Investigating the role of inflammation, progestins and steroid receptors in breast cancer

by
Anishka Eksteen

Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Science at Stellenbosch University

Supervisor: Prof. Donita Africander

Co-supervisor: Dr Nicolette Verhoog

April 2019

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2019

ABSTRACT

Progestins are used by women all over the world in menopausal hormone therapy (HT) and contraception. Numerous clinical trials have, however, reported that some progestins are associated with an increased risk for developing breast cancer. Although multiple progestins with different chemical structures and biological activities have been synthesised, not all progestins have been evaluated for increased breast cancer risk. Obesity-related inflammation is also strongly associated with increased breast cancer risk, particularly amongst postmenopausal women. This increased inflammatory state is characterised by enhanced production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNFα). Both these inflammatory mediators have been implicated in breast cancer development and progression. However, the exact mechanism whereby inflammation and progestins contribute to breast cancer risk is yet to be established. The aim of this study was thus to investigate the effects of different progestins, in the absence and presence of IL-6 or TNFα, on the proliferation, apoptosis, migration and invasion of breast cancer cells. Given that some progestins can interact with multiple steroid receptors, all of which are known to play important roles in breast cancer biology, the first part of the study investigated effects on steroid receptor expression in the T47D breast cancer cell line. Western blot analysis showed that all the progestins decreased the expression of the estrogen receptor (ER)-subtypes, progesterone receptor (PR)-isoforms and the androgen receptor (AR), while no effects were observed on the expression of the glucocorticoid receptor (GR). This study also showed that IL-6 has no effect on steroid receptor expression levels, while TNFα increased the expression of the GR. Results from this study also show that the selected progestins differentially increased cell survival of T47D and MCF-7 BUS breast cancer cells, while all progestins and both inflammatory mediators increased the migration of T47D breast cancer cells. IL-6 had no effect on cell survival of either cell line, while TNFα decreased the survival of the MCF-7 BUS cells. In addition, the progestins medroxyprogesterone acetate (MPA) and drospirenone (DRSP), as well as IL-6, appeared to increase invasion of the MDA-MB-231 breast cancer cell line. In contrast, TNFα appeared to decrease invasion of the MDA-MB-231 cells. This study is the first to show that none of the progestin-mediated effects on steroid receptor expression, proliferation, apoptosis and migration were modulated by either IL-6 or TNFα. On the other hand, TNFα appeared to decrease the progestin-induced effects on the invasion of the MDA MB-231 breast cancer cell line. Taken together, the results show that even though the progestins and the pro-inflammatory cytokines may differentially affect breast cancer growth and survival, all the selected progestins, as well as IL-6 and TNFα, increase migration of T47D breast cancer cells. These results suggest that all these progestins and the inflammatory mediators may possibly increase the risk of developing metastatic breast cancer. Finally, the results obtained in this study indicate that the effects of the progestins on steroid receptor expression and hallmarks of cancer, were not exacerbated by the addition of the inflammatory mediators, suggesting that the combination of inflammation and progestins used in HT does not further increase breast cancer risk.

OPSOMMING

Vroue regoor die wêreld gebruik progestiene in hormoonvervanginsterapie (HVT) en as voorbehoedmiddels. Verskeie kliniese proewe het egter al getoon dat sommige progestiene geassosieer word met ’n verhoogde kans om borskanker te ontwikkel. Hoewel verskeie progestiene met verskillende chemiese strukture en biologiese aktiwiteite reeds gesintetiseer is, is nie alle progestiene vir ’n verhoogde borskankerrisiko geëvalueer nie. Vetsugverwante inflammasie word ook sterk geassosieer met ’n verhoogde kans vir borskanker, veral onder na-menopousale vroue. Hierdie verhoogde inflammatoriese toestand word gekenmerk deur ’n verhoogde produksie van pro-inflammatoriese sitokiene, soos interleukien-6 (IL-6) en tumornekrosefaktor-alfa (TNFα). Albei hierdie inflammatoriese mediators is al geassosieer met die ontwikkeling en progressie van borskanker. Die presiese meganisme waardeur inflammasie en progestiene tot verhoogde borskankerrisiko bydra, is egter nog nie vasgestel nie. Die doel van hierdie studie was dus om die uitwerking van verskillende progestiene, in die afwesigheid en teenwoordigheid van IL-6 óf TNFα, op die proliferasie, apoptose, migrasie en indringing van borskankerselle te ondersoek. Aangesien sommige progestiene met verskeie steroïedreseptore interaksie kan hê, en almal bekend is om belangrike rolle in borskankerbiologie te speel, ondersoek die eerste deel van die studie die uitwerkings op steroïedreseptor-uitdrukking in die T47D borskankersellyn. Western klad-analise het getoon dat al die progestiene die uitdrukking van die estrogeenreseptor (ER)-subtipes, progesteroonreseptor (PR)-isoforms en die androgeenreseptor (AR) verlaag het, terwyl geen effek op die uitdrukking van die glukokortikoïedreseptor (GR) waargeneem was nie. Hierdie studie het ook getoon dat IL-6 geen effek op steroïedreseptor-uitdrukkingsvlakke het nie, terwyl TNFα die uitdrukking van die GR verhoog. Die resultate van hierdie studie toon ook dat die geselekteerde progestiene die seloorlewing van die T47D en MCF-7 BUS borskankerselle differensieel verhoog, terwyl alle progestiene en albei inflammatoriese mediators die migrasie van die T47D borskankerselle verhoog het. IL-6 het geen effek op seloorlewing van enige van die sellyne getoon nie, terwyl TNFα die oorlewing van die MCF-7 BUS selle verlaag het. Daarbenewens het die progestiene medroksieprogesteroonasetaat (MPA) en drospirenoon (DRSP), sowel as IL-6, die indringing van die MDA-MB-231 borskankersellyn verhoog. In teenstelling hiermee het TNFα die indringing van die MDA-MB-231 selle verlaag. Hierdie studie is die eerste om te wys dat geen van die progestien-gemedieerde effekte op steroïedreseptor-uitdrukking, proliferasie, apoptose en migrasie deur óf IL-6 óf TNFα gemoduleer is nie. Aan die anderkant het TNFα die progestien-geïnduseerde effekte op die indringing van die MDA-MB-231 borskankersellyn verminder. Gesamentlik wys hierdie resultate dat hoewel die progestiene en die pro-inflammatoriese sitokiene die groei en oorlewing van borskanker differensieel kan beïnvloed, kon al die geselekteerde progestiene, sowel as IL-6 en TNFα, die migrasie van T47D borskankerselle verhoog. Hierdie resultate dui daarop dat al hierdie progestiene en die inflammatoriese mediators moontlik die risiko van die ontwikkeling van metastatiese borskanker kan verhoog. Laastens, die resultate wat in hierdie studie verkry is, dui daarop dat die uitwerking van die progestiene op steroïedreseptor-uitdrukking en kenmerke van kanker nie vererger word deur die toevoeging van die inflammatoriese mediators nie. Dit dui daarop dat die kombinasie van inflammasie en progestiene wat in HVT gebruik word, nie die risiko vir borskanker verder verhoog nie.

ACKNOWLEDGEMENTS

I would like to thank the following people who made this thesis possible:

Firstly, thank you Prof. Donita Africander for the opportunity to have completed my postgraduate studies under your supervision. The support and guidance you provided over the past few years was immense. I am forever grateful for all the knowledge and experience gained to help me grow and become a fine researcher. You also taught me many life lessons, which I am very grateful for. I will always admire your remarkable passion and dedication towards your work.

Next, I would like to thank my co-supervisor, Dr Nicolette Verhoog. Thank you for your kind heart and your always-willingness to help me. You go the extra mile with all your students and you are an excellent teacher, thank you so much.

Dr Renate Louw-du Toit, who was also my co-supervisor during my BSc honours, thank you for supporting me since day one. You taught me most of what I know about the lab and I am very grateful. Thank you that I could always share my emotions and feelings with you during the difficult times. You always motivated me and told me just to carry on, and you always lead by example.

Danielle, thank you my dear friend. I don't know how I could have done this without you. You showed me how to be a stronger person. Thank you for all the laughs, tears and memories we shared. I am very grateful for our friendship.

To all the other members of the Africander lab who taught me a great deal about science and life in general. Thank you to Meghan Perkins, Angelique Cabral and Megan Cartwright. To our laboratory manager Bradley Khoza, thank you for all the conversations, information and assistance you gave me. Lola, thank you for always caring about me and cheering me up when I needed it the most.

I would like to take this opportunity to thank my parents and all four of my grandparents who were always supporting me and praying for me. I am so very grateful. Mamma and Pappa, thank you for all the love, words of encouragement and reminding me that I can do anything I put my mind to. Thank you for giving me the opportunity to attend university and for everything you have sacrificed so that I could become who I am today. I could not be more grateful for my wonderful parents.

I would also like to give a big thanks to Danie le Roux and his parents for all the love, care and never-ending motivation they have given to me. Danie, thank you for being my number one supporter every single day. You showed me that with hard work, anything is possible. I don't know how I could have done this without you.

Aan my almagtige Vader Jesus, dankie dat U elke dag my rots is. Spreuke 3:5-6.

ALPHABETICAL LIST OF ABBREVIATIONS

AR	Androgen receptor
bp	base pair
DEPC	diethyl pyrocarbonate
Dex	Dexamethasone
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRSP	drospirenone
E_2	17β-estradiol
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EtOH	Ethanol
FCS	Fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GES	gestodene
GR	Glucocorticoid receptor
HRP	Horseradish peroxidase
HT	Hormone Therapy
IL-6	Interleukin 6
LNG	levonorgestrel
MAPK	mitogen-activated protein kinase
Mib	Mibolerone
MPA	medroxyprogesterone acetate
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazolyl)-20-2,5-diphenyltetrazolium bromide

NET-A	norethisterone acetate
NOMAC	nomegestrol acetate
NFκB	nuclear factor kappa B
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PenStrep	penicillin-streptomycin
PR	Progesterone receptor
PR-A	Progesterone receptor-A
PR-B	Progesterone receptor-B
P_4	Progesterone
qPCR	Quantitative real-time polymerase chain reaction
RLU(s)	Relative light unit(s)
RNA	Ribonucleic acid
RO	Reverse osmosis
R5020	Promegestone
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween
TNFα	Tumor necrosis factor alpha
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Breast cancer is a major cause of death among women (Diest *et al.*, 2004; MacCiò and Madeddu, 2011; Hsieh and Huang, 2016). It is a heterogenous disease classified according to receptor content (Hsiao *et al.*, 2010; Turashvili and Brogi, 2017). For example, luminal breast cancer is estrogen receptor-alpha positive ($ERα^+$) and progesterone receptor-positive (PR^+) ($ERα^+/PR^+$) (Dai *et al.*, 2015; Africander and Storbeck, 2018). In contrast, basal breast cancer lacks ERα and PR expression, but overexpresses human epidermal growth factor receptor 2 (HER-2) ($ERα^-/PR^-/HER\text{-}2^+$). Triple negative breast cancer (TNBC) on the other hand, lacks the expression of ERα, PR and HER-2 ($ERα^-/PR^-/HER\text{-}2^-$) (Cheang *et al.*, 2009; Dai *et al.*, 2015; Africander and Storbeck, 2018). More than 70% of breast cancers are $ERα^+$ (Dai *et al.*, 2016; Zahid *et al.*, 2018), and the proliferative effects of estrogen via ERα are considered the main causative factors in breast cancer (Ali and Coombes, 2000; Chang *et al.*, 2006; Williams *et al.*, 2008; Cleary and Grossmann, 2009). It is thus not surprising that current breast cancer therapies include both inhibitors of estrogen production and ER activity (Lanari *et al.*, 2012; Lim *et al.*, 2016; Doan *et al.*, 2017). However, some patients with ER^+ breast tumours become resistant to these therapies (reviewed in Fan *et al.*, 2015), while patients with $ERα^-$ tumours are unlikely to respond to these endocrine therapies (Collaborative Group EBCTC, 1998). In the quest of finding improved breast cancer therapies, other steroid receptors are now also considered as possible therapeutic targets. This is because steroid receptors such as the PR, androgen receptor (AR) and the glucocorticoid receptor (GR) are also present in breast tumours (Conzen, 2008), and emerging evidence indicates that these receptors not only play important roles in breast cancer biology but may in fact influence each other's activity (Rizza *et al.*, 2014; Karamouzis *et al.*, 2015; Thomas and Gustafsson, 2015).

Numerous factors have been associated with increased breast cancer risk, and include the use of menopausal hormone therapy (HT), contraception and being overweight or obese (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; MacCiò *et al.*, 2009; Iyengar *et al.*, 2013). The latter is concerning in South Africa as both breast cancer incidence and the incidence of obesity in women continue to increase (Hruby and Hu, 2015; South Africa Demographic and Health Survey (SADHS), 2016; Lince-Deroche *et al.*, 2017; Van Zyl *et al.*, 2017). One of the mechanisms suggested for the association of obesity with increased breast cancer risk is increased inflammation. However, the mechanism through which inflammation promotes the development of breast cancer is largely unknown (Iyengar *et al.*, 2013). It has however been shown that pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) play important roles in breast cancer progression (Cole, 2009; MacCiò and Madeddu, 2011).

The incidence of obesity-related breast cancer is higher in postmenopausal women than premenopausal women (Marsden, 2003; Surakasula *et al.*, 2014). Postmenopausal women often use HT to alleviate the symptoms of menopause, and is administered as either estrogen-only to women who have had a hysterectomy, or a combination of estrogen and a progestin to women with an intact uterus (Greendale *et al.*, 1999; Ross *et al.*, 2000; Rossouw *et al.*, 2002). Although both estrogen-only and estrogen-progestin combination HT have been associated with increased breast cancer risk, the estrogen-progestin combination is associated with a greater risk (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005, 2008). Progestins are synthetic compounds, designed to have progestational activity with a greater bioavailability and half-life than the natural female hormone, progesterone (P_4) (Speroff and Darney, 1996; Hapgood *et al.*, 2004). Even though progestins were synthesised to act via the PR, numerous studies have shown that some progestins exert effects via other steroid receptors such as the ER, AR and GR (Guerra *et al.*, 2013; Stanczyk *et al.*, 2013; Africander *et al.*, 2014). Multiple progestins with different structures have been synthesised (Schindler *et al.*, 2003; Sitruk-Ware, 2004; Schindler, 2014), and as structure determines function, these progestins may have different biological activities. Not all progestins have been evaluated for an association with breast cancer risk, thus the possibility exists that some may not increase breast cancer risk.

The ability of some progestins to activate multiple steroid receptors, together with the fact that all steroid receptors are expressed in most breast cancers, highlights the complexities of the mechanisms through which progestins may increase breast cancer risk. Comparative studies of progestin effects on breast cancer at the cellular level may provide insight into the mechanisms whereby these hormones influence breast cancer. In addition, increased production of pro-inflammatory mediators such as IL-6 and TNFα, as observed in obesity-related inflammation, is also associated with increased breast cancer risk. The exact mechanisms by which these cytokines contribute to breast cancer development and progression are still poorly understood. Considering that breast cancer is a major cause of death among women, and that the incidence of obesity is increasing, together with the fact that some progestins used in HT are associated with increased breast cancer risk, it is crucial to investigate the link between these factors and increased breast cancer risk. The following sections will provide an overview on the roles of endogenous hormones, progestins, steroid receptors and pro-inflammatory cytokines in breast cancer biology.

1.2. The importance of steroid receptors in breast cancer biology

1.2.1. Mediators of hormone activity

Steroid hormones elicit their biological effects by binding to intracellular steroid receptors (Xu and O'Malley, 2002). These receptors are a subfamily of the nuclear receptor family of transcription factors (DeMayo *et al.*, 2002), and include the PR-isoforms, ER-subtypes, AR, GR and the mineralocorticoid receptor (MR). The well-defined domain organisations of the steroid receptors are depicted in Figure 1.1. These domains include a highly variable N-terminal domain which differs in both amino acid sequence and length, a highly conserved DNA-binding domain (DBD), a hinge region and a C-terminal ligand-binding domain (LBD) which is moderately conserved (McEwan *et al.*, 2007; Africander *et al.*, 2011; Perkins *et al.*, 2018). The N-terminal domain contains an activation function 1 (AF-1) domain, while an AF-2 domain is found in the C-terminal LBD. Both domains are important for inducting the transcriptional activity of steroid receptors. However, the transcriptional activity of AF-2 is dependent on the presence of ligand, while AF-1 transcriptional activity is independent of ligand (Bourguet *et al.*, 2000; Lavery and McEwan, 2005; Africander *et al.*, 2011). Moreover, the AF-2 domain contains specific motifs that allows the interaction with co-factors, while these motifs are absent in the AF-1 domain (Bourguet *et al.*, 2000; Lavery and McEwan, 2005).

Generally, steroid receptors are activated upon hormone binding, after which the receptor undergoes a conformational change and the cytoplasmic hormone-bound receptor translocates to the nucleus (Zhou and Cidlowski, 2005; Griekspoor *et al.*, 2007). The activated steroid receptor can then bind directly to DNA at specific sequences called hormone response elements (HREs) found in the promoter regions of target genes, thereby activating transcription (transactivation) (Kenna *et al.*, 1999; Klinge, 2001; Klinge *et al.*, 2004; Platet *et al.*, 2004). Alternatively, the activated steroid receptor can repress gene expression (transrepression) by tethering to DNA-bound transcription factors such as nuclear factor kappa B (NF-κB) (Rays *et al.*, 1994; Yang, 1995; Paech *et al.*, 1997). Even though all steroid receptors mediate their effects via similar mechanisms, they activate different target genes in different tissues. It is known that steroid receptors play diverse roles in breast cancer biology (Conzen, 2008). As studies investigating the role of the MR in breast cancer is limited, only the roles of the ERs, PRs, AR and GR will be discussed in the following sections.

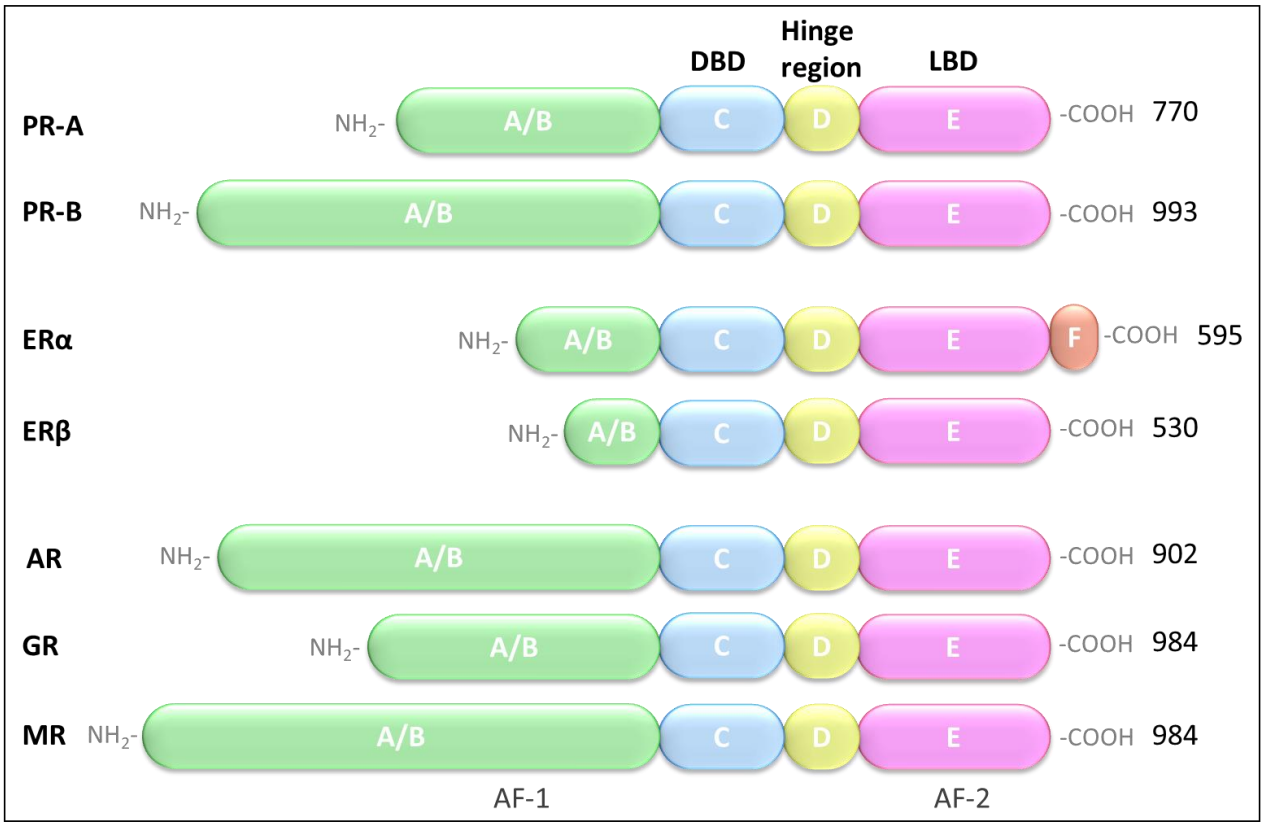


Figure 1.1 Structural overview of steroid receptors. Steroid receptors contain a variable amino-terminal domain (A/B) containing the AF-1 transactivation region, a highly conserved DNA-binding domain (DBD) (C), a hinge region (D) and a carboxy-terminal, ligand-binding domain containing the AF-2 transactivation region (E). A unique carboxy-terminal F domain can be found in ERα. The numbers indicate the length of the steroid receptors in amino acids. Redrawn from (Griekspoor *et al.*, 2007; Africander *et al.*, 2011)

1.2.2. Estrogens and the ER subtypes

The link between estrogen-mediated signalling and breast cancer was recognised as early as the 19th century (Beatson, 1896; Kelsey and Bernstein, 1996; Kuiper *et al.*, 1996; Gustafsson, 1999). However, the exact role of estrogen in breast cancer is complex, and still actively researched. 17β-Estradiol (E_2) is the most biologically active estrogen (Russo *et al.*, 1999; Russo and Russo, 2006), and mediates its physiological effects by binding to the ER (Horwitz & Mcguire 1978; DeMayo *et al.* 2002; Visser *et al.* 2013). Two functional ERs, ERα and ER-beta (ERβ), have been identified. These subtypes are transcribed from different genes (DeMayo *et al.*, 2002; Conzen, 2008), and play opposing roles in breast cancer. For example, ERα promotes the proliferation of breast cancer cells, while ERβ antagonises ERα-mediated proliferation (Kuiper *et al.*, 1996; Barkhem *et al.*, 1998; Gustafsson, 1999; Lazennec *et al.*, 2001; Platet *et al.*, 2004; Ström *et al.*, 2004). However, the role of ERβ is dependent on whether ERα is present, as it has been shown that ERβ increases breast cancer proliferation in ERα$^-$ breast cancer cells (Leygue and Murphy, 2013). The precise role of ERβ in ERα$^-$ breast cancer (TNBC) is still poorly understood, however its potential as a therapeutic target in ERα$^-$

breast cancer is under investigation (Honma *et al.*, 2008; Perkins *et al.*, 2018). Nearly 75% of breast cancers express ERα, and E_2 together with ERα are the main etiological factors associated with breast cancer (Ali and Coombes, 2000; Chang *et al.*, 2006; Williams *et al.*, 2008; Cleary and Grossmann, 2009). Current breast cancer therapies thus target the synthesis of endogenous estrogens and ER activity (Lanari *et al.*, 2012; Lim *et al.*, 2016; Doan *et al.*, 2017). Estrogen synthesis is targeted by inhibiting aromatase, the enzyme responsible for converting androgens to estrogens (Smith and Dowsett, 2003; Chlebowski *et al.*, 2009). A number of therapies are available for the inhibition of the ER, and include selective ER modulators (SERMs) such as tamoxifen (Jordan, 2007), and selective ER down-regulators (SERDs) such as fulvestrant (Ciruelos *et al.*, 2014; Poggio *et al.*, 2016). SERMs are partial ER agonists that prevent E_2-mediated effects by exerting anti-estrogenic actions depending on the tissue type. In contrast, SERDs are ER antagonists that downregulate ER expression (Griekspoor *et al.*, 2007; Ciruelos *et al.*, 2014).

