestrogenic action because they are weaker oestrogens. (Perkins *et al.,* 2017). However, this claim is more applicable on the differing potencies of the oestrogens rather than on differing mechanisms of action. Contrary to menopausal hormone therapies that have received FDA approval, compounded-bioidentical hormone therapy products have not undergone adequate safety and efficacy evaluation in random-controlled trials (RCTs). Thus, leaving only a few observational reports and anecdotal testimonies as the only available scientific evidence (Stuenkel, 2021).

The aim of this study was therefore to investigate the effects of compounded bioidentical formulations with FDA-approved menopausal formulations on breast cancer progression. As there have not been any *in vitro* molecular studies comparing these hormone treatments, this study will compare the mechanisms involved in breast cancer proliferation

and migration of compounded bioidentical formulations with FDA-approved hormone combinations.

4.1 Cell viability

While investigating the effects of the individual hormones on cell viability, we found that bE_3 had the greatest effect on the cell viability of MCF7 cells compared to all the oestrogens at 1 nM (Supplementary Figure 1). This is contrary to the notion that E_3 is a weak oestrogen and leads to a lesser effect compared to E_2. These results are supported by the works of Perkins *et al.* (2019) who observed that E_3 and E_1 along with E_2 are full agonists of oestrogen receptor activation. They concluded that although the oestrogens are full agonists, they display differential potencies for breast cancer cell proliferation. On the other hand, CEE treatment did not elicit any response compared to the control vehicle and other treatment groups (Supplementary Figure 1.). Other studies reported that E_2 is more potent than the major components of CEE, including E_1, Equilin and 17α-Equilin in promoting the proliferation of MCF7 cells (Mueck *et al.,* 2003; Song *et al*., 2013; Atwood & Ekstein, 2019).

Although CEE alone did not elicit any response, cell viability was increased when combined with MPA and NETA (Figure 3.2). Progestogens, including MPA, NETA, P_4 and BP_4, have been shown to have either stimulatory or inhibitory effects on proliferation (Mueck *et al*., 2003; Krämer *et al*., 2005; Chen *et al*., 2011; Atwood and Ekstein, 2019). In our study, MPA, NETA, P_4 and BP_4 all increased cell viability at different concentrations (Figure 3.2.2). In addition, cell viability in MCF7 cells was significantly increased by all treatment combinations with no inhibitory effects. Our findings are in line with the works of Perkins *et al.* (2019) who investigated the effects of biest and triest combinations on cell proliferation. The authors showed that E_3 and E_1 did not antagonize E_2 activity using the MTT cell viability assay. Interestingly, Mueck *et al.* (2003) observed that at low levels of P_4, MPA and norethindrone (NET) stimulated proliferation, when combined with E_2, 17 alpha-dihydroequilin (DHEQ) and equilin, components of CEE, in the range of (0.01 nM – 0.1 μM). Moreover, only supraphysiological concentrations of MPA, P_4, and NET exhibited inhibitory effects in E_2 and equilin, while MPA and NET stimulated DHEQ-induced proliferation (Mueck *et al.,* 2003). It has been proposed that progestational increase of reductive 17β-hydroxysteroid oxidoreductase,

which catalyzes the conversion of E_1 to E_2, is a mechanism that progestogens could increase E_2-induced breast cell proliferation (Mirkin *et al.,* 2006; Atwood & Ekstein, 2019).

In summary these results support the differential potencies each of the hormones had on cell viability and reveal how each component contributed to the combination treatments' increased cell viability. Furthermore, that in combination, all the treatments increased cell viability to the same extent.

4.2 Proliferation and cell cycle progression

To assess proliferation and exclude the possibility that the changes in cell viability were a result of proliferation and not mitochondrial biogenesis, we investigated the effects of the hormone treatments on the cell cycle and proliferation marker MCM2.