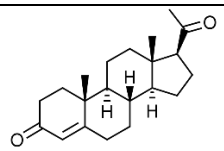
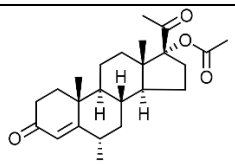
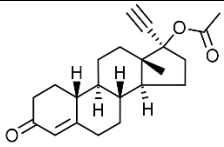
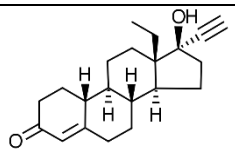
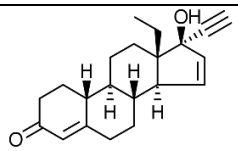
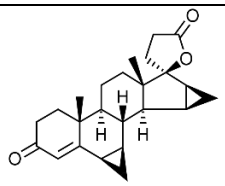
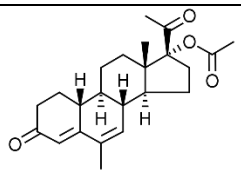
Numerous studies have investigated the effects of E_2 on the proliferation, apoptosis, migration and invasion of breast cancer cells (Furuya *et al.* 1989; van den Brûle *et al.* 1992; Jiang *et al.* 2013; Holton *et al.* 2014; Tian *et al.* 2018). For example, E_2 is known to enhance breast cancer cell proliferation via both genomic and non-genomic mechanisms. Genomic mechanisms refer to the ligand-bound ER modulating gene expression via the ERs (Ali and Coombes, 2000; Tchafa *et al.*, 2013), while non-genomic mechanisms refer to rapid responses entailing the activation of membrane-bound receptors or the activation of protein kinase cascades (Levin, 1999; Lim *et al.*, 2006; Marino *et al.*, 2006; Tchafa *et al.*, 2013). In addition, E_2 increased the mRNA expression of Ki-67, a proliferative marker widely used to evaluate breast cancer cell proliferation (Klinkhammer-schalke, 2013; Dai *et al.*, 2016; Mannell, 2016), via ERα in MCF-7 breast cancer cells (Liao *et al.*, 2014). E_2 is also a known inhibitor of apoptosis in numerous cancer cell lines, such as ovarian, lung and breast cancer cells (Mabuchi *et al.*, 2004; Lewis-Wambi and Jordan, 2009; Grott *et al.*, 2013). In breast cancer, however, the role of E_2 on apoptosis is controversial as it has been shown to elicit both pro- or anti-apoptotic effects (Lewis-Wambi and Jordan, 2009). For example, E_2 induced apoptosis in long-term estrogen-deprived MCF-7 breast cancer cells, but also inhibited apoptosis in MCF-7 and T47D breast cancer cells (Wang and Phang, 1995; Gompel *et al.*, 2000; Song *et al.*, 2001; Fernando and Wimalasena, 2004; Helguero *et al.*, 2005; Lewis-Wambi and Jordan, 2009). Furthermore, it has previously been suggested that E_2 protects against apoptosis, as it enhanced the mRNA expression of the anti-apoptotic Bcl-2 gene in MCF-7 cells, while having no effect on the expression of the pro-apoptotic Bax gene (Wang and Phang, 1995). Several studies have also reported that E_2 enhances the migration and invasion of MCF-7 and T47D breast cancer cells (Albini *et al.*, 1986; van den Brûle *et al.*, 1992; Zheng *et al.*, 2011; Shang *et al.*, 2015), while a recent study showed that is has the ability to repress invasion of T47D

cells (McFall *et al.*, 2018). From the above, it is clear that E_2 has the ability to enhance breast cancer development and progression via multiple mechanisms. Moreover, a recent study by our group showed that estrone (E_1) and estriol (E_3), two endogenous estrogens previously considered as weak estrogens, displayed similar agonist efficacies for transactivation as E_2 via ERα and ERβ (Perkins *et al.*, 2017). However, it is not only endogenous estrogens that are associated with increased breast cancer, but also synthetic estrogens used in postmenopausal HT regimens (Henderson *et al.*, 1988; Pike *et al.*, 1993; Greendale *et al.*, 1999; Chervenak, 2009; Perkins *et al.*, 2018). Further investigation into receptor-mediated effects of endogenous and synthetic estrogens are needed to understand the mechanism whereby these hormones increase breast cancer development and progression.

1.2.3. Progestogens and the PR isoforms

Progestogens are a class of compounds which include endogenous progesterone (P_4) and progestins, which are synthetic hormones designed to mimic the action of P_4 (reviewed in Hapgood *et al.* 2013). Progestins are used in contraception and HT instead of P_4, as it has a greater bioavailability and half-life (Speroff and Darney, 1996; Hapgood *et al.*, 2004). In contraception, progestins are used to prevent pregnancy by inhibiting ovulation (Africander *et al.*, 2011; Guerra *et al.*, 2013). In HT, progestins are combined with estrogen to prevent estrogen-induced endometrial hyperplasia in women with an intact uterus (Johnson, 1998; Greendale *et al.*, 1999; Ross *et al.*, 2000; Cuzick, 2008). To date, a large number of progestins have been synthesised with different chemical structures (Schindler *et al.*, 2003; Sitruk-Ware, 2004; Sitruk-Ware and Nath, 2010; Schindler, 2014). Most progestins are either derived from parent compounds such as progesterone, resulting in 17α-hydroxy-progesterone and 19 nor-progesterone derivatives, or from testosterone, resulting in 19-nor-testosterone derivatives (Sitruk-Ware and Nath, 2010; Africander *et al.*, 2011). One progestin is structurally related to the MR antagonist, spironolactone. All progestins are classified into four consecutive generations, with the fourth-generation progestins designed to have a greater affinity for the PR and display biological effects more similar to P_4 (Sitruk-Ware and Nath, 2010). Examples of progestins from different generations are described in Table 1.1.

Table 1.1 Examples of clinically used progestogens

Progestogen (Abbreviation)	Structure	Generation	Structural derivative	Biological activities via SRs*	Therapy*
Progesterone (P_4)		Natural	-	Progestogenic; anti-androgenic; ± glucocorticoid	Prevention of preterm labour; HT
Medroxy-progesterone acetate (MPA)		1st	17α-hydroxy-progesterone	Progestogenic; ± androgenic; glucocorticoid	HT; PO injectable contraceptive; cancer therapy
Norethisterone acetate (NET-A)		1st	Testosterone	Progestogenic; androgenic;	HT; COC; POP
Levonorgestrel (LNG)		2nd	Testosterone	Progestogenic; androgenic;	HT; POP; COC; IUD; cancer therapy
Gestodene (GES)		3rd	Testosterone	Progestogenic; ± androgenic; ± glucocorticoid	COC
Drospirenone (DRSP)		4th	Spironolactone	Progestogenic; anti-androgenic	HT; COC
Nomegestrol acetate (NoMAC)		4th	19-nor-progesterone	Progestogenic; anti-androgenic	HT; PO contraceptive implant, COC

*HT: hormone therapy; COC: combined oral contraceptive; POP: progestin-only pill; PO: progestin-only; IUD: intrauterine device; steroid receptors (SRs); ±: literature contradictory. Adapted from (Africander *et al.*, 2011; Hapgood *et al.*, 2013; Louw-du Toit, Storbeck, *et al.*, 2016)

Results from a number of clinical studies have indicated that the estrogen-progestin combination HT presents a higher risk for developing breast cancer than estrogen-only HT (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2008), suggesting that the progestin component is responsible for the higher risk. However, the role of P_4 and the progestins in breast cancer is not straightforward. While most studies suggest that P_4 is not associated with increased breast cancer risk (Cordina-Duverger *et al.*, 2013; Stute *et al.*, 2018), there is clinical evidence that

progestins are linked to increased risk of breast cancer (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2008). For example, the Women's Health Initiative (WHI) performed a randomized control trial on postmenopausal women using HT, and reported that the use of MPA in combination with estrogen is associated with an increased risk for developing invasive breast cancer (Rossouw *et al.*, 2002). Additionally, the Million Women Study reported a link between breast cancer and the use of MPA, NET-A and LNG in HT (Million Women Study Collaborators, 2003). Furthermore, the E3N-EPIC cohort study investigated the effects of different HT types, and found that the use of the early-generation progestins MPA, NET-A, as well as the fourth-generation progestin, NoMAC, increased the risk for developing breast cancer (Fournier *et al.*, 2008). This increased risk for developing breast cancer by women using progestins in HT usage is concerning. However, it should be noted that not all progestins have been tested for increased breast cancer risk, and thus further investigation is urgently needed. Moreover, the precise mechanism whereby some progestogens influence breast cancer is yet to be established.

Results from numerous studies investigating the effects of P_4 and progestins in breast cancer cell lines are also not straightforward. For example, it has been shown that P_4, MPA, NET-A, LNG and GES increase the proliferation of the MCF-7 and T47D breast cancer cell lines (Van den Burg *et al.*, 1992; Catherino *et al.*, 1993; Kalkhoven *et al.*, 1994; Schoonen *et al.*, 1995; Franke and Vermes, 2003; Lim *et al.*, 2006; Izzo *et al.*, 2014). In contrast, other studies have reported that P_4, MPA and NET-A can inhibit proliferation of T47D breast cancer cells (Horwitz and Freidenberg, 1985; Musgrove *et al.*, 1991; Botella *et al.*, 1994; Groshong *et al.*, 1997; Formby and Wiley, 1999; Krämer *et al.*, 2006). While NoMAC has been shown to inhibit proliferation of the T47D breast cancer cell line (Botella *et al.*, 1994), it has also been reported to increase proliferation of the MCF-7 breast cancer cell line (Ruan, Schneck, *et al.*, 2012). Most studies investigating the effects of P_4 and the progestins on breast cancer, evaluate effects on proliferation, while only a few studies have evaluated effects on other cancer cell behaviours, such as apoptosis, migration and invasion (Hanahan and Weinberg, 2011). Results from these limited studies are, however, also inconsistent. A study from 20 years ago reported that P_4 can induce apoptosis in the T47D breast cancer cell line (Formby and Wiley, 1998), while two more recent studies in T47D cells, as well as MCF-7 and MDA-MB-231 cells, report that P_4 is anti-apoptotic (Ory *et al.* 2001; Moore *et al.* 2006). The authors of the latter studies also showed that MPA and a synthetic PR agonist, promegestone (R5020), exert anti-apoptotic effects in T47D breast cancer cells (Ory *et al.*, 2001; Moore *et al.*, 2006), whereas others show that MPA and NET-A exert pro-apoptotic effects in the T47D, as well as the MCF-7 breast cancer cell lines (Kandouz *et al.*, 1999; Werner *et al.*, 2005; Sweeney *et al.*, 2014). In addition, P_4, MPA, NES and DRSP can also increase the migration and invasion of T47D breast cancer cells, with MPA causing the highest increase (Fu

et al., 2008, 2010). In light of the above, it is clear that more studies are needed at the cellular level. This will aid in the understanding of the mechanisms by which some progestins increase breast cancer risk, and possibly why others are not associated with such a risk.

Progestins were designed to mimic P_4 by binding to the PR. However, some progestins can also act via other steroid receptors such as the GR, ER and AR (Guerra *et al.*, 2013; Stanczyk *et al.*, 2013; Africander *et al.*, 2014). For example, both MPA and NET-A can bind to the GR, with MPA displaying partial GR agonist activity (Koubovec *et al.*, 2005), and NET-A antagonist activity (Ronacher *et al.*, 2009). Louw-du Toit and co-workers recently reported that none of the progestins described in Table 1.1 could bind to ERβ, while the testosterone-derived progestins, NET-A, LNG and GES, could bind to ERα and display partial agonist activity (Louw-du Toit *et al.*, 2017). Results from the latter study, and an earlier study from our group, showed that MPA, NET-A, LNG and GES can bind to the AR and display androgenic effects similar to the natural androgen dihydrotestosterone (DHT) (Africander *et al.*, 2014; Louw-du Toit *et al.*, 2017). Like P_4, however, NES, DRSP and NoMAC displayed AR antagonist activity (Louw-du Toit *et al.*, 2017). The fact that progestins can interact with steroid receptors other than the PR, may explain some of the adverse effects associated with their clinical use (Koubovec *et al.*, 2005; Africander *et al.*, 2013, 2014; Louw-du Toit *et al.*, 2014). However, it is important to note that the PR itself has also been implicated with increased risk of breast cancer.

The PR is found in 75% of breast cancers and exists as two isoforms, PR-A and PR-B (Horwitz & Mcguire 1978; Conzen 2008; Lanari *et al.* 2009). Although PR-A and PR-B play distinct roles in breast cancer, few studies distinguish between their roles. PR-A and PR-B are transcribed from the same gene, with PR-B having an additional amino-terminal segment of 164-amino acids (Figure 1.1) (Sartorius *et al.*, 1994; Mcfall *et al.*, 2015). While normal breast tissue expresses comparable levels of PR-A and PR-B (Mote *et al.*, 2002), it is known that an imbalance of the isoforms are found in breast cancer, as PR-A is overexpressed (Hopp *et al.*, 2004; Diep *et al.*, 2015; McFall *et al.*, 2018). PR-A is known to inhibit the transcriptional activity of PR-B as well as the ER (Kraus *et al.*, 1995; Hopp *et al.*, 2004). Hopp and co-workers showed that patients with breast tumours that have higher PR-A expression relative to PR-B expression, have an increased risk of relapse, suggesting that these patients are possibly resistant to tamoxifen endocrine therapy (Hopp *et al.*, 2004). However, Lanari and co-workers recently suggested anti-progestins as treatment for patients with breast tumours expressing increased levels of PR-A, as they showed that primary tumours with increased PR-A levels relative to PR-B levels, regress following treatment with the anti-progestin, mifepristone (RU486) (Rojas *et al.*, 2017).

In vitro studies in breast cancer lines have shown that P_4 decreased the proliferation of MDA-MB-231 cells co-transfected with both PR-isoforms, however these authors did not investigate the role of the individual isoforms (Lin *et al.*, 1999). In contrast, a study by Wargon *et al.* showed that the MPA-induced increase in proliferation of T47D breast cancer cells was mediated by PR-B. First, they showed a role for the PR, as the response was inhibited by RU486, a PR antagonist (Wargon *et al.*, 2015), and next they showed that the response was abolished when the expression of PR-B was silenced (Wargon *et al.*, 2015). Interestingly, Giulianelli *et al.* showed that ERα is required for the PR-B-mediated effects of MPA on gene expression and proliferation in T47D breast cancer cells (Giulianelli *et al.*, 2012). Moreover, Daniel and co-workers showed that estrogen responsiveness of MCF-7 breast cancer cells is enhanced by PR-B (Daniel *et al.*, 2015). In contrast, it has been shown that overexpression of PR-A or PR-B in MCF-7 cells decreased E_2-stimulated growth, suggesting that the both PR isoforms can suppress growth in breast cancer cell lines expressing ERα (Zheng *et al.*, 2005). In agreement with the PR being associated with positive effects in breast cancer, it was recently shown that progestin-bound PR forms a complex with ERα, resulting in ERα binding to different chromatin loci and transcriptional sites (Mohammed *et al.*, 2015), resulting in the expression of genes associated with a favourable outcome (Mohammed *et al.*, 2015). However, Mohammed and co-workers only evaluated the effects of P_4 and R5020. Given that progestins have diverse structures and that some progestins have been associated with increased breast cancer risk, it is important to evaluate whether other progestins would initiate similar effects. Taken together, it is evident that the role of the PR in breast cancer is complex, and that it is not only dependent on the presence of ERα, but also on whether the PR is activated by a ligand or not. Understanding the mechanisms of different progestins via PR-A and PR-B is crucial, as it will provide a better understanding of the effects of progestins in breast cancer, while simultaneously aiding in the identification of progestins that can be used in HT without breast cancer risk.

1.2.4. Androgens and the AR

Evidence in the literature suggests that androgens and the AR play important roles in breast cancer biology (Conzen, 2008; Dimitrakakis and Bondy, 2009; Rizza *et al.*, 2014). Their precise function is however controversial. For example, it has been suggested that androgens drive breast cancer development and progression by their conversion to active estrogens (reviewed in Majumder *et al.* 2017). In agreement with androgens having negative effect on breast cancer, two studies have shown that the natural androgen DHT, as well as the synthetic non-metabolizable androgens, mibolerone (Mib) and methyltrienolone (R1881), increase the proliferation of the AR^+ MCF-7 and MDA-MB-453 breast cancer cell lines (Birrell *et al.*, 1995; Doane *et al.*, 2006). In contrast, DHT and Mib are also associated with positive effects on breast cancer, as a number of studies show that both these

androgens inhibit proliferation of MCF-7, T47D and ZR-75-1 breast cancer cells (Birrell *et al.*, 1995; Andò *et al.*, 2002; Aspinall *et al.*, 2004; Lanzino *et al.*, 2013; Rizza *et al.*, 2014). Differences in the effects of androgens on the proliferation of breast cancer cell lines have been attributed to the specific androgen used, its concentration and whether the ER is expressed in the cell line (reviewed in Somboonporn & Davis 2004). Evidence from an observational study suggest that testosterone administration may decrease breast cancer risk (Glaser and Dimitrakakis, 2013), while a prospective case-cohort study suggested that elevated levels of testosterone may lead to an increased breast cancer risk (Cauley *et al.*, 1999).

Androgens mediate their effects via the AR, which is expressed in 55% of ER^- tumours and in 90% of ER^+ tumours (Ogawa *et al.* 2008; Hu *et al.* 2011). Most studies suggest that the AR is associated with a good prognosis in ER^+ breast cancer. Mechanisms for these positive effects have been suggested by studies from Peters *et al.* and Rizza *et al.*, investigating the role of ERα and ERβ, respectively (Peters *et al.*, 2009; Rizza *et al.*, 2014). Peters and co-workers showed that the AR decreases ERα transcriptional activity by competing for the binding to an estrogen response elements (ERE), thereby decreasing E_2-mediated effects on breast cancer cell lines (Peters *et al.*, 2009). Indeed, co-expression of the AR and ERα have been associated with beneficial clinicopathological traits, including decreased tumour size and grade, as well as an increased response to endocrine therapy (Ogawa *et al.* 2008; Hu *et al.* 2011; Lundin *et al.* 2011; Peters *et al.* 2012). Rizza *et al.* showed that the AR-mediated effects of both DHT and Mib lead to an increase in the expression of ERβ, via a mechanism involving recruitment of the AR to an androgen response element (ARE) in the promoter region of ERβ (Rizza *et al.*, 2014). Given that ERβ is known to inhibit the activity of ERα, this mechanism would be associated with a favourable outcome with regards to breast cancer. In ER^- breast tumours, however, the AR is linked to poor prognosis as these tumours proliferate in response to androgens (Doane *et al.* 2006; Hu *et al.* 2011). Mechanistically, this could be explained by the fact that the AR can mimic the role of ERα in ER^- breast tumors, and thus promote breast cancer development and progression (Agoff *et al.*, 2003; Hu *et al.*, 2011; Robinson *et al.*, 2011). Given that the AR is widely expressed in breast tumours and that this steroid receptor is thought to correlate with a favourable prognosis in ER^+ breast cancer, and poor disease outcome in ER^- breast cancer, it is currently under investigation as a potential drug target (Wu and Vadgama, 2017; Giovannelli *et al.*, 2018). For example, use of a non-steroidal, tissue selective AR modulator (SARM), enobosarm (GTx-24) in ER^+ breast cancer patients, is showing promising results in an ongoing phase II clinical trial (Overmoyer *et al.*, 2014; Overmoyer and Cancers, 2015; Giovannelli *et al.*, 2018). A similar phase II clinical trial has shown a 19% clinical improvement in TNBC expressing the AR with the use of the AR antagonist, bicalutamide (Gucalp *et al.*, 2013; Wu and Vadgama, 2017).

Like DHT, the AR agonists MPA and NET-A, also inhibit the transcriptional activity of ERα via the AR (Peters *et al.* 2009; Easter Ndlovu, Stellenbosch University, MSc Thesis, 2016). These results suggest that these progestins may be associated with a favourable outcome in ER^+ breast tumours expressing the AR. This raised the question whether all androgenic progestins would elicit similar effects, and whether it would differ for anti-androgenic progestins. On the other hand, it has also been shown that MPA displays anti-androgenic activity via a mechanism independent of AR binding (Ochnik *et al.*, 2014). Ochnik and co-workers showed that MPA inhibits DHT-induced stabilisation of the AR and the anti-proliferative effects of DHT on proliferation (Ochnik *et al.*, 2014). It is clear that the exact mechanism whereby progestins influence breast cancer via the AR is still poorly understood and that further investigation is warranted.

1.2.5. Glucocorticoids and the GR

The role of glucocorticoids and the GR in breast cancer is relatively understudied when compared to studies for the ER, PR, and AR. However, current evidence suggests that the role of the GR is not straightforward, as some studies report inhibitory effects by glucocorticoids, whilst others report increased breast cancer development and progression (reviewed in Lin & Wang 2016; Africander & Storbeck 2018). The natural glucocorticoid, cortisol, has been reported to increase the proliferation of breast cell lines derived from metastasizing breast tumours (Simon *et al.* 1984), while its reported to decrease the proliferation of MCF-7 breast cancer cells (Ryde *et al.* 1992). Several studies have, however, reported that the synthetic glucocorticoid dexamethasone (Dex) inhibits proliferation of the MCF-7 and T47D breast cancer cell lines (Lippman *et al.* 1976; Ryde *et al.* 1992; Vilasco *et al.* 2011; Courtin *et al.* 2012). Additionally, at least one study has shown that Dex inhibits apoptosis in the MCF-7 and MDA-MB-231 breast cancer cell lines (Wu *et al.*, 2004).

Glucocorticoids elicit their biological effects by binding to the GR, which is expressed in normal breast tissue and breast tumours (Buxant *et al.*, 2010). Interestingly, most breast cancer cell lines also express the GR (Conzen, 2008; Abduljabbar *et al.*, 2015). As with the AR, the role of the GR in breast cancer appears to be dependent on ERα expression. Pan *et al.* reported that high expression of the GR in $ERα^+$ cancers is associated with a good prognosis, whereas a similar expression of the GR in $ERα^-$ cancers is associated with a poor outcome (Pan *et al.*, 2011). Considering that the GR is expressed in about 60% of breast tumours (Abduljabbar *et al.*, 2015), it is important to clarify its exact role in breast cancer biology. Finally, as some progestins are partial GR agonists, while others are GR antagonists (reviewed in Africander *et al.* 2011), further studies are required to determine whether these progestins influence breast cancer development and progression via the GR.

1.3. The role of Inflammation and adipokines in breast cancer

Glucocorticoids are of the most common and effective drugs used for the relief of inflammatory and immune disorders (Barnes, 1998; Straub and Cutolo, 2016). These hormones are generally used for their anti-inflammatory properties whereby they repress genes encoding pro-inflammatory cytokines via the GR (reviewed in Cruz-topete & Cidlowski 2016). However, glucocorticoids can also increase the levels of pro-inflammatory cytokines (Dhabhar, 2002; Busillo *et al.*, 2011; Cruz-topete and Cidlowski, 2016). For example, pro-inflammatory cytokine levels are increased in the central nervous system in response to increased glucocorticoid secretion (O'Connor *et al.*, 2003; Deinzer *et al.*, 2004; Sorrells and Sapolsky, 2007; Cruz-topete and Cidlowski, 2016). Similarly, it has been found that MPA, a progestin with partial glucocorticoid activity, increases the expression of the pro-inflammatory cytokine IL-12, while decreasing the expression of the anti-inflammatory cytokine IL-10, via a GR-dependent mechanism (Louw-du Toit *et al.*, 2014).

There are countless factors that can cause inflammation, and include external environmental stimuli, acute or persistent infections, dysregulation of the immune system and autoimmune diseases (Grivennikov *et al.*, 2011). Depending on the stimuli, an inflammatory response is activated by specific cells from the innate and adaptive immune system. However, chronic activation results in disrupted interactions between the immune cells, which leads to low-grade systemic inflammation. The latter has been linked to the development of various cancers, such as cervical, ovarian and breast cancer (Balkwill and Mantovani, 2001; De Visser *et al.*, 2006; Cole, 2009).