To our knowledge this is the first study to investigate the effects of these combinations on the cell cycle. We found that conventional hormones CEE + MPA and CEE + NETA increased the percentage of cells in the G_0/G_1, while the other treatments had no effects throughout the cycle when compared to the control (Figure 3.3). An increase in cell population in the G_0/G_1 could indicate that the conventional hormones may enter quiescence or facilitate apoptosis. This result was unexpected as prior studies had demonstrated that $E_2 + P_4$ therapy accelerated cell cycle progression by increasing the percentage of cells in the G_2/M phase (Tian *et al*., 2018). In addition, it has been shown that E_2 has a greater propensity to induce proliferation compared to CEE, through increased expression of the proliferation marker Ki67 in earlier studies and through the 5-bromo-2'-deoxyuridine (BrdU) assay (Wood *et al*., 2008; Song *et al*., 2013; Atwood & Ekstein, 2019). Moreover, Diller *et al*. (2014) found that treatment of E_2 increased cell proliferation through the cell cycle. Following treatment, they observed an increase in the expression of proliferation genes cyclin A2, cyclin B1, Ki-67, c-myc and b-myc, offering mechanistic explanations for the observed growth increase. Similarly, Murkes *et al.* (2011), observed a highly significant increase in histological staining of the proliferation marker Ki67 in women treated with CEE + MPA in contrast to an E_2 gel combined with oral micronized progesterone.

As shown by our WST-1 results, our observation could indicate that the progestin component is involved. According to Sutherland *et al.* (1998), progestin treatment on breast

cancer cells induced a bipashic effect on cell cycle progression (Saitoh *et al*., 2005; Lange & Yee, 2008). This is described by an initial acceleration of the cell cycle progression from G_1 to S phase, followed by a decrease in the rate of progression from G_1 to S leading to an accumulation of cells in the G_1 phase and growth inhibition. In a more recent study, an increase in the cell population of MCF7 cells in the G_1 phase after 24 hours of progesterone treatment was observed (Azeez *et al*., 2015). After 48 hours, they observed a mild apoptotic effect indicated by an increase of cells in sub G_1. Authors therefore concluded that progesterone's influence on cell growth was due to the cell cycle. A biphasic effect on the cell cycle has also been reported due to MPA treatment in MCF7 cells. According to these investigations, progestogen-induced inhibition takes place after a specific amount of time (Fedotcheva *et al.,* 2021). The authors argued that progestin-induced proliferation was caused by transitory phosphorylation of Akt and an increase in cyclin D1, and that the cytostatic action of progestins is not linked to a rapid damaging effect on the DNA of the tumour cell (Saitoh *et al.,* 2005).

In addition, we assessed the expression of the proliferation marker, MCM2, following hormone treatments. MCM2 is a key marker used to identify cancer cell proliferation, which plays a key role in DNA replication. MCM2 is highly expressed in the S, G_2 and M phases of the cell cycle, and absent during the G_0 phase. In our study, MCM2 expression was not induced by any of the hormone combinations (Figure 3.4). This was unexpected as both CEE and E_2 alone increased DNA synthesis. Furthermore, we did not observe any inhibitory effects as seen by Seeger *et al.,* (2002) who observed a minimal inhibitory effect of progestogens including P_4 on E_2-induced proliferation. The authors assessed proliferation through the ATP chemosensitivity assay. Similarly, Mueck *et al.,* (2003) demonstrated that the continuous addition of progestogens did not induce any major reduction of proliferative potency of CEE and E_2 using the same assay.

In summary, the combination treatments had a comparable impact in that they did not elicit MCM2 expression in MCF7 cells as we predicted. Moreover, the combination treatments did not increase proliferation through the stimulation of the cell cycle. However, CEE + MPA and CEE + NETA treatment appeared to induce quiescence of MCF7 cells in comparison to the other combination treatments. Hormone action has been demonstrated to occur via the PI3K/Akt pathway, thus, to support our findings we evaluated how the combinations treatments affected this pathway.

4.3 PI3K/Akt signalling pathway activation

Oestrogen and progestogens have the ability to stimulate the PI3K signalling pathway, which is a crucial regulator in the growth of cancer cells via genomic and non-genomic signalling. PI3K/Akt/NF-κB cascade appears to have an important role in several non-genomic actions of oestrogen and has also been associated with progestogen action (Saitoh *et al.,* 2005). This pathway is rapidly and transiently activated, therefore, we investigated the protein expression of regulators and downstream targets involved in the pathway at 5, 10 and 15 minutes after hormone exposure. First, we investigated the protein expression of PTEN, a tumour suppressor that negatively regulates Akt activation. Treatment of the hormone combinations did not activate PTEN phosphorylation. Studies have linked the presence of ER-β to PTEN modulation in breast cancer (Lindberg *et al.,* 2011). Therefore, the absence of ER-β in our MCF7 cells could serve as a possible reason for the lack of PTEN expression.