One of the major causes of chronic inflammation is being overweight and obese (Cleary and Grossmann, 2009; Iyengar *et al.*, 2013; Zahid *et al.*, 2018). Obesity is generally indicated by an increased body mass index (BMI), which is calculated by weight (kg) divided by height in meters squared (m^2). A BMI of 25-29.9 indicates an overweight individual, while a BMI of 30 or more is an indication of obesity (The American Cancer Society, 2015). Mature adipocytes are no longer solely considered as energy-storing cells, but are recognised as active endocrine cells producing growth factors, hormones, cytokines, chemokines and a heterogenous group of pro-inflammatory molecules termed adipokines (MacCiò and Madeddu, 2011; Ouchi *et al.*, 2011; Wang *et al.*, 2015). The increase in adipocytes associated with obesity, leads to dysregulated production of adipokines such as IL-6, IL-1β and TNFα (Vona-Davis and Rose, 2007; Iyengar *et al.*, 2013; Esquivel-vela *et al.*, 2015). These pro-inflammatory mediators leads to increased local inflammation within the breast, which in turn contributes to the development of breast cancer (Iyengar *et al.*, 2013). It has been suggested that the risk for developing ER^+/PR^+ breast tumours is higher in postmenopausal women that are overweight or obese (reviewed in Cleary & Grossmann 2009; Iyengar *et al.* 2013). However, the precise

mechanism by which obesity-related inflammation contributes to breast cancer risk, specifically in postmenopausal women, is still poorly understood.

Several hypotheses have been proposed to describe the association of obesity-related inflammation in postmenopausal breast cancer. For example, increased estrogen biosynthesis by the aromatase enzyme within the adipose tissue of the breast and the tumour itself (Rose *et al.*, 2004; Cleary and Grossmann, 2009). Another hypothesis linking obesity-related inflammation to breast cancer, is that the adipocytes themselves may be involved in the etiopathogenesis of breast cancer (MacCiò *et al.*, 2009). Both *in vivo* and *in vitro* studies have shown that tumour growth is directly influenced by adipocytes (Iyengar *et al.*, 2003). Numerous studies have reported that pro-inflammatory cytokines (adipokines) such as IL-6 and TNFα have the ability to facilitate growth and metastasis of breast cancer cells (Rubio *et al.*, 2006; Sasser *et al.*, 2007; Proietti *et al.*, 2008; Studebaker *et al.*, 2008), suggesting that these two inflammatory mediators play a central role in breast cancer pathogenesis. The exact role that IL-6 and TNFα play in breast cancer development and progression, however, is still unclear (MacCiò and Madeddu, 2011). Both these cytokines have been widely investigated and are also one of the focus points of this study. In the following sections, a brief overview is provided on the role of IL-6 and TNFα in breast cancer biology.

1.3.1 TNFα and breast cancer

TNFα is a crucial protein involved in the inflammatory response, and is implicated in numerous diseases (Cabal-hierro and Lazo, 2012). Although this pro-inflammatory cytokine is primarily synthesised by immune cells such as mast cells, dendritic cell lymphocytes and macrophages, it is also produced by adipocytes (MacCiò and Madeddu, 2011). In fact, TNFα was the first pro-inflammatory adipokine to be recognised as a product of adipocytes (MacCiò and Madeddu, 2011). However, the extent to which TNFα is secreted into circulation is unknown (MacCiò and Madeddu, 2011).

TNFα mediates its responses through two distinct glycoprotein transmembrane receptors known as TNF receptor-1 (TNFR1) and TNFR2 (Cabal-hierro and Lazo, 2012; Waters *et al.*, 2013; Martínez-reza *et al.*, 2017). The expression of these receptors have been shown in breast cancer tissue (Waters *et al.*, 2013; Yang *et al.*, 2017), as well as in breast cancer cell lines such as the T47D, MCF-7 and MDA-MB-231 cells (Zhao *et al.*, 2017). Most biological effects of TNFα occur via activation of TNFR1 (Wajant *et al.*, 2003; Martínez-reza *et al.*, 2017). Generally, TNFα activates three diverse cellular responses which include cell death, cell survival and transcription of other pro-inflammatory genes (reviewed in Waters *et al.* 2013). Evidence on the effects of TNFα on breast cancer cells are not always straightforward. In fact, Wang *et al.* refers to TNFα as a “double-dealer” with regards to

cancer (Wang and Lin, 2008). This is due to the fact that TNFα can a drive cancer cell proliferation, metastasis and tumour angiogenesis, while it can also induce cell death (Wang and Lin, 2008). Results from *in vitro* studies in cells lines have shown that TNFα promotes proliferation in T47D breast cancer cells (Rubio *et al.*, 2006), while inducing apoptosis in the MCF-7 breast cancer cell line (Katerinaki *et al.*, 2003; Wajant *et al.*, 2003; Wang and Lin, 2008). In contrast to the latter, at least two studies have shown that TNFα has no effect on apoptosis in T47D cells (Rubio *et al.*, 2006; Wang and Lin, 2008). Together these results suggest that TNFα exerts cell line-specific effects. In addition, Wolczyk *et al.* showed that TNFα promotes the growth, migration and invasion of both the $ERα^+$ MCF-7, and $ERα^-$ MDA-MB-231, breast cancer cell lines, via a mechanism involving the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway as well as increased expression of matrix metalloproteinases (MMPs) (Wolczyk *et al.*, 2016). MMPs are zinc-containing endopeptidases (Hu and Beeton, 2010) that play an important role in the early stages of cancer invasion, as they cleave the main constituents of the cell basement membrane, namely type IV collagen, elastin and laminin (Toth and Fridman, 2001; Gach *et al.*, 2011; Wolczyk *et al.*, 2016). MMP-2 and MMP-9, also referred to as gelatinase A and gelatinase B, respectively, can hydrolyse denatured gelatin (collagen I) (Murphy and Crabbe, 1995) and their expression and activities are often investigated as a measure of invasion (Toth and Fridman, 2001; Hu and Beeton, 2010). For example, Wolczyk and co-workers showed that TNFα dose-dependently increased the expression of MMP-9, while having no significant effect on MMP-2 expression in MCF-7 and MDA-MB-231 breast cancer cell lines (Wolczyk *et al.*, 2016). As MMP-9 is essential for sustained invasion in tumour cells, these results suggested that TNFα may play a role in enhancing migration and invasion of breast cancer cells (Raghu *et al.*, 2010; Wolczyk *et al.*, 2016). It is evident that the function of TNFα in breast cancer is not yet well understood (Martínez-reza *et al.*, 2017), and that further studies are required to gain clarity on its role in breast cancer pathogenesis. Given the fact that steroid receptors play crucial roles in breast cancer, one possible mechanism that has not been investigated, is whether TNFα may influence steroid receptor expression levels.

1.3.2 IL-6 and breast cancer

TNFα upregulates the expression of IL-6 (Suarez-Cuervo *et al.*, 2003), and increased production of IL-6 has been observed in a variety of chronic inflammatory disorders (Feghali *et al.*, 1997). IL-6 serum levels are also increased in breast cancer patients (Zhang and Adachi, 1999; Vozarova *et al.*, 2001; Slattery *et al.*, 2008) as well as in menopausal women (Morley and Baumgartner, 2004). As IL-6 can be produced by immune cells, tumour cells, adipose cells or a combination of the three (Knüpfer & PreiB 2007; Gyamfi *et al.* 2018), the exact source of the increased IL-6 serum levels in breast cancer patients and menopausal women are unknown. It is, however, known that about a third

of the elevated circulating levels of IL-6 originate from adipose tissue (Fernandez-Real and Ricart, 2003).

IL-6 mediates its effects by binding to the IL-6 receptor (IL-6R), which forms part of the cytokine receptor class I superfamily (Kershaw and Flier, 2004). The receptor exist as two forms, a ± 50 kDa soluble form and a ± 80 kDa membrane-bound form (Kershaw and Flier, 2004), which are both found in breast cancer tissues and cell lines (Chiu *et al.*, 1996; Knüpfer and Preiss, 2007). Although a number of studies have investigated the effects of IL-6 on breast cancer cell lines (Chiu *et al.*, 1996; Badache and Hynes, 2001; Sasser *et al.*, 2007; Studebaker *et al.*, 2008; Gallo *et al.*, 2018), the evidence is not always consistent. Some studies have reported that IL-6 increases proliferation of the MCF-7 breast cancer cell line (Sasser *et al.*, 2007; Studebaker *et al.*, 2008), while another showed that it inhibits the growth of these cells (Chiu *et al.*, 1996). In addition, prolonged treatment with IL-6 can induce apoptosis in the ER^+ MCF-7 cells, but not in the ER^- MDA-MB-231 cells (Chiu *et al.*, 1996). A study by Badache and Hynes reported that IL-6 inhibits the growth of T47D breast cancer cells, while enhancing the migration in this cell line (Badache and Hynes, 2001). Similarly, Gallo and colleagues reported that IL-6 plays an important role in the progression of breast cancer by promoting the migration of MCF-7 and MDA-MB-231 breast cancer cells (Gallo *et al.*, 2018). Results from an *in vivo* study has also showed that increased levels of IL-6 are linked to enhanced metastasis and increased breast tumour size (Dirat *et al.*, 2011). Moreover, like TNFα, IL-6 stimulates estrogen production by increasing aromatase expression within malignant breast tissue as well as adipose tissue (Purohit *et al.*, 1995, 2002; Suarez-Cuervo *et al.*, 2003; Irahara *et al.*, 2006). Overall, the current evidence in the literature suggests that IL-6 may contribute to breast cancer risk via several mechanisms. As discussed for TNFα, the influence of IL-6 on the expression levels of steroid receptors in breast cancer cells is unknown and may be a possible mechanism whereby this cytokine influences breast cancer risk.

1.4 Conclusion

Progestins, in combination with estrogen, are widely used by postmenopausal women in HT (Johnson, 1998; Greendale *et al.*, 1999; Ross *et al.*, 2000; Cuzick, 2008; Africander *et al.*, 2011; Guerra *et al.*, 2013). These estrogen-progestin HT regimens have been associated with increased risk for developing breast cancer (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2008), that are notably higher than risk with estrogen-only regimens. Although progestins were designed to mimic P_4 by binding to the PR, some progestins bind to other steroid receptors, such as the GR, ER and AR (Guerra *et al.*, 2013; Stanczyk *et al.*, 2013; Africander *et al.*, 2014). Thus, it is possible that multiple steroid receptors may be implicated in the mechanism for

increased breast cancer risk by progestins. For example, MPA has been shown to increase migration and invasion of breast cancer cells via the PR (Fu *et al.*, 2008), while it has also been reported that ERα is required for MPA-induced breast cancer proliferation via PR-B (Giulianelli *et al.*, 2012). Mechanisms other than direct steroid receptor binding may also be involved, as MPA has previously been shown to inhibit DHT-induced stabilisation of the AR and the anti-proliferative effects of DHT in normal breast epithelial cells, without activating the AR itself (Ochnik *et al.*, 2014). Multiple generations of progestins have been synthesised with different chemical structures (Schindler *et al.*, 2003; Sitruk-Ware, 2004; Sitruk-Ware and Nath, 2010; Schindler, 2014), and only a few have been evaluated for their risk in terms of breast cancer.

Low-grade chronic inflammation is also strongly linked to the development and progression of breast cancer (Cleary and Grossmann, 2009; Cole, 2009; Pierce *et al.*, 2009), with obesity being one of the major contributing factors associated with an increased inflammatory state (Ouchi *et al.*, 2011). It is believed that the elevated levels of circulating pro-inflammatory cytokines, also known as adipokines when produced by adipocytes, contribute to the onset and development of breast cancer (Vona-Davis and Rose, 2007; Iyengar *et al.*, 2013). Two specific pro-inflammatory cytokines, IL-6 and TNFα, are found within both adipose tissue and breast tumours, and have been reported to facilitate tumour growth and metastasis (Rubio *et al.*, 2006; Sasser *et al.*, 2007; Proietti *et al.*, 2008; Studebaker *et al.*, 2008). However, the exact mechanism linking chronic inflammation and breast cancer is still poorly understood. Whether effects of cytokines such as IL-6 and TNFα on steroid receptor expression levels in breast cancer cells may also be a possible mechanism is not known. This is important as all steroid receptors have been implicated in breast cancer pathogenesis (Conzen, 2008).

Although a number of studies have investigated the effects of IL-6, TNFα and some progestins at the cellular level, results from these studies are often contradictory. Considering that many questions on the effects of IL-6 and TNFα on breast cancer remain unanswered, and that a wide range of progestins are available for clinical use, it is clear that more molecular studies are required to compare the effects of these cytokines and different progestins on breast cancer risk. Postmenopausal women have a greater risk for developing breast cancer associated with obesity-related inflammation, than premenopausal women (MacCiò and Madeddu, 2011). As postmenopausal women often use HT to relieve menopausal symptoms, the question arises whether there is any direct association between inflammation, progestins used in HT and breast cancer. Studying these factors at a molecular level will aid in our understanding of what may be driving inflammation- and HT-associated breast cancer.

1.5 Hypothesis and Aims

It is evident that obesity-related inflammation and some progestins used in HT are associated with an increased risk of breast cancer (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2008; MacCiò and Madeddu, 2011; Iyengar *et al.*, 2013) (Figure 1.2). However, not all progestins have been evaluated for this risk. Moreover, the precise mechanism for the association between obesity-related inflammation and breast cancer, as well as progestins and breast cancer, are not known.

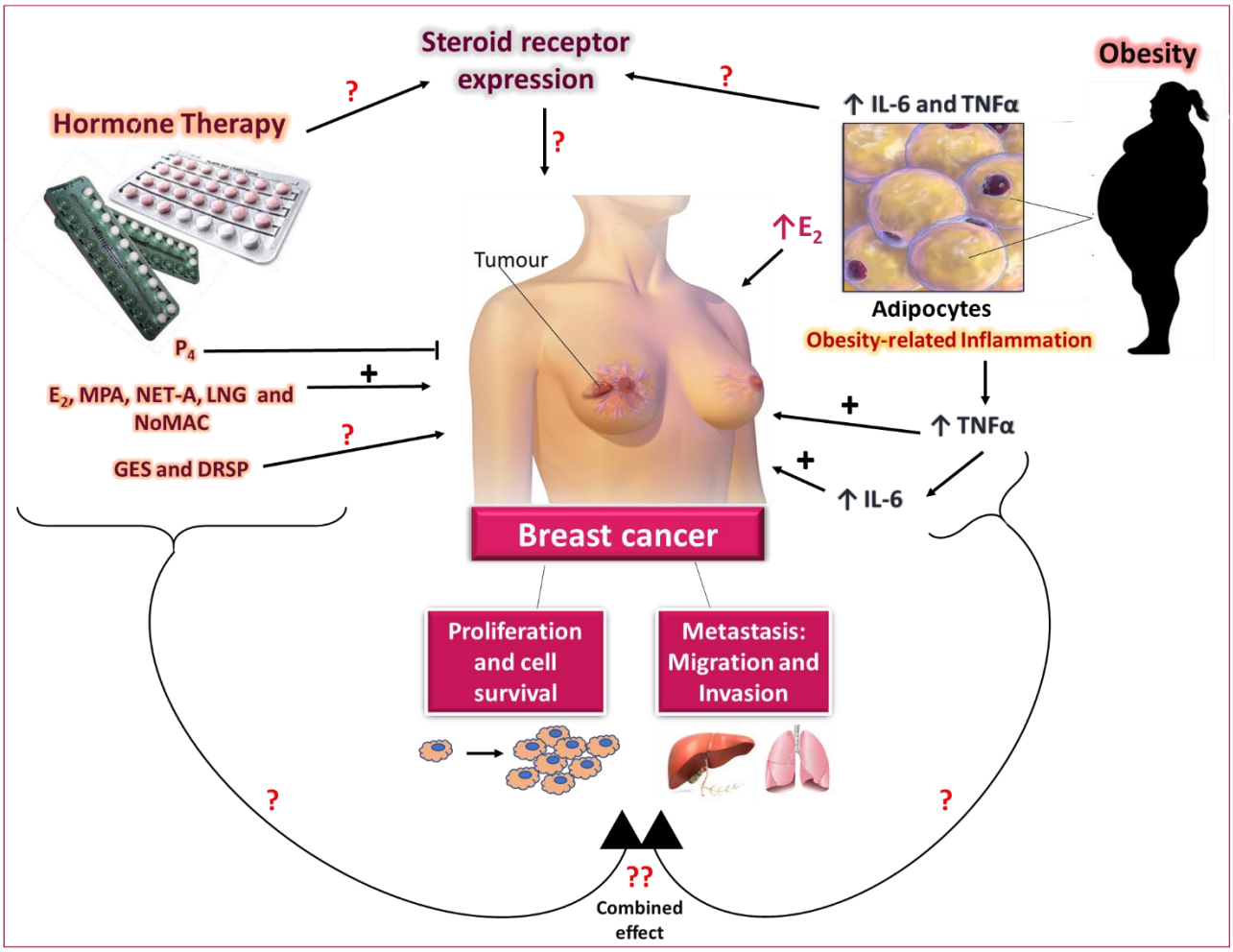


Figure 1.2 Illustration of the known and unknown links between HT, inflammation and breast cancer. (Figure compiled by author (A. Eksteen); images sourced through www.google.com – licenced for non-commercial reuse)

The primary hypothesis of this study was that the selected progestins and natural female hormones would elicit differential effects on steroid receptor expression and hallmarks of breast cancer. Considering that pro-inflammatory mediators such as IL-6 and TNFα are also associated with increased breast cancer risk (Nojek *et al.*, 2006; Sasser *et al.*, 2007; Proietti *et al.*, 2008; Studebaker

et al., 2008), it was hypothesised that these differential effects of the progestins would be exacerbated in the presence of IL-6 and TNFα.

Using various breast cancer cell lines as *in vitro* model systems for breast cancer, such as the human $ER\alpha^+$ MCF-7 BUS and T47D breast cancer cell lines, as well as the human $ER\alpha^-$ MDA-MB-231 cells, the aims of the study were as follows:

1. To directly compare the effects of selected progestins and the natural female hormones, as well as IL-6 and TNFα on steroid receptor expression using western blotting.
2. To directly compare the effects of selected progestins and the natural female hormones, as well as IL-6 and TNFα on the following hallmarks of cancer:
   A. Proliferation, using a combination of cell viability assays and quantitative real-time polymerase chain reaction (qPCR) analyses. The latter is for the evaluation of the mRNA expression of the well-known marker of proliferation, Ki-67.
   B. Apoptosis, using the Caspase-Glo 3/7 apoptosis detection assay kit as well as qPCR analyses for the mRNA expression of the pro- and anti-apoptotic markers, Bax and Bcl-2.
   C. Migration, using the scratch wound-healing assay.
   D. Invasion, using the gelatin zymography and transwell assays.
3. To determine whether IL-6 and TNFα modulate the effects of the progestins and the natural female hormones on steroid receptor expression as well as on breast cancer cell proliferation, apoptosis, migration and invasion.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell lines and mammalian tissue culture

The MCF-7 BUS human breast cancer cell line, a gift from Prof. A. Soto (Tufts University, Massachusetts, USA), was maintained in phenol-red Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa (SA)) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Separations, SA) and 1% penicillin-streptomycin (PenStrep) (50 IU/mL penicillin and 50 µg/mL streptomycin) (Sigma-Aldrich, SA). The T47D human breast cancer cell line, obtained from Prof. I. Parker (University of Cape Town, SA), was maintained in phenol-red DMEM supplemented with 10% FCS and 1% PenStrep. The MDA-MB-231 human breast cancer cell line, a kind gift from Prof. A. Edkins (Rhodes University, SA), were maintained in the same medium as the T47D breast cancer cell line, plus 2 mM L-glutamine (Sigma-Aldrich, SA). The cell lines used in this study were selected based on their cellular properties such as steroid receptor content, their ability to respond to hormones as well as their metastatic potential (see Table 2.1). Cells were regularly tested for mycoplasma infection by means of Hoechst staining (Chen, 1977) and only mycoplasma negative cells (Addendum B, Figure B1) were used for experiments. All cells were maintained in 75 cm^2 tissue culture flasks (Bio-Smart Scientific, SA) at 37°C, with 90% humidity and 5% CO_2.

Table 2.1 Table of cell lines used in this study.

Cell line	Endogenous steroid receptor expression	Other properties
MCF-7 BUS	ERα, ERβ, PR-A, PR-B, AR, GR (Horwitz *et al.*, 1975; Singer *et al.*, 2003)	Estrogen-dependent, proliferative, non-metastatic, poorly invasive (Horwitz *et al.*, 1978; Lee *et al.*, 2015)
T47D	ERα, ERβ, PR-A, PR-B, AR, GR (Horwitz *et al.*, 1978; Singer *et al.*, 2003)	Proliferative, non-metastatic, poorly invasive (Gordon *et al.*, 2003; Ström *et al.*, 2004)
MDA-MB-231	ERβ, GR (Horwitz *et al.*, 1978; Vladusic *et al.*, 2000)	Estrogen-independent, proliferative, metastatic, highly invasive (Cailleau *et al.*, 1974; Kang *et al.*, 2003)

2.2. Test compounds

The natural female hormones, 4-Pregnene-3,20-dione [Progesterone; P_4] and 17β-estra-1,3,5(10)-etriene-3,17 diol [17-β-estradiol; E_2], as well as the progestins, 6α-methyl-17α-hydroxyprogesterone acetate [medroxyprogesterone acetate; MPA], 17α-ethynyl-19-nortesterone 17β-acetate [norethisterone acetate; NET-A], 13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one [levonorgestrel; LNG], 17α-

ethinyl-17β-hydroxy-18-methylestra-4,15-dien-3-one [gestodene; GES], 6β, 7β, 15β, 16β-dimethylene-3-oxo-17α-pregn-4-ene-21,17 carbolactone [drospirenone; DRSP] and 17α-acetoxy-6-methyl-19-norpregna-4,6-dione-3,20-dione [nomegestrol acetate; NOMAC], as well as the synthetic glucocorticoid dexamethasone (Dex) and the selective estrogen receptor modulator, tamoxifen, were purchased from Sigma-Aldrich, SA. The synthetic PR ligand promegestone, R5020, was purchased from PerkinElmer, SA, while the synthetic androgen mibolerone (Mib), was obtained from Steraloids, (United States of America). TNFα and IL-6 were purchased from ThermoFisher, SA. Lyophilised TNFα was reconstituted in sterile, distilled water to a concentration of 0.1 mg/mL, whereas IL-6 was reconstituted in 100 mM acetic acid to a concentration of 1 mg/mL. Further dilutions of TNFα and IL-6 were made using phenol red-free DMEM supplemented with 10% FCS. All test compounds were prepared using absolute ethanol and stored at −20˚C in light-protective vials. For all experiments the compounds were diluted 1000 times in phenol red-free DMEM (Sigma-Aldrich, SA) resulting in a final ethanol (EtOH) concentration of 0.1% (v/v). Thus, 0.1% (v/v) EtOH was used as a solvent control for all experiments.

2.3. Western Blot Analysis

T47D breast cancer cells were seeded into 12-well plates at a density of $2x10^5$ cells per well. The next day, cells were treated for 24 hours with either 0.1% (v/v) EtOH or 100 nM of the hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα in unsupplemented phenol red-free DMEM. Following treatment, the medium was aspirated and the cells lysed with 80 µL 2X Laemmli SDS sample buffer (Addendum A). The cell lysates were then boiled at 95°C for 10 minutes and denatured protein lysates were stored at -20°C until analysis. Protein samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 15 minutes, followed by 200 V for 1 hour in 1X SDS-PAGE running buffer (Addendum A). Following electrophoresis, the separated proteins were transferred to nitrocellulose membranes (AEC-Amersham Biosciences, SA) using 1X transfer buffer (Addendum A). The membranes were subsequently blocked for 90 minutes in 10% (w/v) fat-free milk powder made in Tris-buffered saline (TBS) containing 0.01% (v/v) Tween-20 (TBST) (Addendum A). The membranes were subsequently washed in TBST once for 15 minutes, twice for five minutes, followed by another five-minute wash in TBS at room temperature with agitation. The membranes were then probed with specific primary antibodies (see Table 2.2) at 4°C for 16 hours. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. Following the incubation with the primary antibody, membranes were washed as previously described and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 90 minutes at room temperature with agitation (Table 2.2). The secondary antibody dilutions were prepared in 10% (w/v) fat-free milk powder made in

TBST. The membranes were subsequently washed with TBST as described above and the proteins of interest visualized by means of chemiluminescence using the Clarity™ Western ECL Substrate (Bio-Rad, SA) and the MyECL Imager (Thermo Scientific, SA). Images were analysed using the MyImageAnalysis™ software version 2.0 (Thermo Scientific, SA).

Table 2.2 Specific antibodies and dilutions used for Western blot analyses.