We then investigated PDK-1, which phosphorylates Thr308 on Akt. Despite there being no significant changes of PDK-1 activation compared to the control, increased phosphorylated PDK-1 protein trends were observed when comparing hormone treatments of E_2 + P_4, BE_2 + BP_4, E_2 + E_3 and BE_2 + BE_3 at 10 minutes (Figure 3.6). Moreover, no significant changes in Akt activation were found when treated with the hormone combinations (Figure 3.7). These findings are unexpected as oestrogens play a critical role in the proliferation of breast cancer cells through Akt activation. In addition, it is hypothesized that CEE + MPA- and CEE + NETA-induced proliferation is related to the activation of signalling pathways like PI3K/Akt and MAPK. Interestingly, Wood *et al.* (2013) found that increased breast tissue proliferation of CEE + MPA treatment was highly associated with signal transducer and activator of transcription 5 (STAT5), EGFR and RANK/RANKL pathways. Song and colleagues (2013) showed that both CEE and E_2 alone increased DNA synthesis and reduced apoptosis with activation of MAPK, Akt, and p70S6K and upregulation of anti-apoptotic factors such as Bcl-2, survivin and X-linked inhibitor of apoptosis protein (XIAP). Literature also indicated that MPA and P_4 induced proliferation and migration in T47-D cells through the PI3K/Akt/NF-κB cascade (Saitoh *et al.,* 2005; Wang & Lee, 2016). Similar results were observed in MCF7 cells which were transfected with the PGMRC1 (Zhang *et al*., 2022). In this study, The PI3K/AKT pathway was shown to be upregulated by NET + E_2 and induced significant proliferation (Zhang *et al.,* 2022). In contrast, the inhibition of TGF-

β production by high-dose progesterone is associated with PI3K/AKT signalling inhibition since this pathway is also activated by TGF-β (Tzavlaki & Moustakas, 2020; Fedotcheva *et al*., 2021).

GSK-3β, a downstream target of many signalling pathways including Akt, was also assessed. Akt activation negatively regulates GSK-3β, which is also implicated in cell cycle progression. Our results showed that E_2 + E_3 treatment resulted in the deactivation of GSK-3β (Figure 3.8). It was previously shown that E_2 treatment led to the rapid activation of Akt and a resultant phosphorylation and deactivation of GSK-3β that resulted in breast cell proliferation in MCF7 cells containing luciferase reporter gene (Medunjanin *et al.,* 2005). Since we observed no significant changes in the Akt signaling pathway, it is possible that the E_2 + E_3 treatment promotes proliferation as a result of Wnt signalling. A study conducted by Matthew *et al.,* (2014), demonstrated that E_2 weakly induced WNT4 expression in MCF7 cells stimulating proliferation.

In summary, the hormone treatments did not induce breast cancer cell proliferation through the modulation of the PI3K signalling pathway as expected. Although assessing the rapid non-genomic actions of the hormone treatments on breast cancer cells may be challenging, further investigation of other signalling pathways such as Wnt and MAPK signalling pathways, as a mechanism for hormone treatment induced proliferation is warranted. Furthermore, we also assessed the effects of the hormone treatments on metastasis.

4.4 Metastasis: Epithelial-to-mesenchymal transition

We evaluated the expression of EMT related proteins and performed a migration assay to examine the effects of various hormone combinations on breast cancer metastasis. The treatment of E_2 is known to induce migration, whereas progestogens inhibit EMT and metastatic spread in endometrial cancer cells (Santoro *et al*., 2016; Goyette *et al*., 2017). We demonstrated that the various hormone treatments had varying impacts on the induction of EMT in MCF7 cells compared to each other. The treatment of BE_2 + BP_4 ($p<0.05$) clearly indicated mesenchymal characteristics of EMT, which are described by a decrease in E-cadherin and an increase in N-cadherin (Figure 3.9), while E_2 + P_4 and E_2 + E_3 treatment only increased N-cadherin expression. Treatment of E_2 + E_3 and BE_2 + BE_3 did not induce EMT, rather, treatment of E_2 + E_3 ($p<0.05$) and BE_2 + BE_3 ($p<0.05$) showed an increase in E-cadherin