Protein	Primary Antibody	Secondary Antibody
ERα	1: 1000 (ERα HC-20) [1]	1: 1000 (goat anti-rabbit)
ERβ	1: 500 (EPR3777) [2]	1: 1000 (goat anti-rabbit)
PR-A/B	1: 1000 (PR-A/B H-150) [3]	1: 2000 (rabbit anti-mouse)
AR	1: 1000 (AR 441) [1]	1: 3000 (rabbit anti-mouse)
GR	1: 3000 (GR H-300) [1]	1: 4000 (goat anti-rabbit)
GAPDH	1:3000 (GAPDH 0411) [1]	1: 4000 (rabbit anti-mouse)

[1] ERα, AR, GR, GAPDH, goat-anti-rabbit, rabbit anti-mouse: Santa Cruz Biotechnology, Inc, SA
[2] ERβ: Abcam, SA
[3] PR-A/B: Leica Biosystems, SA

2.4. Cell viability assays

MTT (3-(4,5-dimethylthiazolyl)-20-2,5-diphenyltetrazolium bromide) cell viability assays were conducted to evaluate effects on proliferation of breast cancer cell lines as previously described, but with a few modifications (Mosmann, 1983; Verhoog *et al.*, 2007). Briefly, the MCF-7 BUS cells were serum starved in phenol red-free DMEM, supplemented with 5% heat-inactivated charcoal-stripped FCS (Addendum A) and 1% PenStrep for one week, before seeding the cells into 96-well plates at a density of 5 x10^3 cells per well using phenol red-free DMEM supplemented with 5% heat-inactivated charcoal-stripped FCS and 1% PenStrep. The T47D and MDA-MB-231 cell lines were maintained as described in Section 2.1 before seeding at 1x10^4 cells per well. The next day, cells were treated for 72 hours with 0.1% (v/v) EtOH (vehicle control) or 100 nM of the hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα in supplemented phenol red-free DMEM at 37˚C in 90% humidity and 5% CO_2. Following the incubation, the medium was aspirated and the cells were re-treated with new/fresh test compounds for a further 48 hours. Four hours prior to the end of the total 120-hour period, 50 µL of the colorimetric MTT (Sigma-Aldrich, SA) solution (Addendum A) and 150 µL phenol red-free DMEM was added to each well. The yellow MTT thiazole solution was reduced by metabolically active cells to form purple, insoluble formazan crystals. The solution was carefully aspirated and the formazan crystals solubilised in 200 µL dimethyl sulphoxide (DMSO).

The absorbance was measured at 550 nm in the BioTek® Power Wave 340 microplate spectrophotometer (Analytical and Diagnostic Products, SA). Results were expressed as fold induction relative to 0.1% (v/v) EtOH (vehicle) set as one.

2.5. Apoptosis Assay

To measure apoptosis the Caspase-Glo 3/7 apoptosis detection assay kit was used (Promega, Anatech, SA). It measures the activities of the caspase-3 and caspase-7 enzymes, which are known to drive apoptosis. This specific assay relies on caspase-3 and caspase-7 cleaving the caspase-3/7 pro-substrate containing a DEVD tetrapeptide sequence, which is the recognition site for these enzymes. After cleaving of the substrate, aminoluciferin is released which reacts with luciferase and produces measurable light. The amount of luciferase measured is proportional to the amount of caspase activity.

The MCF-7 and T47D breast cancer cell lines were maintained as described in Section 2.1. The cells were seeded into High Binding Isoplate–96 HB, white framed 96-well plates (PerkinElmer, Massachusetts, USA) at a density of 1 x 10^3 cells per well and left to settle. The following day, the cells were treated with 0.1% (v/v) EtOH or 100 nM of the hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα in unsupplemented phenol red-free DMEM for 6 or 24 hours for the MCF-7 BUS and T47D breast cancer cells, respectively. After the incubation period has lapsed, 100 µL of the Caspase-Glo 3/7 substrate was added to the cells without aspirating the medium. The plates were then covered with plate sealers and lids, before the contents of the wells were gently mixed on a shaker at 30 rpm for 30 seconds. The plates were incubated at room temperature for a further 2 hours before the luminescence of each sample was read using a luminometer (Veritas, Whitehead Scientific, SA). The following controls were included in each experiment: cell culture medium without cells (blank) and cell culture medium with vehicle (0.1% (v/v) EtOH) treated cells (vehicle control). The data presented is the fold caspase 3/7 activity, where the blank was subtracted from each sample value, and the relative light units (RLU) of each sample was set relative to the vehicle treated cells set as one.

2.6. Migration (Scratch wound-healing) assay

The T47D breast cancer cells were maintained as described in Section 2.1 and seeded into 24-well plates at a density of 2.5 x 10^5 cells per well in supplemented phenol red-free DMEM. Cells were grown to confluence and treated with 5 µg/mL Mitomycin C (Sigma-Aldrich, SA), an inhibitor of proliferation, for 2 hours (Tomasz, 1995). The medium was subsequently aspirated and a thin "scratch/wound" was made on the cell monolayer using a 200 µL pipette tip. Cell debris was removed by washing the cells with pre-warmed 1X phosphate buffered saline (PBS), prior to treatment with

0.1% (v/v) EtOH or the test compounds for 72 hours. Using an inverted microscope (Olympus IX81 inverted fluorescent microscope at 4X magnification), an image indicating the initial wound was captured directly after treatment (zero hours), followed by capturing of images every 24 hours over a period of 72 hours. Image J software was used to quantify the wound area and the percentage migration of the cells relative to the initial wound area was calculated.

2.7. Invasion assays

2.7.1. Gelatin zymography assay

T47D breast cancer cells were maintained as in Section 2.1 and seeded into 6-well plates at a density of $2.5x10^5$ cells per well in phenol-red free DMEM supplemented with 10% CS-FCS and 1% PenStrep. The following day, the cells were incubated with the test compounds in unsupplemented phenol red-free DMEM for 24 hours. After the incubation period, the supernatants containing secreted proteins, were collected in 2 mL microcentrifuge tubes and kept on ice. To remove any cell debris, the microcentrifuge tubes containing the supernatant were centrifuged at 400 x g for 5 minutes at 4°C. After centrifugation, the supernatant was removed and placed into clean microcentrifuge tubes. The samples were diluted with 4X sample buffer (Addendum A) and 15 µL was loaded in the same order onto two 10% acrylamide gels containing 1% gelatin. Gel electrophoresis was performed at 90 V for approximately two hours using 1x SDS-PAGE running buffer (Addendum A). One gel (the total protein loading control) was stained with Coomassie blue stain solution (Addendum A) for 30 minutes on a shaker at room temperature and rinsed with a destain solution (Addendum A) until clear blue bands were visible. To assess gelatin degradation, 100 mL of renaturing buffer (Addendum A) was added to the second gel and incubated for 30 minutes at room temperature with agitation. The buffer was removed and the step repeated. Thereafter, the gel was incubated with 100 mL developing buffer (Addendum A) in a sealed container overnight at 37°C. The following day, the gel was stained and destained as above. Photographs of the gels were captured with the Bio-Rad Molecular Imager Gel Doc XR+ with Image Lab Software system. The intensity of the bands on both gels were quantified according to Hu & Beeton (Hu and Beeton, 2010). The value of each sample was then divided by the total protein of that specific sample and set relative to the vehicle control.

2.7.2. Transwell assay

For the cell invasion assay, 24-well polycarbonate transwell inserts containing 8.0 µm pore filter membranes (Corning, Inc., Corning, NY) were placed into the wells of a 24-well tissue culture plate (Wang and Rao, 2014). The filter membrane was coated with 100 µL of 100 µg/mL Corning matrigel matrix (BD Biosciences). The matrigel was allowed to dry for 6 hours at 37°C, with 90% humidity

and 5% CO_2. The MDA-MB-231 cell line was maintained as in Section 2.1 and $2x10^5$ cells in unsupplemented phenol red-free DMEM gently added into the upper chamber (insert) of each well. The plates were incubated for 30 minutes at 37°C and 5% CO_2 to allow the cells to settle. Thereafter, the cells were treated with either 0.1% (v/v) EtOH (control) or 100 nM MPA or DRSP in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα. Phenol red-free DMEM supplemented with 10% charcoal-stripped FCS (chemo-attractant) was added to the bottom chamber of each well and the plates incubated for 24 hours. After the 24-hour incubation, the transwell insert was removed and excess cells that did not migrate removed by carefully swabbing the inside of the insert with a cotton swab. These inserts were then washed twice with 1X PBS before fixing the cells by placing the inserts into 1 mL 70% (v/v) EtOH for 10 minutes. The inserts were again washed twice with 1X PBS and left to air dry. The cells on the transwell inserts were subsequently stained with 1 mL 0.2% crystal violet for 20 minutes at room temperature. Excess crystal violet was carefully removed by washing the transwell insert with 1X PBS and allowing it to dry. A widefield confocal microscope (Carl Zeiss LSM780 with ELYRA PS1 Super-resolution platform) was used to capture images of the cells on the membrane. For quantification of migration, the cells on the membrane were dissolved in 33% glacial acetic acid and the absorbance measured at 590 nm.

2.8. Isolation of total RNA

T47D and MCF-7 BUS breast cancer cell lines were maintained as described in Section 2.1 and seeded into 12-well plates at a density of $1x10^5$ cells per well in supplemented phenol-red free DMEM and allowed to settle for 24 hours. The cells were then treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM E_2 or MPA in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα in unsupplemented phenol red-free DMEM for 24 hours. Following a single PBS wash, total RNA was isolated from the cells using Tri-Reagent (Sigma-Aldrich, SA) according to the manufacturer's instructions. Briefly, cells were lysed by adding 400 µL Tri-Reagent. To facilitate lysis, the cells were placed at -80°C and then thawed. The thawed cell lysates were then transferred into 1.5 mL microcentrifuge tubes and incubated at room temperature for 5 minutes. Total RNA was extracted by adding 80 µL chloroform, vortexing for 15 seconds and incubating the samples at room temperature for 2-3 minutes. The samples were then centrifuged at 14000 xg for 15 minutes at 4°C. The aqueous phase containing the RNA was transferred into a clean microcentrifuge tube. An equal volume of ice-cold isopropanol was added to the microcentrifuge tube containing the aqueous phase and this was vortexed for 15 seconds. The samples were then incubated for 15 minutes at room temperature, followed by centrifugation at 14000 xg for 15 minutes at 4°C to pellet the RNA. The pellets were washed with 500 µL 75% (v/v) EtOH (diluted using diethyl pyrocarbonate (DEPC) water) and vortexed for one minute before centrifugation at 8 000 xg for 5 minutes at 4°C. The supernatant was

removed and the RNA pellets air-dried for 10 minutes on ice. RNA pellets were subsequently dissolved in DEPC-treated water and the concentration and purity assessed by measuring the A_{260}/A_{280} ratio using a NanoDrop (ND-100 Spectrophotometer). The integrity of the RNA was confirmed by assessing the presence of intact 28S and 18S ribosomal RNA subunits on a 1% w/v denaturing agarose gel (Aranda *et al.*, 2012) (Addendum B, Figure B2). RNA samples were stored at -80°C.

2.9. cDNA synthesis

Total RNA was reverse-transcribed using the ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. Briefly, for each RNA sample 0.5 µg RNA and 33.3 µM oligo(dT) primer was added into a thin-walled PCR tube. Nuclease-free water was subsequently added to a final volume of 2.5 µL and the mixture was incubated for 5 minutes at 70°C to denature the RNA. Thereafter, the samples were placed on ice for 5 minutes. The following master mix of the rest of the components needed for the reverse transcription reaction was prepared per RNA sample: 3.5 µL nuclease free water, 2 µL ImProm-II 5X reaction buffer, 1 mM $MgCl_2$, 0.5 nM dNTPs, 10 U recombinant RNasin ribonuclease inhibitor and 80 U ImProm-II reverse transcriptase. A volume of 7.5 µl of the master mix was added to each sample. Samples were incubated for 5 minutes at 25˚C to allow annealing of the oligo(dT) primers. This was followed by incubation for 1 hour at 42˚C for extension. Samples were then placed at 70˚C for 15 minutes to inactivate the reverse transcriptase enzyme and the cDNA samples stored at -20˚C.

2.10. Quantitative real-time polymerase chain reaction (qPCR)

Realtime qPCR was performed using the LightCycler 96 system (Roche Applied Science, SA) according to the manufacturer's instructions. The reaction master mix was prepared as follows per sample: 0.5 µM forward and reverse primers, 5 µL SYBR Green (Kapa SYBR Fast qPCR master mix; Roche Applied science, SA) and 2 µL sterile PCR-grade water. For the reaction in the LightCycler Multiwell plates (Roche Diagnostics (Pty) Ltd, SA), a volume of 9 µl of the master mix was added to each well, and 1 µl of either cDNA or the sterile PCR-grade water (non-template negative control). The details of the forward and reverse primers used in this study are shown in Table 2.3. A melting curve analyses was performed to confirm the presence of a single amplicon (Addendum B, Figure B3), while the amplicon size was confirmed by agarose gel electrophoresis (Addendum B, Figure B4). GAPDH was used as the internal control, and the relative transcript levels of the target genes was normalised to the relative transcript levels of this gene. Target genes were quantified using the mathematical equation described by Pfaffl (Pfaffl, 2001).

Table 2.3 Details of primers used for quantitative analysis of gene expression.

Gene	Primers (5' - 3')	Strand	Length of amplicon (bp)	Annealing temp (°C)	Primer Efficiency	Reference
Ki67	CGGACTTTGGGTGCGACTT	Forward	203	57	1.83	(Chottanapund *et al.*, 2013)
	GTCGACCCCGCTCCTTTT	Reverse				
Bax	GAGAGGTCTTTTTCCGAGTG	Forward	234	56	1.88	(Ding *et al.*, 2014)
	GGTGAGGAGGCTTGAGGAGT	Reverse				
Bcl-2	GCTACCTAAGAAAAACCTGG	Forward	132	56	1.91	(Ding *et al.*, 2014)
	CAAGAAACAAGGTCAAAGGG	Reverse				
GAPDH	TGAACGGGAAGCTCACTGG	Forward	307	60	2	(Ishibashi *et al.*, 2003)
	TCCACCACCCTGTTGCTGTA	Reverse				

2.11. Data and Statistical Analysis

Visual representation and statistical analysis were conducted using the GraphPad Prism® v 5.00 software package, for Windows (GraphPad Software, USA). For Western Blot analysis, one-way ANOVA with Bonferroni's (compare all pairs of columns) post-test was used. One-way ANOVA with Newman-Keuls (compare all pairs of columns) post-test was used for MTT assays and qPCR experiments. For apoptosis and zymography assays, one-way ANOVA with either Dunnett's (compare all columns vs control column) or Bonferroni's (compare all pairs of columns) post-test were used for statistical analyses. For all assays, data with two or more data sets were analysed using two-way ANOVA with Bonferroni's post-test. Statistical significance were indicated either by letters, with values statistically different being assigned different letters, or by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, while no statistical significance (ns) indicates $p>0.05$. The error bars indicate the standard error of the mean (SEM) of three independent experiments, except for the transwell invasion assay where only a single experiment was performed.

CHAPTER 3

RESULTS

3.1. All progestogens downregulated the expression of both PR-isoforms in T47D breast cancer cells, while the effects were not influenced by IL-6 or TNFα.

Steroid hormones mediate their physiological effects by binding to steroid receptors, most of which have been implicated in breast cancer pathogenesis (Birrell *et al.*, 1998; Conzen, 2008; Peters *et al.*, 2009; Thomas and Gustafsson, 2015; Wellberg *et al.*, 2017; McFall *et al.*, 2018). It is well known that the cognate hormone of a steroid receptor can regulate its expression (Kathryn B. Horwitz and Mcguire, 1978; Dong *et al.*, 1988; Y. Zhang *et al.*, 2009; Chang *et al.*, 2013). This study investigated whether the natural female hormones, E_2 and P_4, as well as selected progestins (MPA, NET-A, LNG, GES, DRSP and NoMAC) could also influence the expression levels of steroid receptors. Moreover, the effects of these hormones on steroid receptor expression, in the absence and presence of either IL-6 or TNFα, were also investigated. This investigation was done in the T47D breast cancer cell line as it endogenously expresses high levels of the PR isoforms, the ER subtypes, AR and GR (Nardulli and katzenelenbogen, 1988; Satrorius *et al.*, 1994; Jacobsen and Horwitz, 2012). Western blot analyses were performed with lysates of the T47D breast cancer cell line treated with either 0.1% (v/v) EtOH or 100 nM of the hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα. These specific concentrations of IL-6 and TNFα were previously used in studies investigating effects of these cytokines on breast cancer cell proliferation (Chiu *et al.*, 1996; Rubio *et al.*, 2006; Sasser *et al.*, 2007).

As the test compounds in this study are primarily PR ligands, we firstly investigated the effects of the test compounds on the expression levels of the PR isoforms. A representative western blot for both the PR isoforms is shown in Figure 3.1A. The quantification of the blot shows that promegestone (R5020), a potent synthetic agonist for the PR, downregulated the expression of both PR-A and PR-B (Figure 3.1B and D), while E_2 caused an increase in the expression of both isoforms. Both these results are consistent with previous findings in MCF-7 and T47D breast cancer cell lines (Read *et al.*, 1988; Lauber *et al.*, 2004; Tung *et al.*, 2006; Chen *et al.*, 2013). Interestingly, our results in the T47D breast cancer cell line show that all the progestogens decreased the expression of both the PR-isoforms to the same extent, and neither IL-6 nor TNFα could modulate these effects (Figure 3.1C and E).

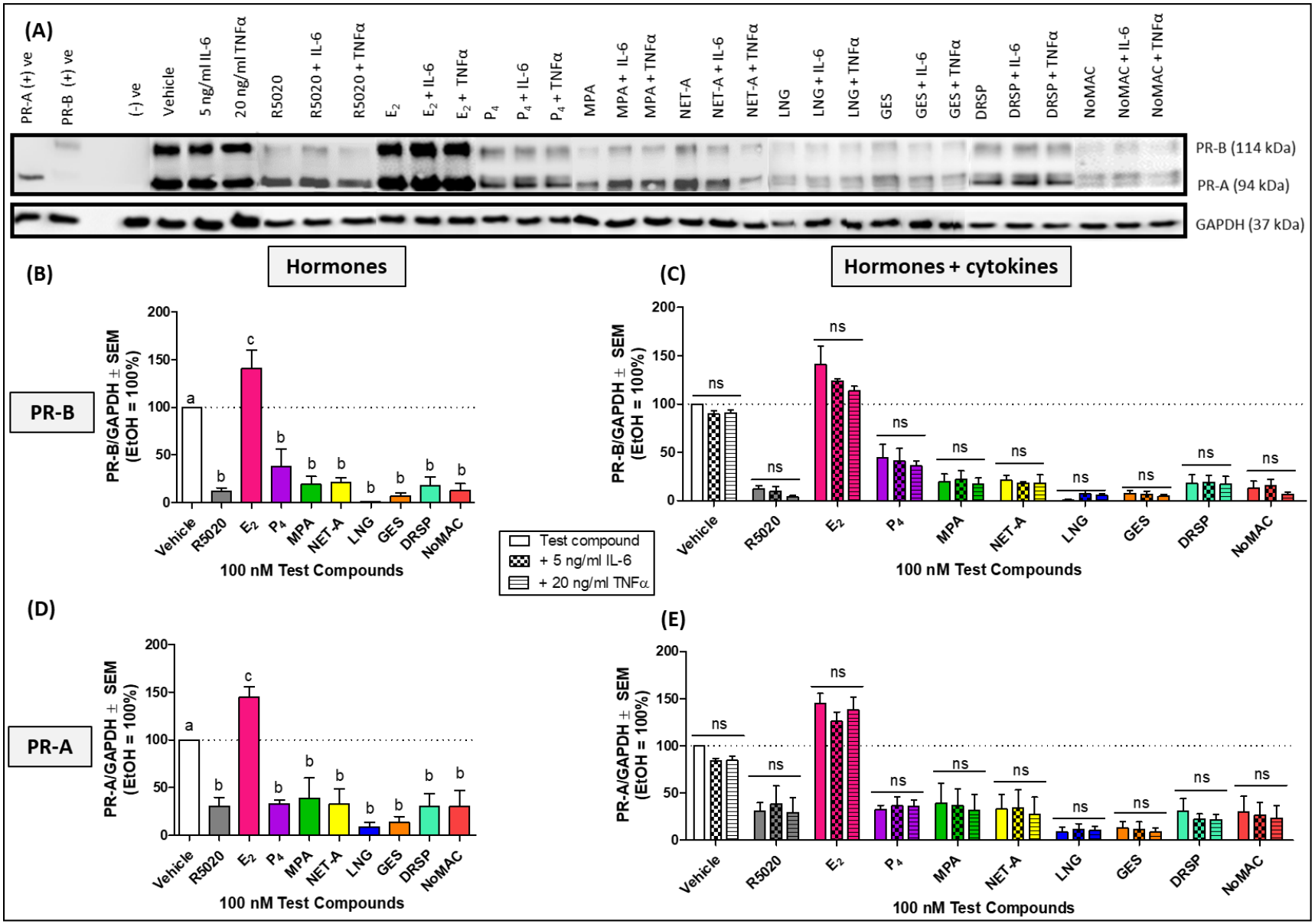


Figure 3.1 legend on next page.

Figure 3.1 All the progestogens downregulated the expression of PR-A and PR-B in T47D breast cancer cells, and the effects were not influenced by IL-6 or TNFα. Total protein from the human T47D breast cancer cell line treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones, in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 hours, was harvested for western blotting. Steroid receptor expression was detected using antibodies specific for the progesterone receptor-A/B (PR-A/B) and GAPDH, and quantified using MYImage Analysis. (A) The western blot shown is representative of 3 independent experiments for PR-A and PR-B. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test were used to compare the effects of the hormones on the expression of (B) PR-B and (D) PR-A. Two-way ANOVA with Bonferroni's post-test was used to compare the effects of hormones on the expression of (C) PR-B and (E) PR-A, in the absence and presence of IL-6 or TNFα. For the one-way ANOVA analysis, statistically significant differences are indicated by letters, with values statistically different being assigned different letters, while for two-way ANOVA statistically significant differences are indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

3.2. Like E_2, all the progestins downregulated the expression of both ER-subtypes, and the effects were not modulated by IL-6 or TNFα.

It is well-known that some progestins can interact with other members of the steroid receptor family such as the AR, GR and ERα (Koubovec *et al.*, 2005; Africander *et al.*, 2014; Louw-du Toit *et al.*, 2017). Moreover, it has been shown that ERα is required for MPA-induced gene expression via the PR (Giulianelli *et al.*, 2012), while progestin (R5020)-bound PR can modulate the transcriptional activity of ERα (Mohammed *et al.*, 2015).

Western blot analyses for ERα as well as the ERβ subtype were thus next performed using lysates of the T47D breast cancer cell line treated as in Section 3.1. As shown in Figure 3.2B, all hormones, except P_4, decreased the expression of ERα, with all progestins decreasing ERα expression to a similar extent. Although all hormones decreased the expression of ERβ, P_4, DRSP and NoMAC decreased expression to a lesser extent (Figure 3.2D). Consistent with the literature, E_2, the cognate ligand for ERα and ERβ, downregulated the expression of both isoforms (Y. Zhang *et al.*, 2009; Yeh *et al.*, 2013). Neither IL-6 nor TNFα modulated the above-mentioned effects on ER protein expression levels (Figure 3.2C and E).

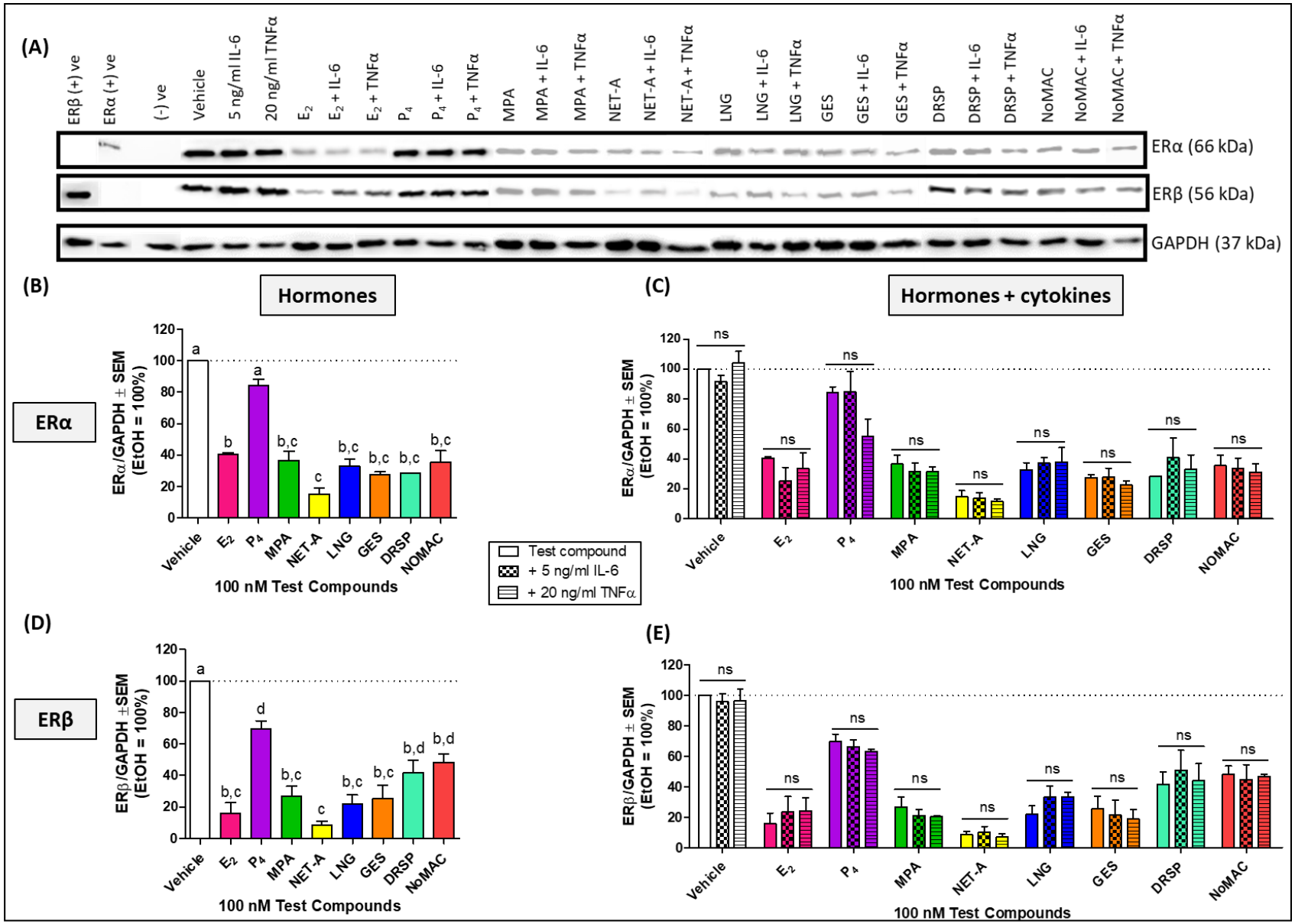


Figure 3.2 legend on next page.

Figure 3.2 All hormones, except P_4, downregulated the expression of both ERα and ERβ in T47D cells, and the effects were not influenced by IL-6 or TNFα. Total protein from the human T47D breast cancer cell line treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones, in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 hours, was harvested for western blotting. Steroid receptor expression was detected using antibodies specific for ERα, ERβ and GAPDH, and quantified using MYImage Analysis. (A) The western blot shown is representative of 3 independent experiments for ERα and ERβ. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test were used to compare the effects of the hormones on the expression of (B) ERα and (D) ERβ. Two-way ANOVA with Bonferroni's post–test was used to compare the effects of hormones on the expression of (C) ERα and (E) ERβ, in the absence and presence of IL-6 or TNFα. For the one-way ANOVA analysis, statistically significant differences are indicated by letters, with values statistically different being assigned different letters, while for two-way ANOVA statistically significant differences are indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

3.3. All progestogens and E_2 downregulated the expression of the AR, but not the GR, and the effects were not modulated by IL-6 or TNFα.

Next we investigated the effects of the progestins on the expression levels of the AR and GR as these receptors are also implicated in breast cancer pathogenesis and it is known that a number of progestins bind to these receptors (Koubovec *et al.*, 2005; Africander *et al.*, 2014; Louw-du Toit *et al.*, 2017). Figure 3.3B shows that the synthetic androgen mibolerone (Mib) downregulates the expression of the AR, while all the progestogens decreased AR expression to the same extent as each other (Figure 3.3B). E_2 also decreased the expression of the AR, albeit to a lesser extent. As expected, the synthetic glucocorticoid, dexamethasone (Dex), decreased the expression of the GR (Figure 3.3D) (Dong *et al.*, 1988; Burnstein& *et al.*, 1990; Burnstein *et al.*, 1994; Webster *et al.*, 2001; Xue *et al.*, 2014). None of the progestogens or E_2 had a significant effect on GR expression levels in the T47D breast cancer cells compared to the vehicle (Figure 3.3D). Although IL-6 had no effect on the expression of either the AR (Figure 3.3C) or the GR (Figure 3.3E) in T47D breast cancer cells, GR protein expression was increased in response to TNFα (Figure 3.3E). The increased GR expression in response to TNFα, correlates with a study reporting that TNFα upregulated the expression of the GR in human cervical adenocarcinoma (HeLaS3) and human lymphoid (CEMC7) cells (Webster *et al.*, 2001). Importantly, none of the effects of the hormones on steroid receptor expression were modulated by the addition of the pro-inflammatory mediators.

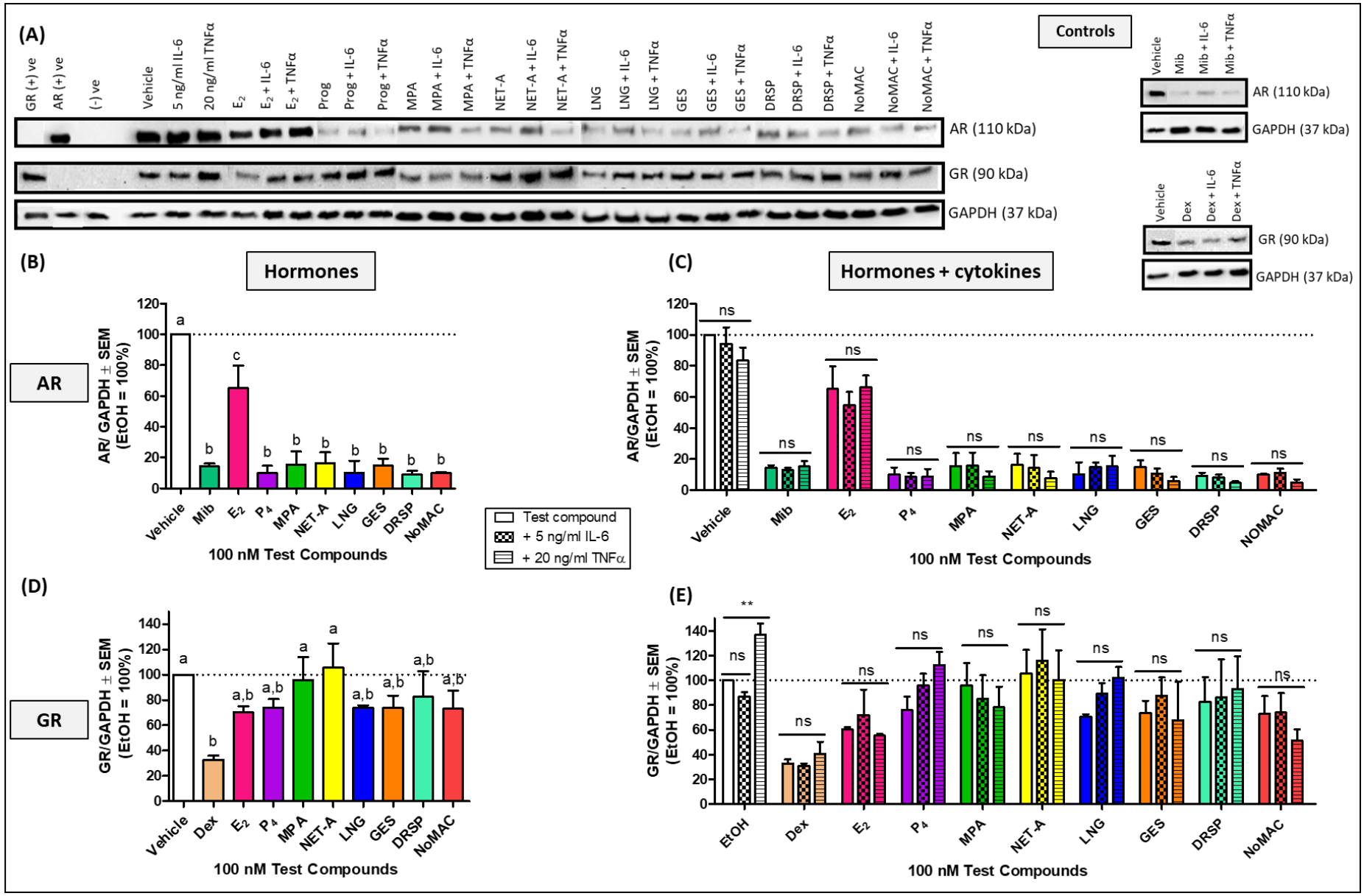


Figure 3.3 legend on next page.

Figure 3.3 All hormones evaluated downregulated the expression of the AR, but not the GR in the T47D and the effects were not influenced by IL-6 or TNFα. However, TNFα on its own increased the expression of the GR. Total protein from the human T47D breast cancer cell line treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones, in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 hours, was harvested for western blotting. Steroid receptor expression was detected using antibodies specific for the androgen receptor (AR), glucocorticoid receptor (GR) and GAPDH, and quantified using MYImage Analysis. (A) The western blot shown is representative of 3 independent experiments for AR and GR protein expression. One-way ANOVA with Bonferroni's (compares all pairs of columns) post-test were used to compare the effects of the hormones on the expression of (B) AR and (D) GR. Two-way ANOVA with Bonferroni's post–test was used to compare the effects of hormones on the expression of (C) AR and (E) GR, in the absence and presence of IL-6 or TNFα. For the one-way ANOVA analysis, statistically significant differences are indicated by letters, with values statistically different being assigned different letters, while for two-way ANOVA statistically significant differences are indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

3.4. Progestogens display differential effects on proliferation in both the T47D and MCF-7 BUS breast cancer cell lines.

The evidence in the literature regarding the effects of the progestins on proliferation of breast cancer cells are contradictory, with effects appearing to be cell line-specific. For example, at least two studies show that MPA increases the proliferation of T47D breast cancer cells (Liang *et al.*, 2006; Izzo *et al.*, 2014), while another study has shown that MPA has no effect on the proliferation of MCF-7 breast cancer cells (Catherino *et al.*, 1993). NET-A, GES and LNG have been shown to increase the proliferation of MCF-7 breast cancer cells (Van den Burg *et al.*, 1992; Schoonen *et al.*, 1995; Franke and Vermes, 2003), but NET-A have been shown to inhibit proliferation of the T47D breast cancer cell line (Botella *et al.*, 1994) suggesting cell-line specific effects. Thus, this study directly compared the effects of different progestins on the proliferation of both the T47D and MCF-7 BUS breast cancer cell lines. T47D cells were seeded at $1x10^4$ cells per well while the MCF-7 BUS cells were serum-starved for one week before seeding at $5x10^3$ cells per well. These cell numbers were previously optimised in the Africander laboratory. Both cell lines were treated for 72 hours with either 0.1% (v/v) EtOH (vehicle control), 100 nM E_2 or progestogen. The medium was subsequently aspirated, and the cells retreated with the same hormones for a further 48 hours. Results in Figure 3.4A show that E_2 increased the proliferation of the T47D breast cancer cell line, while all progestogens, except DRSP and NoMAC, increased proliferation of the T47D breast cancer cell line to a similar extent. Interestingly, P_4, MPA, NET-A and GES differentially increased the proliferation of the MCF-7 BUS cell line, while LNG, DRSP and NoMAC had no effect on proliferation (Figure 3.4B). To confirm whether the effects of the progestins are indeed cell-specific, the data from Figure 3.4A and B were replotted and are shown in Figure 3.4C. The analysis shows that while E_2, P_4, MPA, NET-A and GES increase proliferation of both cell lines, the increase is significantly higher in the MCF-7 BUS cell line. Interestingly, neither

DRSP or NoMAC influenced cell proliferation in either cell line. Surprisingly, LNG increased proliferation of only the T47D cells.

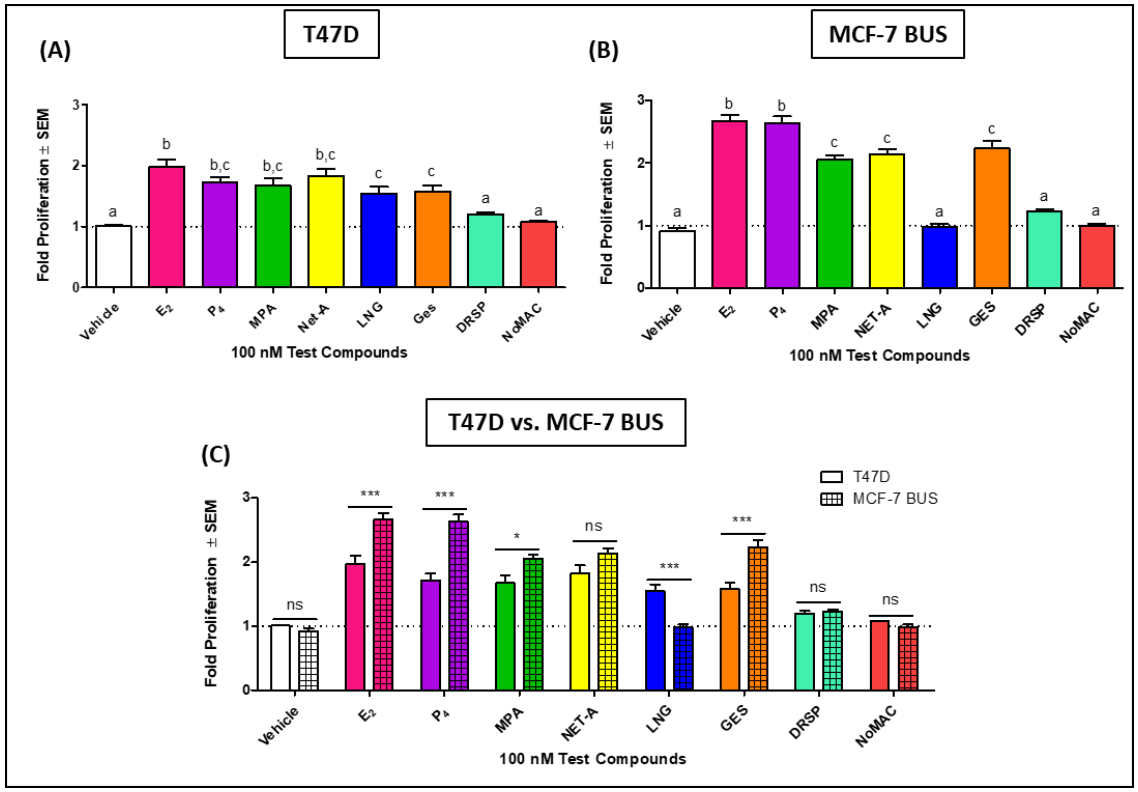


Figure 3.4 E_2 and the progestogens differentially regulate proliferation of the T47D and MCF-7 BUS breast cancer cell lines. (A) T47D and (B) MCF-7 BUS breast cancer cells were treated with either 0,1% (v/v) EtOH (vehicle) or 100 nM hormones for 72 hours. Thereafter, the cells were retreated with the same test compounds for 48 hours and cell proliferation quantified using the MTT cell viability assay. Proliferation is shown as fold proliferation with 0.1% EtOH (vehicle) set as 1 and all other test compounds set relative to this. The result shown represents the average of three independent experiments performed in triplicate. One-way ANOVA with Newman-Keuls (compares all pairs of columns) post-test were used for statistical analysis. (C) Data of (A) and (B) were replotted as grouped data, and statistical analysis performed using two-way ANOVA and Bonferroni's post-test. Statistical significance is indicated by either letters, where values that differ significantly are assigned different letters, or using *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

3.5. IL-6 had no effect on progestogen-induced proliferation of the T47D or MCF-7 BUS breast cancer cell lines, while proliferation of the MCF-7 BUS breast cancer cells was significantly decreased by TNFα.

Having shown that the progestins differentially regulate breast cancer cell proliferation, with some in a cell line-dependent manner, we next investigated whether these responses would be influenced by the inflammatory mediators, IL-6 and TNFα. The T47D and MCF-7 BUS breast cancer cell lines were thus treated with either 0.1% (v/v) EtOH or 100 nM of the hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα.

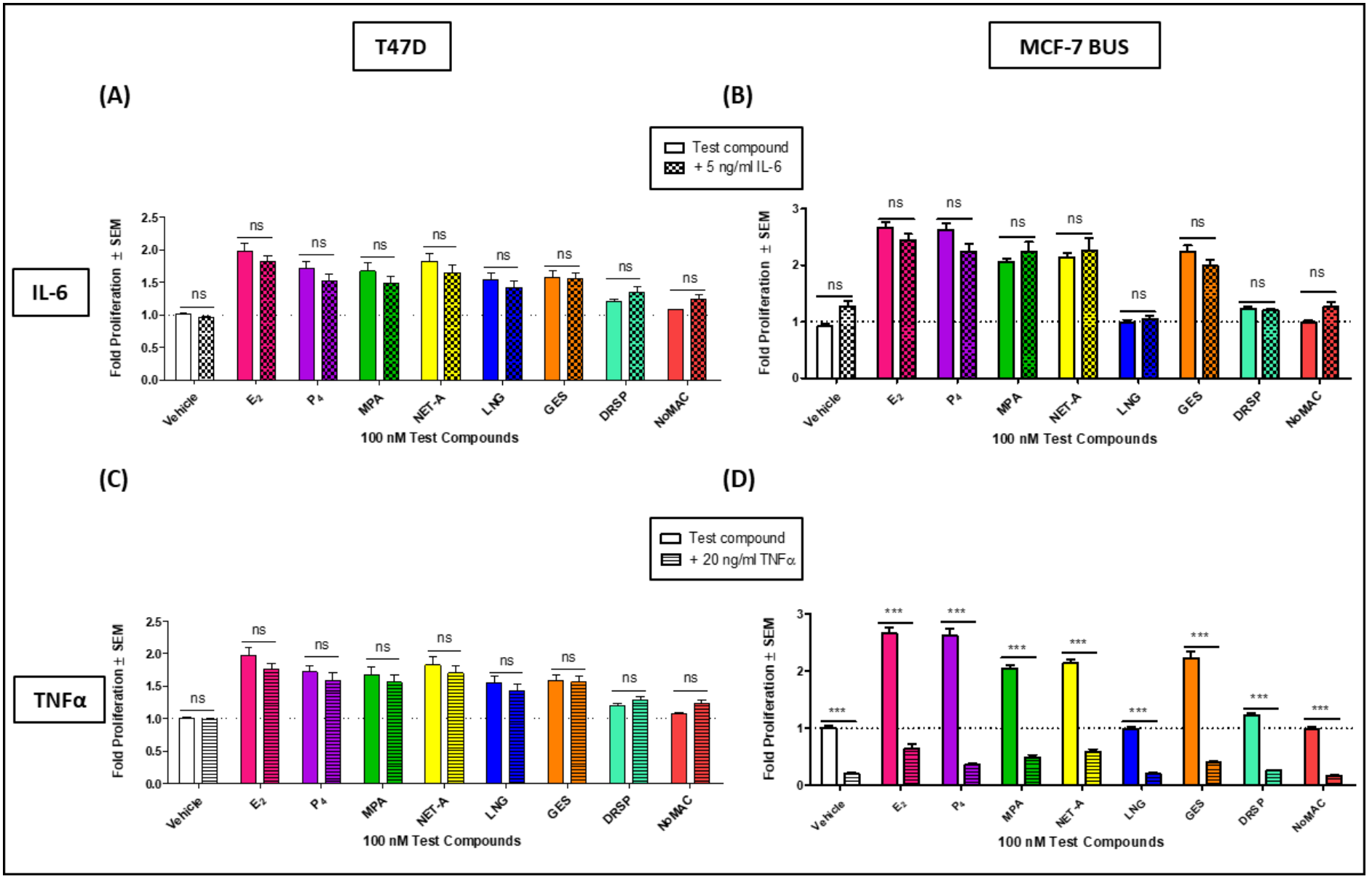


Figure 3.5 legend on next page.

Figure 3.5 IL-6 did not modulate hormone-induced proliferation of the MCF-7 BUS or T47D breast cancer cell lines, while TNFα decreased proliferation in only the MCF-7 BUS cells. (A and C) T47D and (B and D) MCF-7 BUS breast cancer cells were treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα for 72 hours. Thereafter, the cells were retreated with the same test compounds for 48 hours. Proliferation is shown as fold proliferation with 0.1% EtOH set as 1 and all other test compounds set relative to this. The result shown is the average of three independent experiments performed in triplicate. Two-way ANOVA with Bonferroni's post–test was used for statistical analyses. Statistical significance is indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

Results showed that IL-6 did not modulate the progestogen-induced proliferation in either the T47D (Figure 3.5A) or MCF-7 BUS (Figure 3.5B) breast cancer cell lines. Although 20 ng/ml TNFα also had no effect on progestogen-induced proliferation of the T47D breast cancer cells (Figure 3.5C), it displayed anti-proliferative effects in the MCF-7 BUS breast cancer cells (Figure 3.5D). Consistent with these results, the real-time qPCR results in Figure 3.6 shows that both IL-6 and TNFα had no significant effect on the mRNA expression of Ki-67, a well-studied marker of proliferation (Klinkhammer-schalke, 2013; Dai *et al.*, 2016; Mannell, 2016), in the T47D cells (Figure 3.6A), while its expression in the MCF-7 BUS cells was significantly decreased by TNFα (Figure 3.6B). Also in line with the proliferation assay (Figure 3.4), E_2 and MPA upregulated Ki-67 expression in both cell lines (Tan *et al.*, 2009; Wood *et al.*, 2013; Holton *et al.*, 2014).

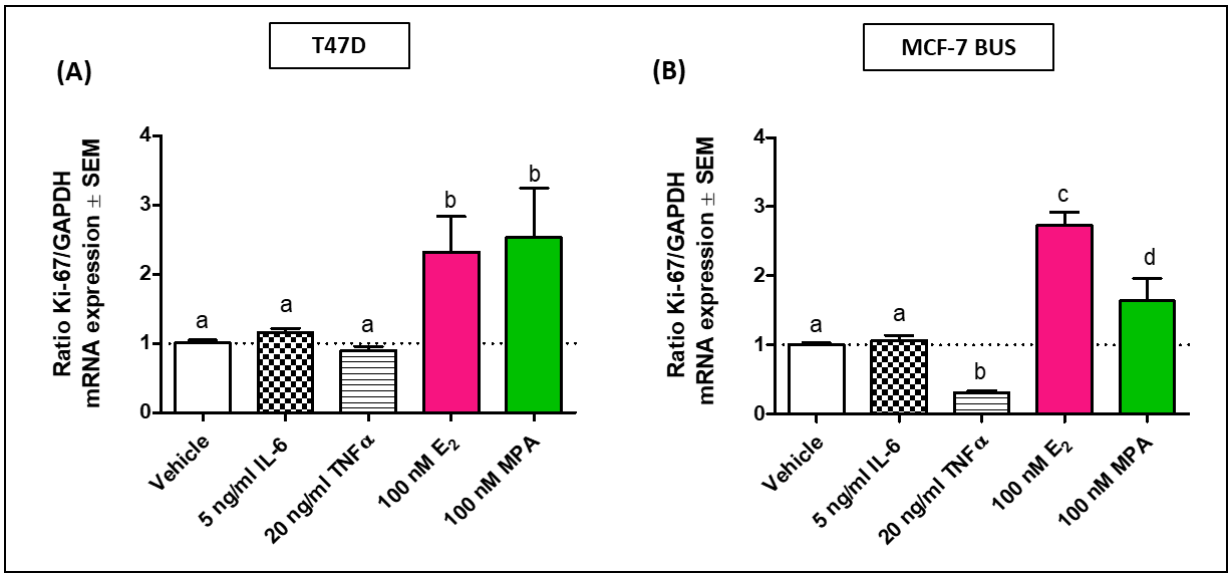


Figure 3.6 TNFα decreased the mRNA expression of Ki-67 in the MCF-7 BUS, but not the T47D, cell line. (A) T47D and (B) MCF-7 BUS breast cancer cells were treated with either 0.1% (v/v) EtOH, 5 ng/ml IL-6, 20 ng/ml TNFα, 100 nM E_2 or MPA for 24 hours. Total RNA was isolated, cDNA synthesised and the mRNA expression levels of Ki-67 and GAPDH determined by qPCR. GAPDH was used as the internal standard and the ratio of Ki-67 mRNA/GAPDH mRNA of the treated samples were calculated relative to that of the vehicle. Results represent the average of three independent experiments. One-way ANOVA with Newman-Keuls (compare all pairs of columns) post-test were used for statistical analysis. Statistical significance is indicated by letters, where values that differ significantly are assigned different letters.