treatment compared to BE_2 + BP_4. Furthermore, BE_2 + BE_3 treatment significantly increased E-cadherin ($p<0.05$) protein expression compared to CEE + MPA. Our results indicated that E_3 in combination with E_2 does not stimulate EMT compared to oestrogens combined with progestogens. In addition, none of the hormone treatments activated protein expression of slug, or β-catenin (Figure 3.10). Our findings are in contrast with Kim *et al.,* (2017), who observed the inhibition of E_2-induced EMT processes by P_4 by increasing mRNA and protein expression of E-cadherin in MCF7 clonal variant cells. This inhibition could be attributed to a higher concentration of P_4. Meanwhile a recent study showed that E_2 does not activate the gene expression profile of EMT (Qureshi *et al.,* 2022). Rather that E_2 represses SNAI2 activation by recruiting Nuclear receptor corepressor 1 (N-cor1) with ERα. Interestingly, these authors demonstrated that exposure to E_1 and increased conversion of E_2 to E_1, upregulated EMT transcriptional profiles, promoting tumour invasion *in vivo*.

In summary, we observed that the hormone treatments had different effects on EMT. We hypothesis that the differences we observed in our study could be due to the differential effects between progestogens and their efficacy at different concentrations. With regards to the effect of E_2 + E_3, we hypothesised that they would have similar effects as E_2 + P_4, inducing EMT. Taking our results into consideration we therefore suggest further study of E_1, E_2 and E_3, individually and in combination and their association with EMT. In addition, we advise further investigation on additional proteins implicated in EMT as well as the use of the trans-well migration- and invasion assay to evaluate the differential migratory ability of the treatment strategies.

The results from the migration assay showed that none of the treatments increased the migratory capacity or migratory rate of MCF7 cells. We did, however, observed that treatment of E_2 + P_4 at 6 hours, had a significantly lesser wound closure than the control vehicle ($p<0.05$) (Figure 3.). A vast majority of studies revealed that E_2 stimulated migration in MCF7 cells (Kim *et al.,* 2017; Park *et al.,* 2016; Qureshi *et al.,* 2022). Although the mechanisms are not fully understood, progestogen treatment in breast cancer has been demonstrated to both promote cell migration and to inhibit it. Our findings are in contrast to research on the topic. A comparative study conducted by Fu and colleagues (2008), discovered that E_2 stimulated migration in T47-D cells and that there was no additive effect when combined with natural and

synthetic progesterone's. However, Kim *et al., (*2017) showed that E_2-induced migration and invasion was inhibited by P_4 through the reduction of proteolytic enzymes such as MMP-9 and cathepsin B in MCF7 clonal variant cells. This is also supported by Wang & Lee (2016) in both MCF7 and T47-D breast cells. Meanwhile research conducted by Qureshi *et al.* (2022), who demonstrated using a wound-closure assay that oestrogen-starved MCF7 cells migrated as a result of E_2. Interestingly, they also showed that E_1 stimulated a greater migration in the wound-closure assay than E_2. However, one study observed P_4 induced migration in both MCF7 and T47-D breast cells at 50 nM (Wang & Lee 2016).

In summary, the hormone treatments induced differential effects on the EMT processes but elicited a similar effect on the migration of MCF7 cells. This suggests that while the hormone therapies may act on the same pathways, their efficacies may differ. We hypothesized that the hormone treatments would stimulate the migration of MCF7 cells, however our findings did not compare to the findings of other research, where our results indicated no stimulation or repression of migration of MCF7 cells. To clarify the roles of hormones in combination, more research on the individual effects of hormone treatment on migration is advised.

5. CONCLUSION

The aim of this study was to elucidate the relationship of the biest compounded bioidentical formulation on breast cancer progression, assessing its effects on proliferation and metastasis in comparison to FDA-approved hormone formulations. We reported that all hormone combinations promoted cell viability to the same degree after treatments. Our results indicated for the first time, that the biest combinations do not induce proliferation and metastasis in the MCF7 cells. Furthermore, we have demonstrated that biest combinations had contrasting effects on initiating EMT compared to E_2 + P_4 therapy, but comparable effects on the cell cycle. Overall, our findings demonstrated that the breast cancer progression-related pathways stimulated by the FDA-approved formulations were not stimulated by the biest combinations. Future research should focus on additional compounded formulations addressing various pathways and mechanisms associated with breast cancer risk and progression.

LIMITATIONS AND FUTURE RECOMMENDATIONS

More technical repeats would ideally provide a better indication of the outcomes for the western blot examination. Furthermore, we advise using more time points when analyzing the Akt signalling pathway.