3.6. IL-6 and the hormones had no effect on apoptosis in either the T47D or MCF-7 BUS cell line. TNFα on the other hand, induced apoptosis in the MCF-7 BUS cell line, but not in the T47D cell line.

It has been shown that TNFα promotes apoptosis in MCF-7 cells (Simstein *et al.*, 2003), but not in T47D cells (Nojek *et al.*, 2006). Having shown that TNFα decreases the viability of the MCF-7 BUS cells, but not the T47D cells (Figure 3.5), we hypothesised that this decrease was due to TNFα inducing apoptosis of the MCF-7 BUS cells. We thus treated the T47D and MCF-7 BUS breast cancer cell lines with either 5 ng/ml IL-6 or 20 ng/ml TNFα, and measured apoptosis using the Caspase-Glo 3/7 apoptosis detection assay kit (Promega, Anatech, SA). First, the optimal cell density was determined to be $1x10^3$ cells per well for both cell lines (Addendum B, Figure B5). The treatment time for these experiments were based on the fact that Mooney *et al.* showed that the mechanism of apoptosis differs between the T47D and MCF-7 cell lines, with the MCF-7 cell line being an early apoptotic cell line, as compared to the T47D cell line (Mooney *et al.*, 2002). Thus, we treated the T47D breast cancer cells with test compound for a much longer time period (24 hours) than the MCF-7 BUS cells (6 hours). The results in Figure 3.7B confirms that TNFα induced apoptosis in the MCF-7 BUS cells, but not in the T47D cells (Figure 3.7A). IL-6 on the other hand, had no effect on apoptosis in either cell line (Figure 3.7). The lack of apoptosis induction was not due to the assay not working in the T47D breast cancer cells, as Tamoxifen, a known inducer of apoptosis (Li *et al.*, 2008; Vivian *et al.*, 2011), induced apoptosis in these cells (Addendum B, Figure B5).

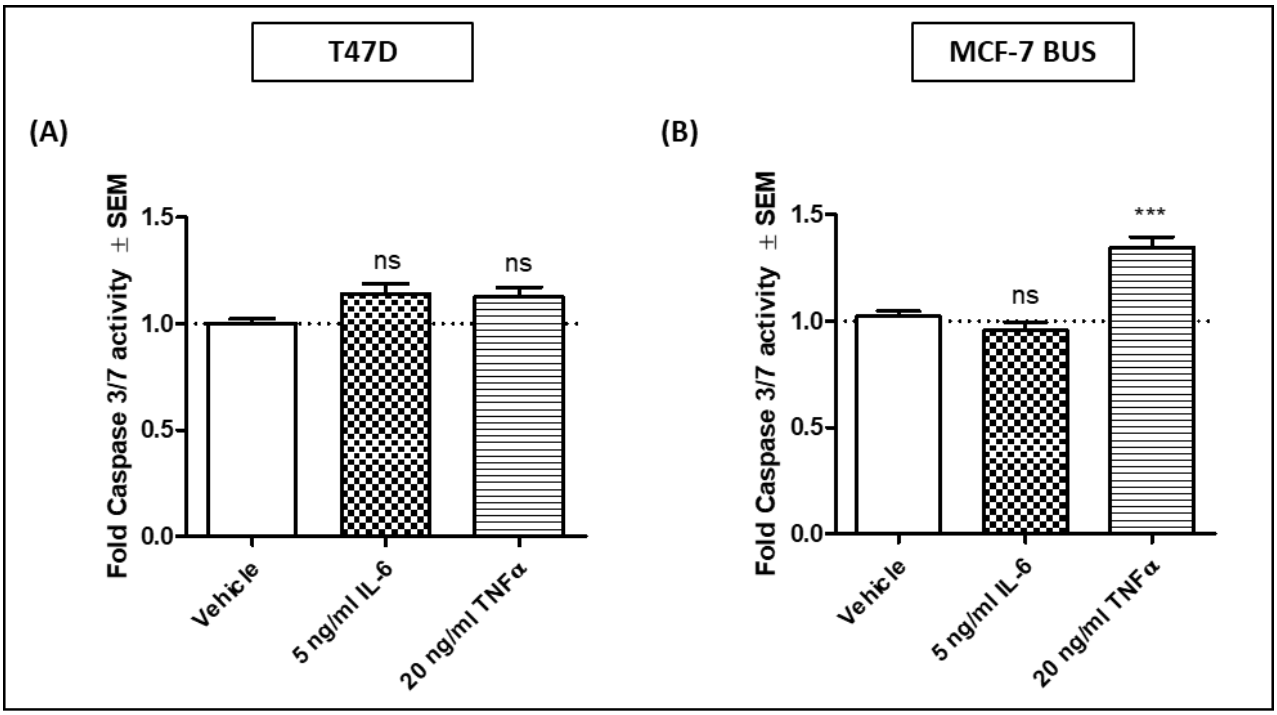


Figure 3.7 legend on next page

Figure 3.7 Only TNFα induced apoptosis in the MCF-7 BUS breast cancer cell line, while neither IL-6, nor TNFα induced apoptosis in the T47D breast cancer cell line. (A) T47D and (B) MCF-7 BUS breast cancer cells were treated with 0.1% (v/v) EtOH (vehicle), 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 and 6 hours, respectively. The Caspase-Glo 3/7 luminescent assay was used to determine caspase-3 and 7 activities, as a measure of apoptosis. Results are expressed as the fold caspase 3/7 activity relative to EtOH (vehicle) set as 1 and represent the average of three independent experiments performed in triplicate. One-way ANOVA with Dunnett's (compare all columns vs. control column) post-test were used for statistical analyses. Statistical significant differences are indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

In contrast to the above-mentioned effects using the caspase kit, both IL-6 and TNFα increased the mRNA expression of the pro-apoptotic Bax gene in the T47D cells (Figure 3.8A), while having no effect on Bax expression in the MCF-7 BUS cells (Figure 3.8C). Both TNFα and IL-6 also upregulated the mRNA expression of the anti-apoptotic Bcl-2 gene in T47D cells (Figure 3.8B), while neither IL-6 nor TNFα had any effect on Bcl-2 in MCF-7 BUS cells (Figure 3.8D).

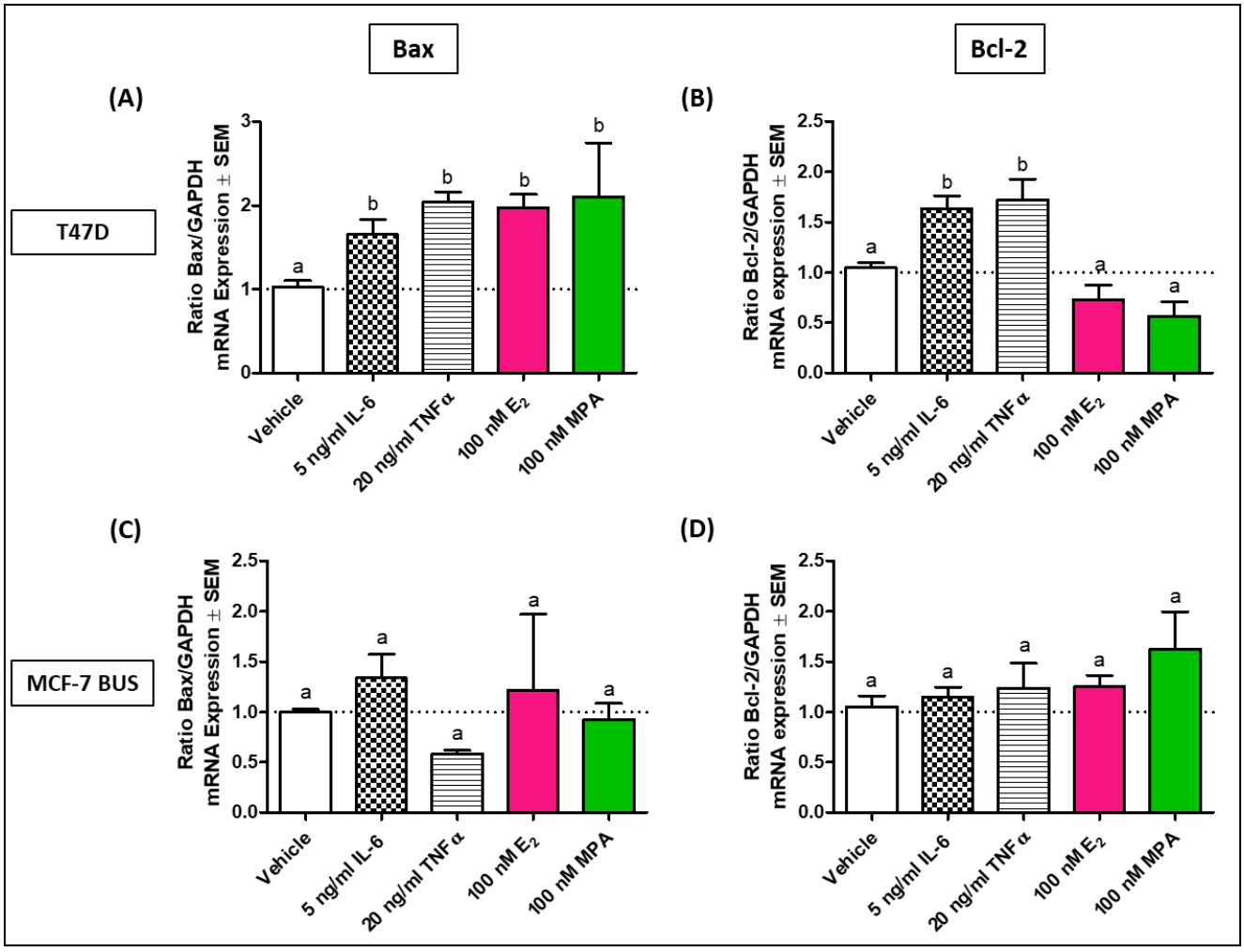


Figure 3.8 legend on next page

Figure 3.8 IL-6 and TNFα significantly upregulated the mRNA expression of both the pro-apoptotic, Bax, and anti-apoptotic, Bcl-2, gene in only the T47D cells, while having no effect on the MCF-7 BUS cells. (A and B) T47D and (C and D) MCF-7 BUS cells were treated with either 0.1% (v/v) EtOH, 5 ng/ml IL-6, 20 ng/ml TNFα, 100 nM E_2 or MPA for 24 hours. Total RNA was isolated, cDNA synthesised and mRNA expression levels of Bax, Bcl-2 and GAPDH were determined by qPCR. GAPDH was used as the internal standard and the ratio of Bax mRNA/GAPDH mRNA of the treated samples were calculated relative to EtOH. Results represent the average of three independent experiments. One-way ANOVA with Newman-Keuls (compare all pairs of columns) post-test were used for statistical analyses. Statistical significance is indicated by letters, where values that differ significantly are assigned different letters.

Having shown the effects of IL-6 and TNFα on both proliferation and apoptosis, and that E_2 and the progestogens differentially regulate breast cancer cell proliferation, we next investigated the effects of these hormones on apoptosis, and whether the effects are influenced by IL-6 or TNFα. Investigating the effects on both proliferation and apoptosis is important as these processes are opposing determinants of breast cancer cell survival and growth (Evan and Vousden, 2001). Results from the Caspase-Glo 3/7 assay showed that none of the hormones, in the absence or presence of IL-6, induced apoptosis in either the T47D (Figure 3.9A and C) or MCF-7 BUS (Figure 3.9B and D) breast cancer cell lines. Similarly, apoptosis was not induced in T47D cells treated with both hormone and TNFα (Figure 3.9C). Although an increase in apoptosis was observed in MCF-7 BUS cells treated with hormone and TNFα (Figure 3.9D), this was likely due to the increase in apoptosis observed for TNFα only.

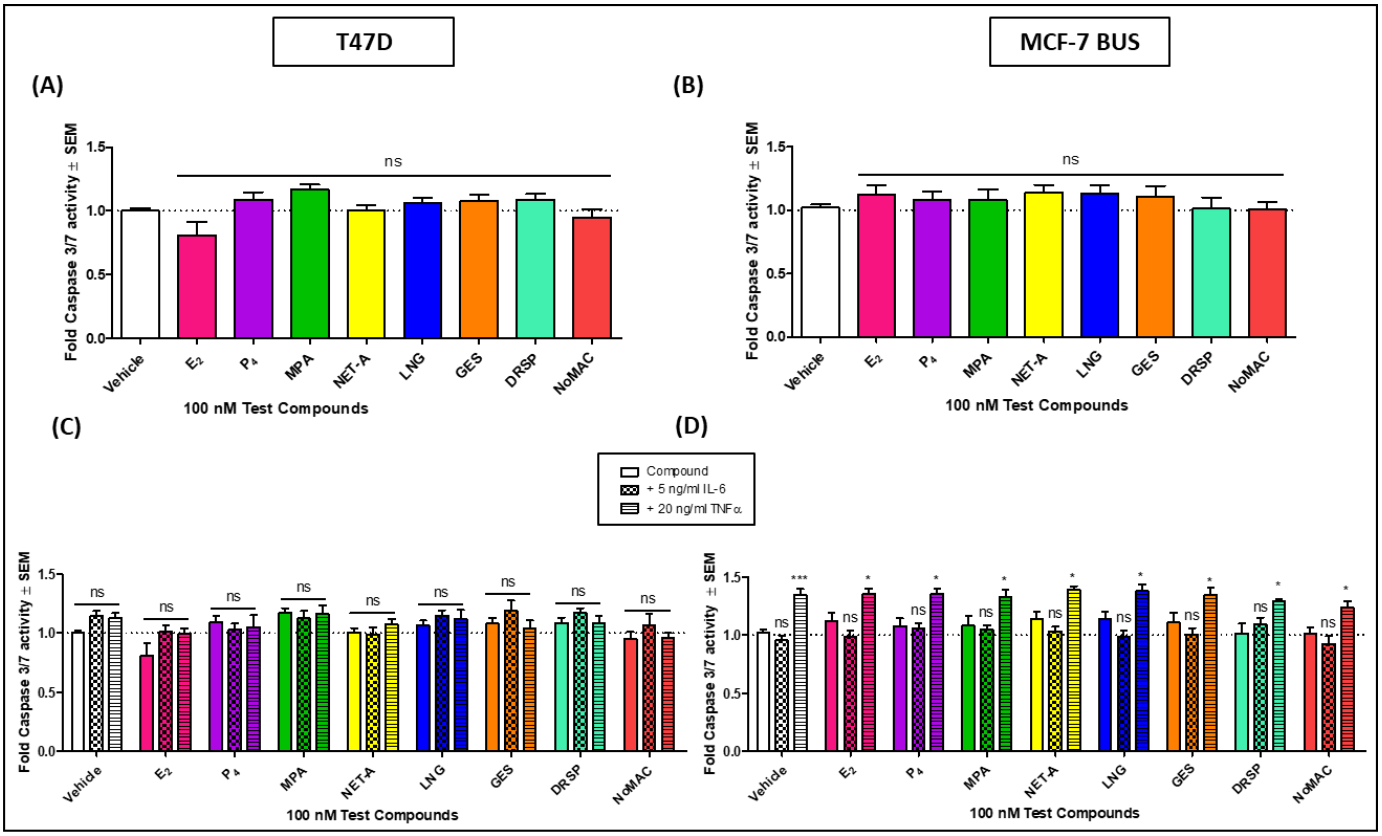


Figure 3.9 legend on next page

Figure 3.9 Neither E_2 nor the progestogens induced apoptosis in T47D and MCF-7 BUS breast cancer cell lines. (A and C) T47D and (B and D) MCF-7 BUS breast cancer cells were treated with 0.1% (v/v) EtOH (vehicle) or 100 nM hormones in the (A and B) absence and (C and D) presence of 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 and 6 hours, respectively. The Caspase-Glo 3/7 luminescent assay was used to determine the caspase-3 and 7 activities, as a measure of apoptosis. Results are expressed as fold caspase 3/7 activity relative to EtOH (vehicle) set as 1 and represent the average of three independent experiments performed in triplicate. One-way ANOVA (A and B) and two-way ANOVA (C and D) with Bonferroni's post–test was used for statistical analyses. Statistically significant differences are indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

3.7. The effect of the hormones and the inflammatory mediators on the migration of T47D breast cancer cells

In addition to the uncontrolled cell growth of cancer cells, these cells can also metastasize by a multi-step process that includes migration and invasion of cancer cells (Hanahan and Weinberg, 2011; Martin *et al.*, 2013). Having shown that the selected progestins differentially increased breast cancer cell proliferation (Figure 3.4), we next investigated their effects on the migration and invasion of the breast cancer cells. Considering that IL-6 and TNFα are known to enhance the migration of breast cancer cells (Wolczyk *et al.*, 2016; Gallo *et al.*, 2018), we also investigated effects of different concentrations of these inflammatory mediators on migration, as well as whether any hormone-mediated effects would be influenced in their presence. As we had previously shown that TNFα is cytotoxic to the MCF-7 BUS cells (Figure 3.5D), via the induction of apoptosis (Figure 3.7B), migration was evaluated only in the T47D breast cancer cell line. The frequently used wound scratch assay was used to measure the migration of T47D cells treated with the hormones, the inflammatory mediators or combinations of these over a 72-hour time period.

3.7.1. All concentrations of IL-6 evaluated similarly increased migration of the T47D breast cancer cells, whereas 20 ng/ml TNFα showed the highest increase in migration of this cell line.

First, we wanted to confirm previous reports that IL-6 and TNFα increase migration of breast cancer cells (Badache and Hynes, 2001; Wolczyk *et al.*, 2016; Gallo *et al.*, 2018). T47D cells were thus incubated with increasing concentrations of IL-6 (5, 20, 50 and 100 ng/ml) and TNFα (2, 20 and 200 ng/ml). Figure 3.10A and Figure 3.11A show photographs of the wounds captured at 0, 24, 48 and 72 hours using an inverted microscope, while Figure 3.10B-D and Figure 3.11B-D show the quantification of these photographs. Figure 3.10B and Figure 3.11B show the increase in migration over time in the presence of different concentrations of IL-6 and TNFα, respectively. The data in Figure 3.10B and Figure 3.11B were replotted as grouped bar graphs to show the statistical significance of the increase in migration over time in response to a specific concentration of IL-6 and TNFα (Figure 3.10C and

Figure 3.11C), and the statistical significance of the effects of the different concentrations of IL-6 and TNFα on migration at a specific time point (Figure 3.10D, and Figure 3.11D). Taken together, these results show that migration of the T47D breast cancer cells was increased in a time-dependent manner by all concentrations of IL-6 (Figure 3.10B and C). Surprisingly, all concentrations of IL-6 increased the migration of the T47D cells to similar extent at 24, 48 and 72 hours (Figure 3.10D). Thus, 5 ng/ml IL-6 was used in subsequent assays evaluating migration, as this was also the concentration used in the proliferation and apoptosis assays. Figure 3.11C shows that all concentrations of TNFα significantly increased the migration of T47D breast cancer cells at 72 hours, while Figure 3.11D shows that only 20 ng/ml TNFα significantly increased migration of T47D breast cancer cells at all time-points. Interestingly, 20 ng/ml TNFα was also the concentration used in the MTT and apoptosis assays.

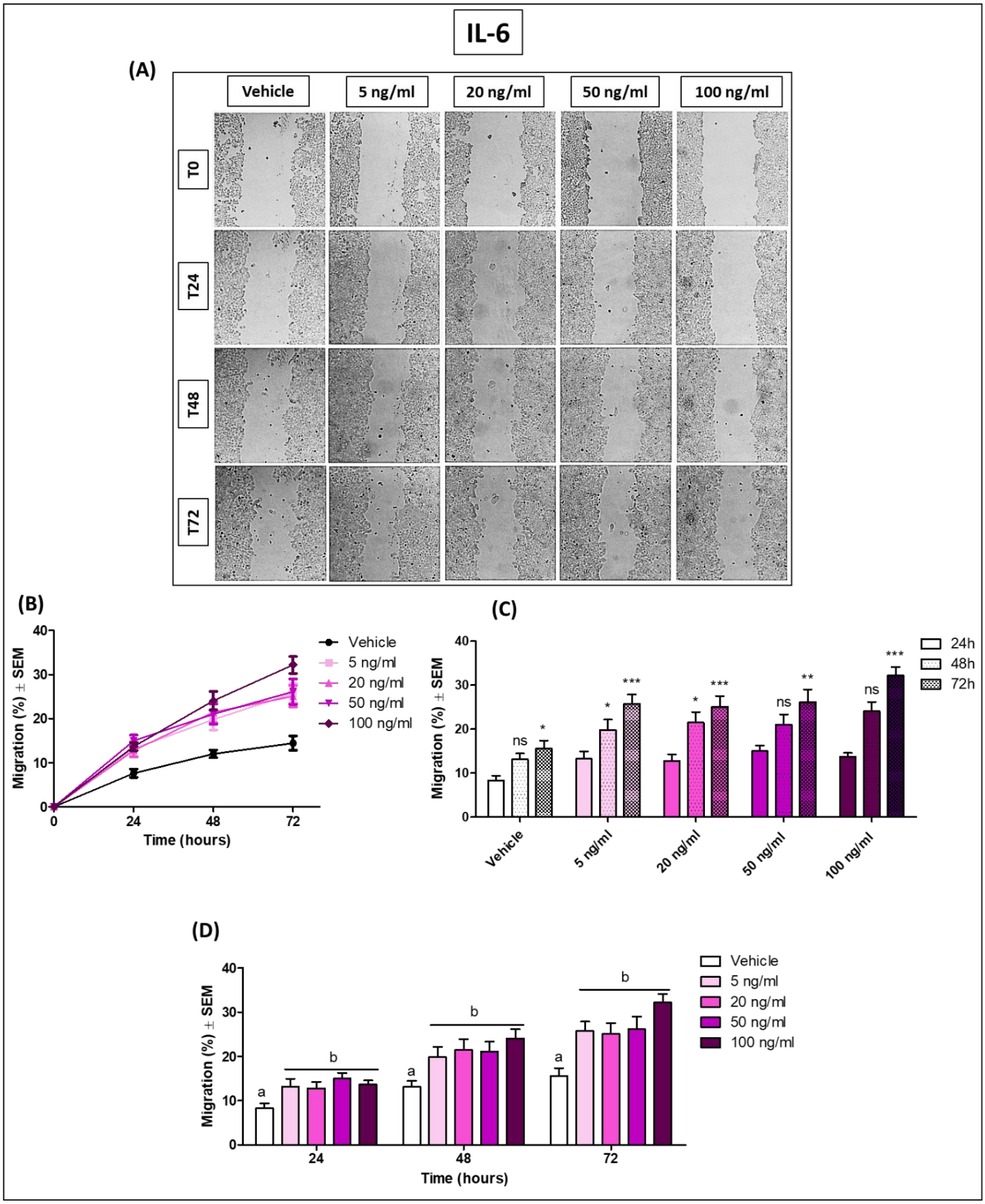


Figure 3.10 legend on next page.

Figure 3.10 The increase in migration of T47D breast cancer cells in response to IL-6 was time but not concentration dependent. T47D breast cancer cells were treated with either 0.1% (v/v) EtOH (vehicle) or 5, 20, 50 and 100 ng/ml IL-6. A wound scratch assay was used to measure the migration of the cells in response to treatment. Photographs of the wound were captured at t=0 (0 hours), 24, 48 and 72 hours using an inverted microscope and representative images are shown in (A). Image J was used to measure the width of the wound at each time point and the relative percentage (%) migration relative to the initial wound area was calculated (B). The data in (B) was replotted as bar graphs and show the statistical significance of the (C) time-dependent increase in migration in response to IL-6, and (D) migration in response to increasing concentrations of IL-6 at specific time points. Results represent the average of three independent experiments. Two-way ANOVA with Bonferroni's (compares each column to all other columns) post-test were used for statistical analysis. Statistical significance is indicated either by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$, or by letters, with values statistically different being assigned different letters.