It is common practice when investigating hormones in breast cell lines to exclude normal breast cell lines as a control, due to the absence of hormone receptors. However, we surmise this as a limitation in our study. We recommend using both MCF12-A and MCF10 breast cell lines in future hormone studies as well as the T47-D breast cell line to explore other possible mechanisms.

Study limitations include not investigating the individual hormones in conjunction with the combination therapies to identify the hormone responsible for the alterations seen in the various assays we carried out. Thus, to identify the components causing any observed activity, we advise further research into the individual hormones alongside the combination therapies. Furthermore, we recommend investigating protein expression alongside gene expression through polymerase chain reaction.

To investigate invasion and migration, we recommend using automated software to detect wound closure and migration rate for more accurate data acquisition. Furthermore, to utilise the transwell migration assay as it provides sensitive detection of migration.

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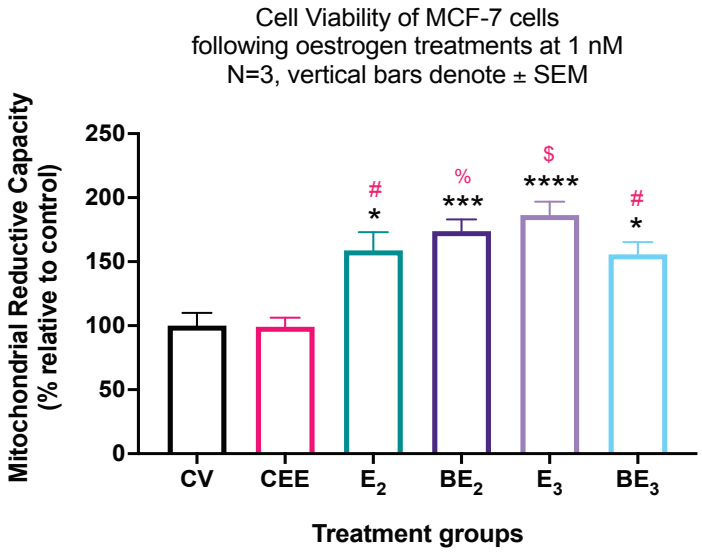
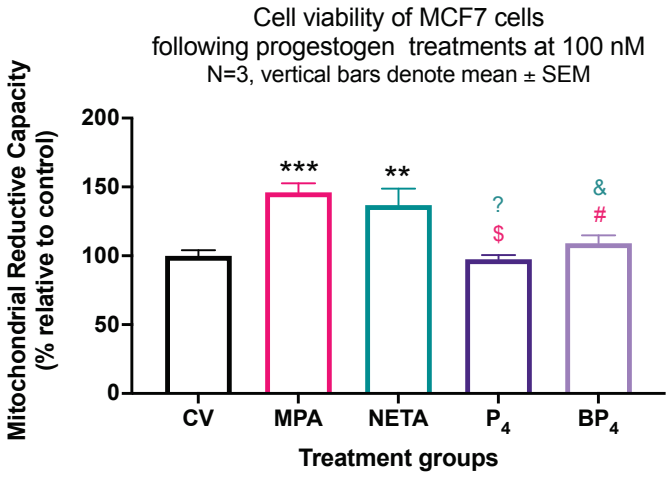
SUPPLEMENTARY DATA



Supplementary Figure 1. Cell viability of MCF7 cells following oestrogen treatments at 1 nM for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BE_2 – bioidentical oestradiol, BE_3 – bioidentical estriol, CEE – estrone 3-Sulfate salt, CV – control vehicle, E_2 – oestradiol, E_3 – estriol; * p<0.05 vs control vehicle; * p<0.05 vs control vehicle; *** p<0.001 vs control vehicle; **** p<0.0001 vs control vehicle; # p<0.01 vs CEE; % p<0.001 vs CEE; $ p<0.0001 vs CEE



Supplementary Figure 2. Cell viability of MCF7 cells following progestogen at 100 nM treatments for 72 hours. Values expressed as a percentage of the control vehicle. Vertical bars denote mean ± SEM. The results are representative of three independent experiments. Abbreviations: BP_4 – bioidentical progesterone, CV – control vehicle, MPA – medroxyprogesterone acetate, NETA – norethisterone acetate; ** p<0.01 vs control vehicle; *** p<0.001 vs control vehicle; $ p<0.0001 vs MPA, # p<0.01 vs MPA, ? p<0.001 vs NETA, & p<0.05 vs NETA.