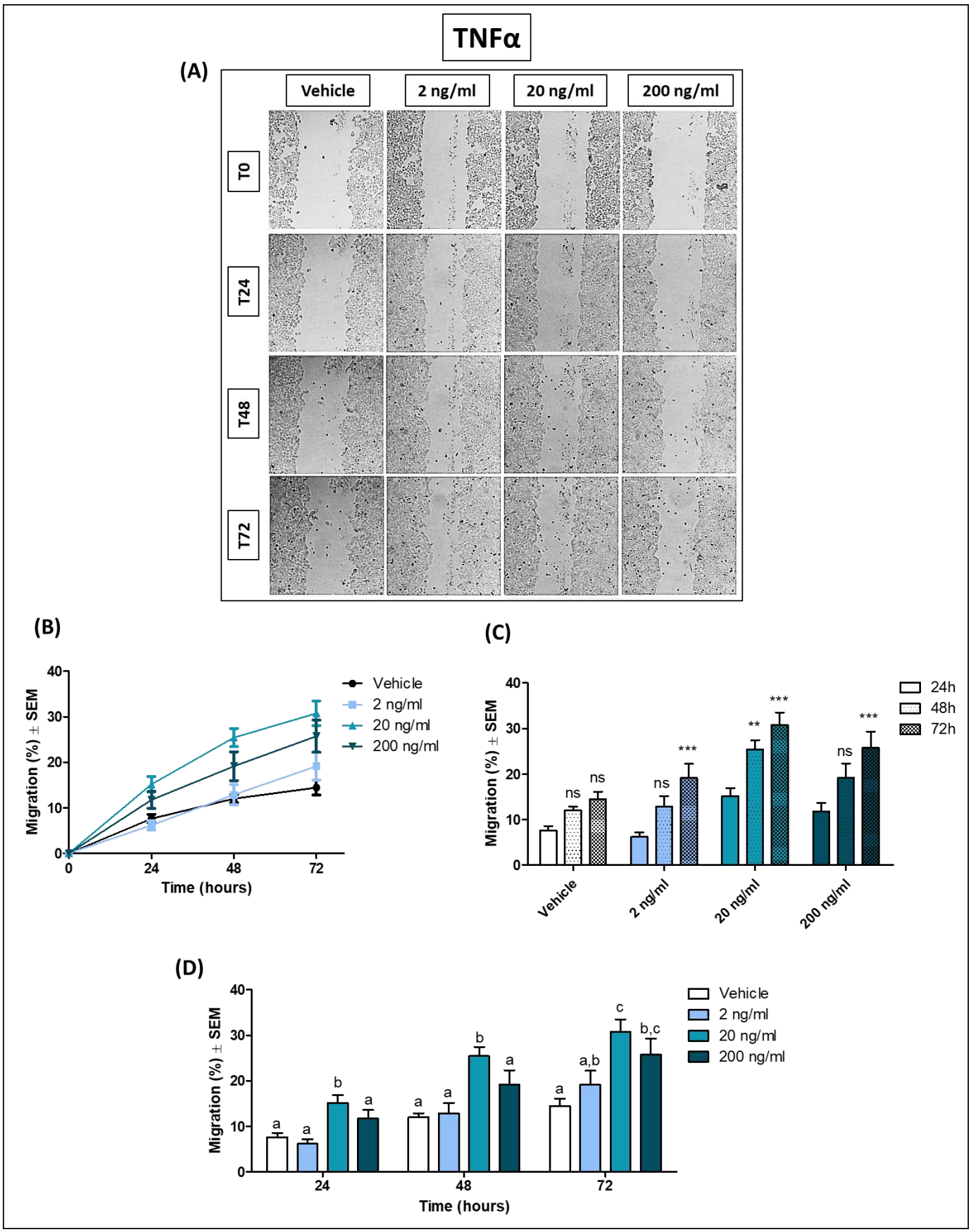


Figure 3.11 legend on next page.

Figure 3.11 Migration of T47D breast cancer cells in response to TNFα was optimal at 20 ng/ml at all time points evaluated. T47D breast cancer cells were treated with either 0.1% (v/v) EtOH (vehicle) or 2, 20, and 200 ng/ml TNFα. A wound scratch assay was used to measure the migration of the cells in response to treatment. Photographs of the wound were captured at t=0 (0 hours), 24, 48 and 72 hours using an inverted microscope and representative images are shown in (A). Image J was used to measure the width of the wound at each time point and the relative percentage (%) migration relative to the initial wound area could be calculated. (B). The data in (B) was replotted as bar graphs and show the statistical significance of the (C) time-dependent increase in migration in response to TNFα, and (D) migration in response to increasing concentrations of TNFα at specific time points. Results represent the average of three independent experiments. Two-way ANOVA with Bonferroni's (compares each column to all other columns) post-test were used for statistical analysis. Statistical significance is either indicated *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$, or by letters, with values statistically different being assigned different letters.

3.7.2. Unlike P_4, E_2 and all the progestins increased the migration of T47D cells.

Results for the evaluation of the effects of E_2 and the progestogens on the migration of the T47D breast cancer cells are shown in Figure 3.12. Photographs of the wounds captured at 0, 24, 48 and 72 hours are shown in Figure 3.12A. Quantification of photographs are shown in Figure 3.12B and results are represented as % migration over time. The same data in Figure 3.12B was replotted in Figure 3.12C-E to show the statistical significance of the migration in response to the hormones at specific time points. The results show that migration of T47D breast cancer cells in response to E_2 and all the progestins was already evident after 24 hours (Figure 3.12C), and all hormones, except P_4, increased migration of these cells to the same extent at 24, 48 and 72 hours (Figure 3.12C to E). P_4 was the only hormone that had no significant effect on the migration of T47D breast cancer cells over a time-period of 72 hours.

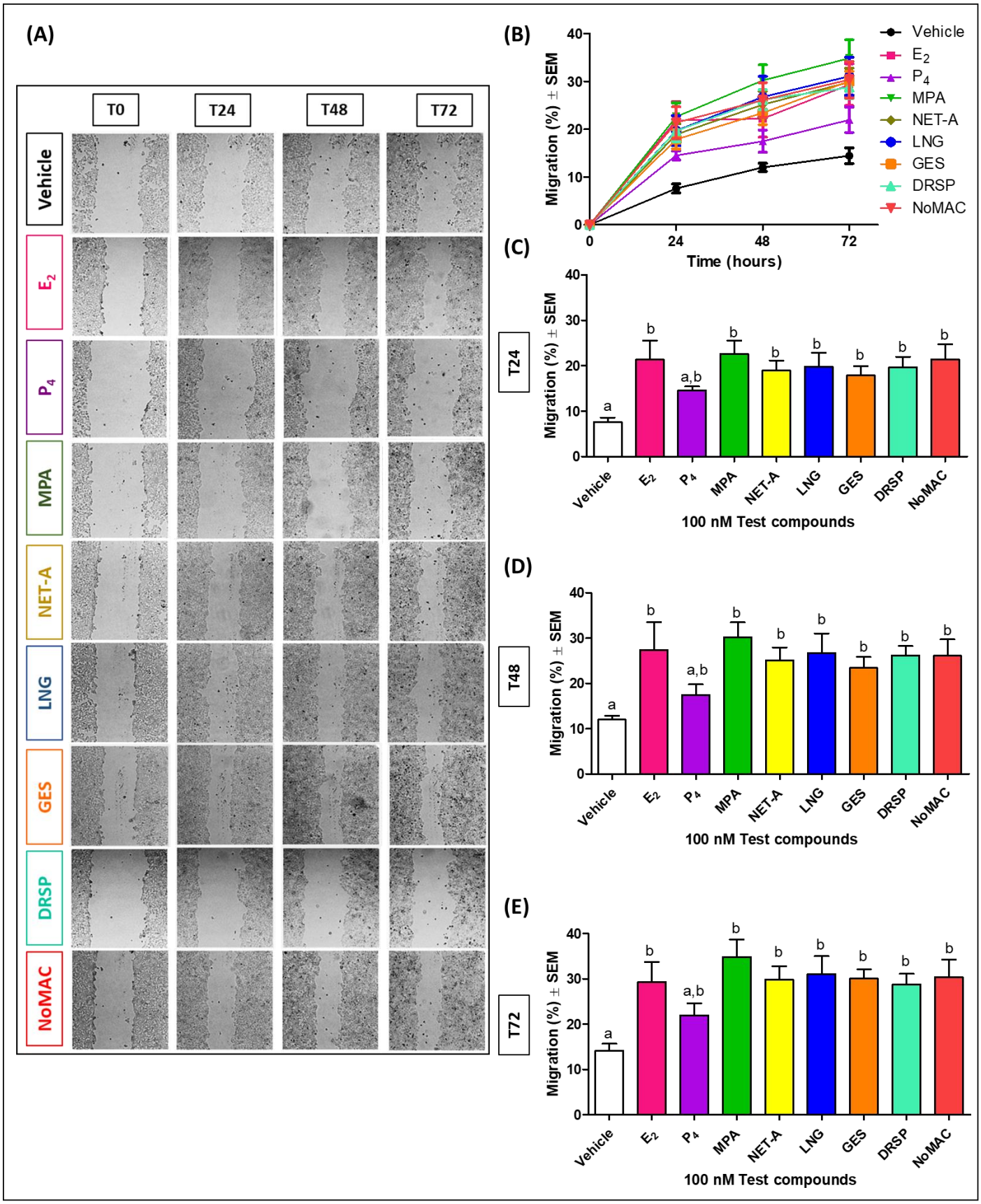


Figure 3.12 legend on next page.

Figure 3.12 All hormones evaluated, except P_4, increased the migration of T47D cells to the same extent. T47D breast cancer cells were treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones. A wound scratch assay was used to measure the % migration of the cells in response to treatment. Photographs of the wound were captured at t=0 (0 hours), 24, 48 and 72 hours using an inverted microscope and representative images are shown in (A). Image J was used to measure the width of the wound at each time point and the relative percentage migration relative to the initial wound area was calculated and (B) shows percentage migration over time. The data in (B) was replotted as bar graphs in (C, D and E) and show the statistical significance of the migration in response to the hormones at 24 hours, 48 hours and 72 hours, respectively. Results represent the average of three independent experiments. Two-way ANOVA with Bonferroni's (compare all pairs of columns) post-test were used for statistical analyses. Statistical significance is indicated with letters, with values statistically different being assigned different letters (C-E).

3.7.3. Neither of the two inflammatory mediators modulated the effects of the hormones on migration of the T47D breast cancer cell line.

Having shown that both IL-6 and TNFα (Figure 3.10 and Figure 3.11), as well as the progestins and E_2 (Figure 3.12), increased migration of the T47D breast cancer cell line, we next evaluated migration in the presence of a combination of IL-6 or TNFα with these hormones. T47D breast cancer cells were treated with either 0.1% (v/v) EtOH or 100 nM hormones in the absence and presence of either 5 ng/ml IL-6 (Figure 3.13) or 20 ng/ml TNFα (Figure 3.14). Representative images for T47D cells treated with the hormones in the absence and presence of IL-6 or TNFα are shown in Figure 3.13A and Figure 3.14A, respectively. As results were similar at all the time points, the quantification of the effects of the hormones in combination with either IL-6 (Figure 3.13B) or TNFα (Figure 3.14B) are shown only for the 72 hour time point. Interestingly, neither IL-6 (Figure 3.13B) nor TNFα (Figure 3.14B) could modulate the effects of the hormones on the migration of the T47D breast cancer cell line.

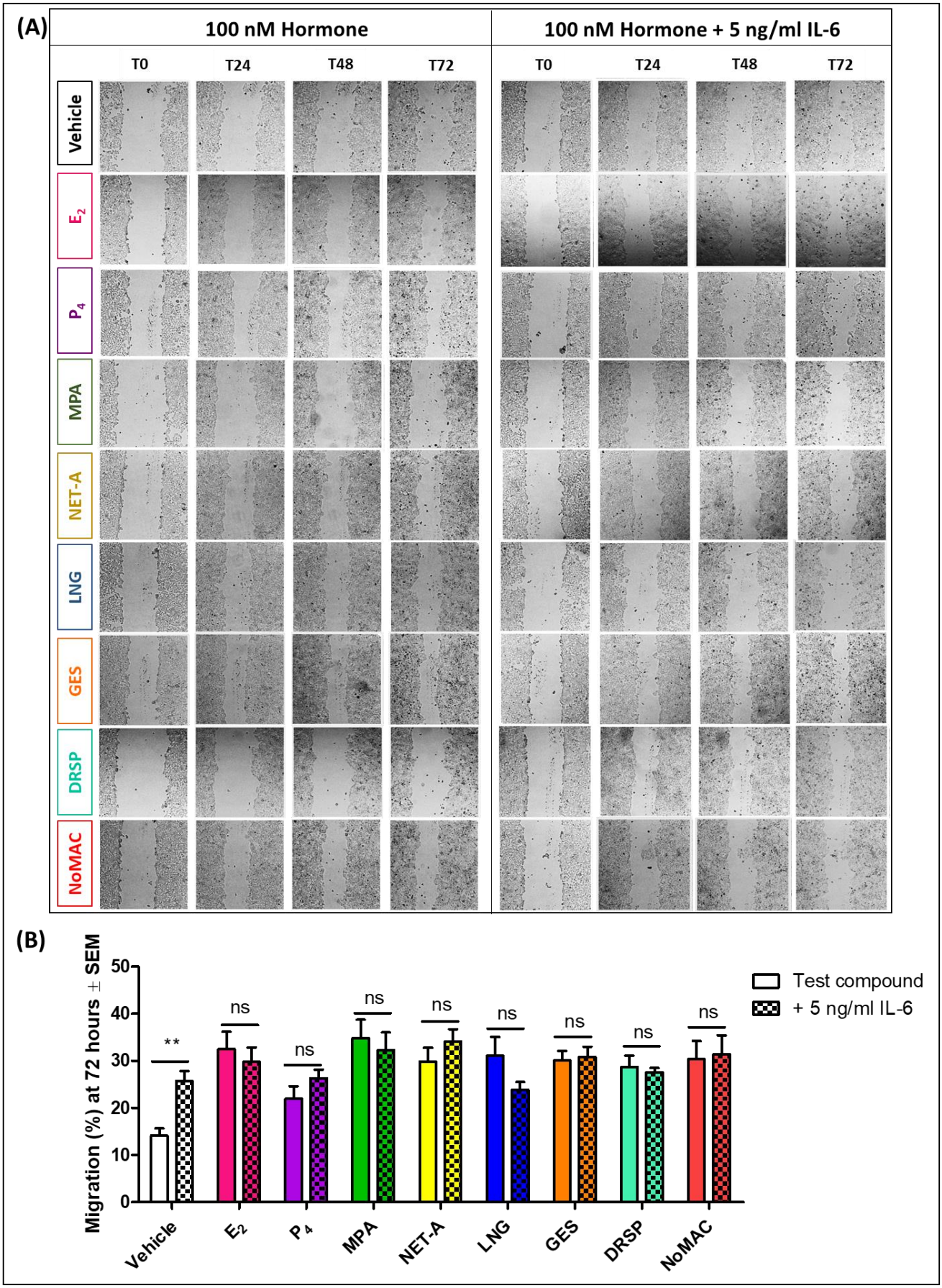


Figure 3.13 legend o next page.

Figure 3.13 IL-6 did not modulate the hormone-induced increase in migration of the T47D breast cancer cells. T47D cells were treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones in the absence and presence of 5 ng/ml IL-6. A wound scratch assay was used to measure the percentage (%) migration of the cells in response to treatment. Photographs of the wound were captured at t=0 (0 hours), 24, 48 and 72 hours using an inverted microscope and representative images are shown in (A). Image J was used to measure the width of the wound at each time point and the relative percentage migration relative to the initial wound area was calculated. A representative result of the average of three independent experiments at 72 hours is shown in (B). Two-way ANOVA with Bonferroni's (compare all pairs of columns) post–test was used for statistical analyses. Statistical significance is indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

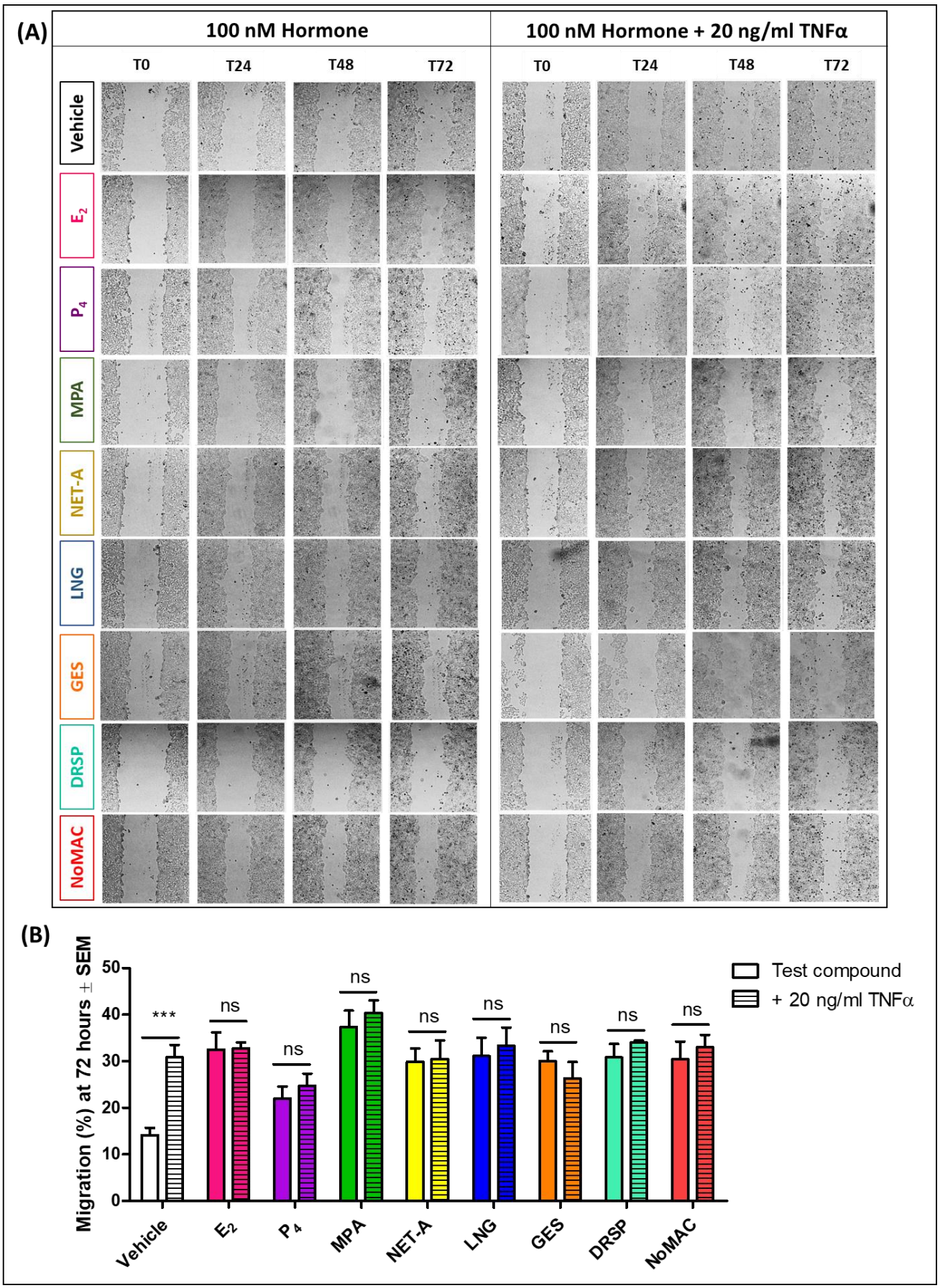


Figure 3.14 legend on next page.

Figure 3.14 TNFα did not modulate the hormone-induced increase in migration of the T47D breast cancer cells. T47D cells were treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones in the absence and presence of 20 ng/ml TNFα. A wound scratch assay was used to measure the percentage (%) migration of the cells in response to treatment. Photographs of the wound were captured at t=0 (0 hours), 24, 48 and 72 hours using an inverted microscope and representative images are shown in (A). Image J was used to measure the width of the wound at each time point and the relative percentage migration relative to the initial wound area was calculated. A representative result of the average of three independent experiments at 72 hours is shown in (B). Two-way ANOVA with Bonferroni's (compare all pairs of columns) post–test was used for statistical analyses. Statistical significance is indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

3.8. None of the hormones or inflammatory mediators regulated MMP-2 expression in the T47D breast cancer cell line.

Having shown that IL-6 (Figure 3.10) and TNFα (Figure 3.11), as well as E_2 and the progestins (Figure 3.12), increased the migration of T47D breast cancer cells, we next investigated the effects of these test compounds on invasion. It has previously been shown that one of the possible mechanisms by which IL-6 and/or TNFα promote invasion of breast cancer cells is by increasing extracellular matrix metalloproteases (MMPs) in tumours (Hagemann *et al.*, 2005; Ibrahim *et al.*, 2016). MMP-2 and MMP-9 are gelatinases that play key roles in degrading extracellular matrices and promoting tumour invasion and metastasis (Toth and Fridman, 2001; Wolczyk *et al.*, 2016). A gelatin zymography assay was used to evaluate the secretion of MMP-2 and MMP-9 in T47D breast cancer cells (Toth and Fridman, 2001) treated with either 0.1% (v/v) EtOH or 100 nM hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 hours. Although both MMP-9 and MMP-2 could be detected (Figure 3.15A), the bands for MMP-9 were too feint to be quantified. Quantification of MMP-2 showed that neither IL-6, TNFα (Figure 3.15C), the selected hormones (Figure 3.15D), nor combinations of these (Figure 3.15E), had any effect on MMP-2 expression levels in the T47D breast cancer cell line.

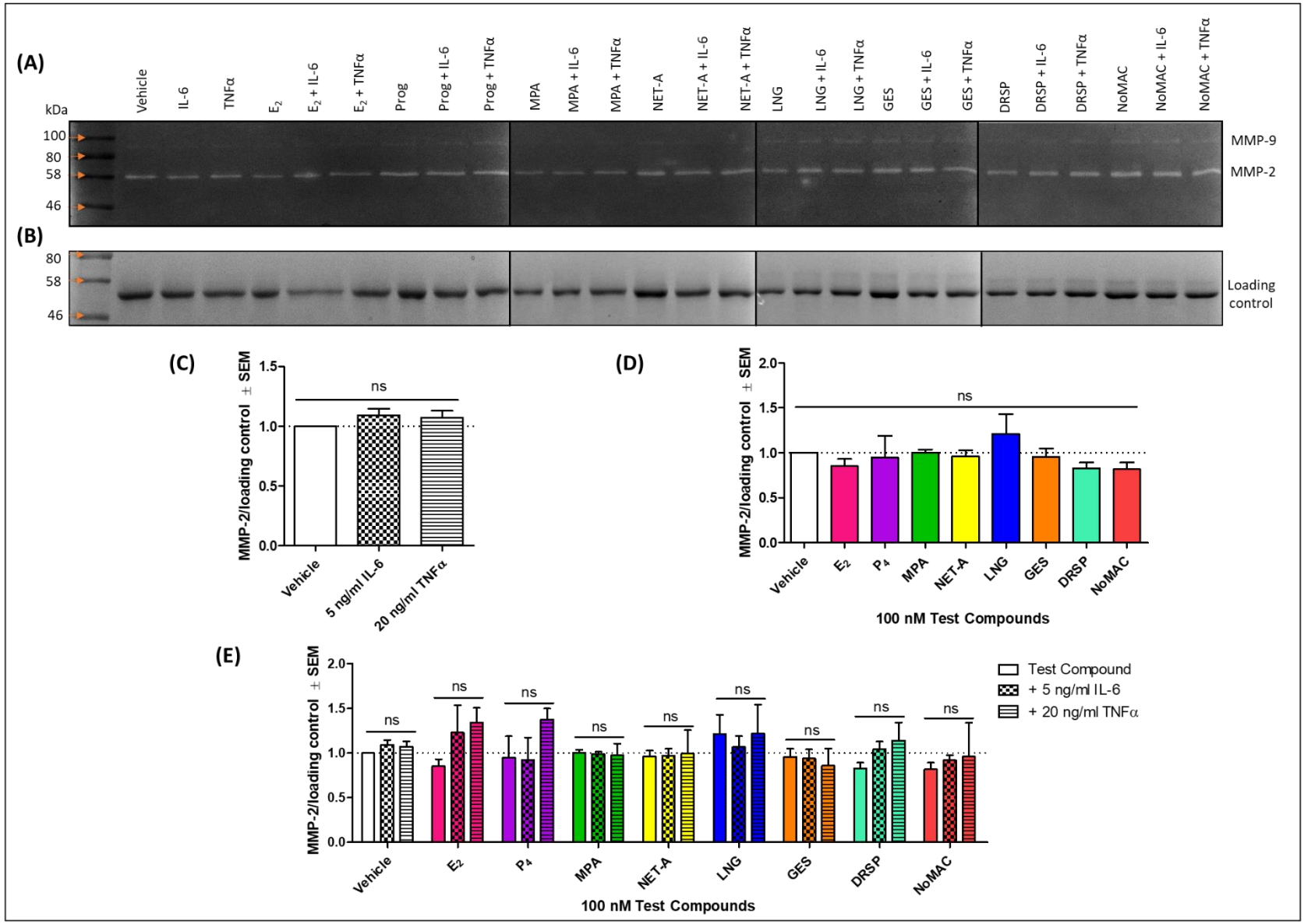
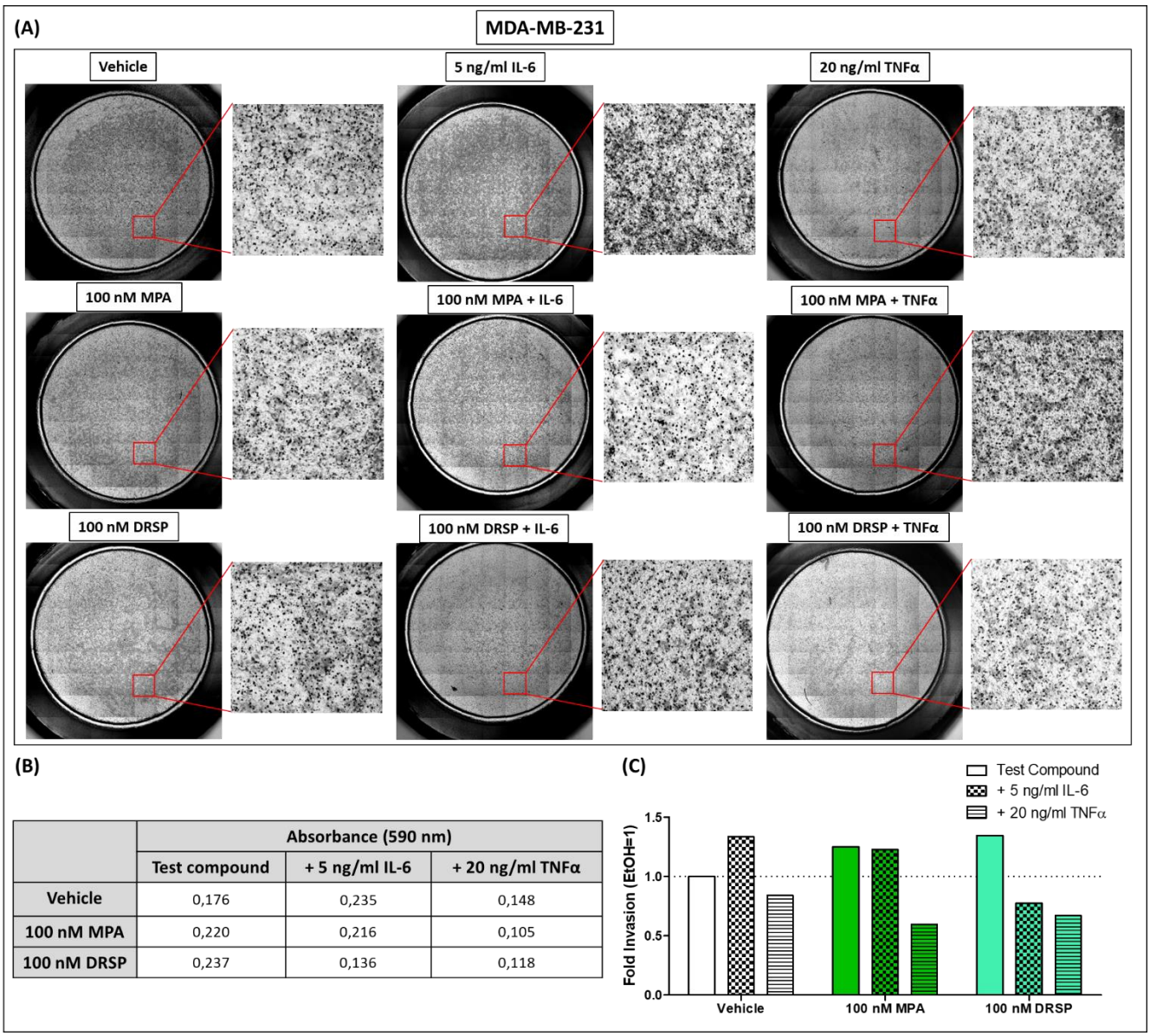


Figure 3.15 legend on next page.

Figure 3.15 Gelatin zymography showing that none of the hormones or the inflammatory mediators influenced MMP-2 expression in the T47D breast cancer cell line. Supernatants from T47D cells treated with either 0.1% (v/v) EtOH (vehicle) or 100 nM hormones in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 hours was harvested for gelatin zymography. Representative gels of three independent experiments indicating (A) MMP-9 at 84 kDa and MMP-2at 62 kDa and (B) the total protein loaded, are shown. Densitometric analyses of the bands were done using Image J (Hu and Beeton, 2010). One-way ANOVA with Dunnett's (compare all columns to the vehicle) and two-way ANOVA with Bonferroni's (compare all pairs of columns) post–tests were used for statistical analyses. Statistical significance is indicated by *, ** or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$; no statistical significance (ns) indicates $p>0.05$.

3.9. TNFα inhibited the MPA and DRSP-induced increase in invasion of the MDA-MB-231 breast cancer cells.

As no significant increase in the MMP expression of the T47D breast cancer cell line was seen, the effects of the test compounds on invasion was next evaluated using the Boyden-chamber transwell invasion assay as described in Section 2. After two failed attempts in the T47D breast cancer cell line, and one in the MCF-7 BUS cell line, we questioned whether this was due to a sub-optimal technique or whether it was due to the specific cell lines used. In terms of the latter, the T47D and MCF-7 breast cancer cell lines are known to be less invasive than the MDA-MB-231 TNBC cell line (Gordon *et al.*, 2003; Sarnataro *et al.*, 2006; Sun *et al.*, 2016). For this reason, the highly invasive MDA-MB-231 breast cancer cell line was next treated with the older first-generation progestin, MPA, and the newer fourth generation progestin, DRSP, in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα. Figure 3.16A shows confocal images of the cells on the filter membranes. The images could not be quantified due to the high proportion of invading cells, and thus quantification was achieved by dissolving the crystal violet stained cells in 33% glacial acetic acid and measuring the absorbance at 590 nm as previously described (Liu *et al.*, 2016). The relative absorbance values (Figure 3.16B) were plotted as fold invasion relative to the vehicle control set as 1 (Figure 3.16C). Results show that invasion of this cell line appears to be increased by MPA, DRSP, and IL-6, while the TNFα in the absence and presence of the hormones appear to decrease invasion. Interestingly, IL-6 also appears to decrease the effect of DRSP on invasion of the MDA-MB-231 breast cancer cell line, while the effect of MPA on invasion is unchanged in the presence of IL-6. However, it should be noted these results were from a single experiment, and thus no definitive conclusions can be drawn at this time.



	Absorbance (590 nm)		
	Test compound	+ 5 ng/ml IL-6	+ 20 ng/ml TNFα
Vehicle	0,176	0,235	0,148
100 nM MPA	0,220	0,216	0,105
100 nM DRSP	0,237	0,136	0,118

Figure 3.16 MPA, DRSP, and IL-6 increased the invasion of MDA-MB-231 breast cancer cells, while TNFα decreased invasion in the absence and presence of the progestins. The MDA-MB-231 breast cancer cells were treated with either 0.1% EtOH (control), 100 nM MPA or DRSP in the absence and presence of 5 ng/ml IL-6 or 20 ng/ml TNFα and cell invasion assayed with the matrigel invasion assay. (A) Representative confocal images of the invading cells on the membranes are shown, as well as the colorimetric quantification of the cells on the membrane (B and C). (B) The absorbance readings at 590 nm are tabulated and (C) the results, expressed as fold invasion relative to EtOH (vehicle) set as 1, are plotted. Results represent a single experiment.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4.1. Introduction

Breast cancer is the most prevalent cancer in women (Gordon *et al.*, 2003; Sarnataro *et al.*, 2006; MacCiò *et al.*, 2009; Sun *et al.*, 2016). Numerous risk factors contribute to breast cancer development and progression such as progestins used in menopausal HT and contraception, as well as being overweight or obese (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2005; Iyengar *et al.*, 2013; Zahid *et al.*, 2018). Although there is a tendency to group effects of progestins as a class, it is important to note that a diverse range of progestins are clinically used and that not all progestins have been investigated for an association with increased breast cancer risk. Progestins were designed to exert biological effects similar to P_4 by binding to the PR. However, some progestins are able to bind to multiple steroid receptors which may lead to off-target effects (Guerra *et al.*, 2013; Stanczyk *et al.*, 2013; Africander *et al.*, 2014). It is well recognised that hormones and steroid receptors play important roles in breast cancer development and progression (Conzen, 2008). For example, ERα causes E_2-mediated cell proliferation (Ali and Coombes, 2000; Chang *et al.*, 2006; Williams *et al.*, 2008; Cleary and Grossmann, 2009), while ERβ antagonises ERα-mediated cell proliferation (Kuiper *et al.*, 1996; Barkhem *et al.*, 1998; Gustafsson, 1999; Lazennec *et al.*, 2001; Platet *et al.*, 2004; Ström *et al.*, 2004). This suggests that ERβ is associated with good prognosis when co-expressed with ERα (Leygue and Murphy, 2013). Emerging evidence has also highlighted roles for the AR and GR, and show that whether these receptors are associated with either good or bad prognosis is dependent on whether ERα is expressed or not (Hopp *et al.*, 2004; Doane *et al.*, 2006; Hu *et al.*, 2011; Diep *et al.*, 2015; McFall *et al.*, 2018). Similarly, progestin-bound PR has been shown to form a complex with ERα, and redirects ERα chromatin binding, leading to the expression of genes that are associated with a favourable outcome in ER^+ breast cancer (Mohammed *et al.*, 2015), whereas in ER^- breast cancer, the PR causes cell proliferation (Thomas and Gustafsson, 2015). The ratio of PR-A:PR-B is also upregulated in breast cancer which is associated with a poor prognosis and increased invasiveness of breast cancer (Hopp *et al.*, 2004; Diep *et al.*, 2015; McFall *et al.*, 2018).

In terms of the obesity risk factor, growing evidence suggests that excessive production of pro-inflammatory cytokines such as IL-6 and TNFα, may be one of the mechanisms by which obesity contributes to breast cancer development and progression (Cole, 2009; MacCiò and Madeddu, 2011). The exact mechanism by which IL-6 and TNFα cause increased breast cancer risk is still poorly understood. It has however been suggested that these cytokines increase aromatase expression and activity, and thus estrogen signalling, as well as enhance tumour growth and metastasis (Purohit *et al.*, 1995, 2002; MacCiò and Madeddu, 2011; Morris *et al.*, 2012). Whether other mechanisms, such as the disruption of steroid receptor levels, may also be involved, is not known. Considering that the

incidence of obesity and its associated risk factors continues to grow (Iyengar *et al.*, 2013; Zahid *et al.*, 2018), and that progestins are widely used in HT, it is crucial to understand whether the combination of these risk factors would further increase breast cancer risk. In the light of the above, we thus investigated the effects of selected progestins relative to the natural female hormones, in the absence and presence of the inflammatory mediators, IL-6 and TNFα, on steroid receptor expression in breast cancer cells, as well as on specific hallmarks of cancer cells. These included the growth (proliferation) and survival (apoptosis), as well as the migration and invasion of breast cancer cell lines (Hanahan *et al.*, 2000; Hanahan and Weinberg, 2011).

4.2. E_2 and the progestogens differentially downregulated the expression of all steroid receptors, except the GR, in T47D breast cancer cells.

Steroid hormones mediate their effects by binding to steroid receptors (Xu and O'Malley, 2002), which all play a role in breast cancer pathogenesis. Thus, we evaluated the effects of the natural female hormones, E_2 and P_4, as well as selected progestins used in menopausal HT and contraception, on the expression of the PR-isoforms, ER-subtypes, the AR and GR. The T47D breast cancer cell line was used as a model system as it endogenously expresses all these steroid receptors (Nardulli & Katzenelenbogen 1988; Satrorius *et al.* 1994; Jacobsen & Horwitz 2012), and all results are summarised in Table 4.1. Western blot analyses confirmed the well-established fact that the cognate ligand or synthetic analogues thereof, decrease the expression of the PR, ER and GR (Figure 3.1-Figure 3.3) (Dong *et al.* 1988; Read *et al.* 1988; Zhang *et al.* 2009; Yeh *et al.* 2013). Results from this study showed that AR expression was decreased in response to the synthetic androgen, Mib (Figure 3.3B). This was surprising as it is has been shown that Mib and the natural androgen, DHT, increase AR protein expression in LNCaP prostate cancer and MDA-MB-453 breast cancer cell lines (Krongrad *et al.*, 1991; Lee and Chang, 2003; Moore *et al.*, 2006; Guo *et al.*, 2007; Wang *et al.*, 2009). A possible reason for the discrepancy between our results and that in the literature, may be due the fact that LNCaP and MDA-MB-453 cell lines contain a mutant AR (Taplin *et al.*, 1995; Yeap *et al.*, 1999; Moore *et al.*, 2012), while the wild-type AR is expressed in the T47D breast cancer cell line. The mutation in the AR found in the LNCaP and MDA-MB-453 cells are single point mutations in the ligand binding domain (Yeap *et al.*, 1999; Moore *et al.*, 2012). Given that a ligand induces conformational changes in a steroid receptor upon ligand binding (Lee and Chang, 2003), it may be possible that the androgens induce different conformations in the wild-type AR as compared to the mutant AR, which may result in degradation of the wild-type AR, but stabilisation of the mutant AR.

When investigating the effects of E_2 and the progestogens on the expression of these receptors, we show that the progestogens similarly decreased the expression of the AR and both PR-isoforms

(Figure 3.1 and 3.3). Considering that high affinity PR agonists, like R5020 and P_4, are known to decrease PR expression (Read *et al.*, 1988), it was not completely surprising that the progestins, designed to have agonist activity via the PR, did the same. Given that the PR has been shown to be associated with a favourable outcome in ER^+ breast cancer (Mohammed *et al.*, 2015), a decrease in PR expression may lead to ERα chromatin binding and gene expression profiles that enhance tumourigenicity, thus resulting in a poor prognosis. Furthermore, our results showing that E_2 upregulates the expression of both PR-isoforms is also consistent with the well accepted fact that the PR is a classical target gene for the ER in breast cancer cells (Kastner *et al.*, 1990; Chen *et al.*, 2013). In terms of the AR, it is known that all the progestogens evaluated in this study bind to the AR, with MPA, NET-A, LNG and GES displaying agonist properties similar to DHT, while DRSP and NoMAC display antagonist properties (Louw-du Toit *et al.*, 2017). It has been shown that AR agonists increase AR expression, while antagonists decrease its expression (Zhu *et al.*, 2000; Rathkopf and Scher, 2013). It was thus surprising that all progestins similarly decreased the expression of the AR in this study (Figure 3.3B). E_2 also decreased the expression of the AR, albeit to a lesser extent than the progestogens. This is in agreement with at least one study showing that E_2 significantly downregulates AR protein levels in T47D breast cancer cells (Ariazi *et al.*, 2007). In ER^+ breast cancer, activation of the AR is associated with a good prognosis as it can inhibit ERα signalling, and decrease breast cancer cell proliferation by increasing ERβ expression (Lanzino *et al.*, 2005; Peters *et al.*, 2009; Rizza *et al.*, 2014). In light of this, the result showing that all progestogens decreased the expression of the AR in this ER^+ breast cancer cell line, may suggest an unfavourable outcome in terms of breast cancer.

All the progestins decreased the expression of both ERα and ERβ, albeit to different extents, while P_4 surprisingly had no effect on ERα expression, but decreased ERβ expression (Figure 3.2B and D). These results were surprising, as none of the progestogens can bind to ERβ, while only NET-A, LNG and GES can bind to ERα (Louw-du Toit *et al.*, 2017). This suggests that the effects of MPA, DRSP and NoMAC on ERα expression, and P_4 and all the progestins on ERβ expression, does not occur via a mechanism involving binding of the progestogens to ERα or ERβ, but rather via an indirect mechanism. It has been shown that crosstalk exists between ERα and the PR (Giulianelli *et al.*, 2012; Daniel *et al.*, 2015), and that progestin-bound PR can regulate the transcriptional activity of ERα (Mohammed *et al.*, 2015). Given that the T47D breast cancer cells also express the PR, it may be possible that the progestin-induced effects on ER protein expression are in fact due to crosstalk between the progestin-bound PR and the ER subtypes. ERα-PR crosstalk in the presence of R5020 and P_4 has recently been reported to be associated with a good prognosis in terms of breast cancer (Mohammed *et al.*, 2015). However, more studies investigating possible crosstalk between ERβ and

the PR are lacking. Considering our results which indicates that progestins can decrease the expression of both ERα and ERβ, and knowing that ERβ plays an important role in antagonising ERα-mediated proliferation in breast cancer cells, such investigations are crucial.

In terms of the effects of E_2 and the progestogens on GR expression, none of these hormones altered GR expression levels in the T47D breast cancer cells (Figure 3.3D), unlike the GR agonist Dex. Although, P_4, MPA and GES are known partial agonists for the GR (Koubovec *et al.*, 2005; Hapgood *et al.*, 2013), a number of studies have previously shown that P_4 and MPA do not modulate GR levels (Ronacher *et al.* 2009; Avenant *et al.* 2010; Nicolette Verhoog, University of Cape Town, PhD Thesis, 2010; Renate Louw-du Toit, Stellenbosch University, PhD Thesis, 2013).These studies also showed that NET-A, which has GR antagonist properties, does not influence GR levels. In agreement with our results, at least one other study reported that E_2 has no effect on GR protein levels (Yong Zhang *et al.*, 2009). In contrast, two other studies have shown that E_2 causes a decrease in GR protein levels (Krishnan *et al.*, 2001; Kinyamu and Archer, 2003). As with most other steroid receptors, the function of the GR in breast cancer depends on whether it is expressed in the absence or presence of ERα (Pan *et al.*, 2011). When the GR is co-expressed with ERα, it is associated with a good prognosis in terms of breast cancer, while in the absence of ERα, this steroid receptor is associated with an unfavourable outcome (Pan *et al.*, 2011). The fact that we show no effects on GR expression with E_2 and the progestogens suggests that the GR will be able to be activated by glucocorticoids, which suggests a favourable outcome in terms of ER^+ breast cancer.

Table 4.1 Summary of the effects of E_2, the progestogens and the inflammatory mediators on steroid receptor expression levels of T47D breast cancer cells

Test Compound	Change in steroid receptor expression levels					
	PR-A	PR-B	ERα	ERβ	AR	GR
E_2	↑	↑	↓	↓	↓	-
P_4	↓	↓	-	↓	↓	-
MPA	↓	↓	↓	↓	↓	-
NET-A	↓	↓	↓	↓	↓	-
LNG	↓	↓	↓	↓	↓	-
GES	↓	↓	↓	↓	↓	-
DRSP	↓	↓	↓	↓	↓	-
NoMAC	↓	↓	↓	↓	↓	-
IL-6*	-	-	-	-	-	-
TNFα*	-	-	-	-	-	↑

↑ Increased protein expression ↓ Decreased protein expression - No effect on protein expression

*Neither IL-6 nor TNFα modulated the effects of the hormones on steroid receptor expression levels

In summary, it is possible that E_2, as well as all the progestogens may influence breast cancer development and progression by downregulating steroid receptors such as the ER-subtypes, PR-isoforms and the AR, but not the GR in T47D breast cancer cells.

4.3. Although neither IL-6 nor TNFα influenced steroid receptor expression levels in the T47D breast cancer cells, TNFα upregulated the expression of the GR.

While it is known that steroid receptors as well as pro-inflammatory mediators, play important roles in breast cancer biology, the precise mechanisms are not known (Lazennec *et al.*, 2001; Cops *et al.*, 2008; Tan *et al.*, 2009). To the best of our knowledge, this is the first study to evaluate whether IL-6 and TNFα influence steroid receptor expression in a breast cancer cell line, and whether they influence hormone-induced effects on steroid receptor expression. Neither IL-6 nor TNFα influenced the expression levels of either PR-isoforms, or the ER-subtypes or the AR in T47D breast cancer cells, while TNFα upregulated the expression of the GR (Figure 3.1-Figure3.3; Table 4.1). Moreover, neither of these cytokines modulated the effects of the hormones on any of these steroid receptors (Figure 3.1-Figure3.3). The increase in GR protein expression in response to TNFα correlates with a previous study that showed the same effect in the human cervical adenocarcinoma (HeLaS3) and human lymphoid (CEMC7) cells (Webster *et al.*, 2001). These authors showed that TNFα increases GR expression in the HeLaS3 and the CEMC7 cells via a mechanism involving the binding of NF-κB to its DNA binding site upstream to the human GR promoter (Webster *et al.*, 2001). Thus, it is likely that TNFα increases GR expression in T47D breast cancer cells in a similar manner. As GR expression in ER^+ breast cancer has been associated with a good prognosis (Pan *et al.*, 2011), an increase in GR expression by TNFα may suggest a favourable outcome in ER^+ breast cancer.

4.4. E_2 and the progestogens may lead to the survival of breast cancer cells.

Cancer cells can sustain proliferative signalling while evading cell death signals, ultimately contributing to the successful survival of the disease (Hanahan *et al.*, 2000; Evan and Vousden, 2001; Hanahan and Weinberg, 2011). The literature regarding the effects of the progestins on breast cancer cell survival are contradictory. In this study, a direct comparison of the effects of E_2, P_4 and selected progestins showed that these hormones differentially regulated proliferation of two ER^+ breast cancer cell lines (Figure 3.4 and summarised in Table 4.2). The increase in proliferation of the T47D and MCF-7 BUS cells in response to P_4 (Figure3.4A and B) is consistent with two other studies (Liang *et al.*, 2006; Tian *et al.*, 2018), whilst others have shown that P_4 inhibits proliferation of T47D and MCF-7 cells (Groshong *et al.*, 1997; Formby *et al.*, 1998; Ruan *et al.*, 2012). Interestingly, the results of the current study showed that DRSP and NoMAC had no effect on the proliferation of both cell lines,

while the proliferation by all ligands except LNG, were higher in the estrogen-sensitive MCF-7 BUS cell line (Figure 3.4C). Furthermore, LNG was the only progestin eliciting cell line-specific effects, indicated by the fact that it increased proliferation of the T47D cells, while having no effect on proliferation of the MCF-7 BUS cells (Figure 3.4C). Considering that at least two studies have previously shown that LNG increases the proliferation of MCF-7 cells (Van den Burg *et al.*, 1992; Schoonen *et al.*, 1995), a similar response was expected in the MCF-7 BUS cells, as this highly estrogen-responsive cell line was originally cloned from the MCF-7 breast cancer cell line (Villalobos *et al.*, 1995). Thus, the lack of proliferation in response to LNG in the MCF-7 BUS cells are surprising and hard to interpret, especially since MPA, NET-A, LNG and GES have similar activities via the PR, AR and ERα (Africander *et al.*, 2011, 2014; Louw-du Toit *et al.*, 2017). Importantly, results showing that the fourth-generation progestins, DRSP and NoMAC, have no effect on proliferation of either T47D or MCF-7 BUS breast cancer cell lines (Figure3.4), suggest that these hormones may be a safer HT option than the earlier-generation progestins in terms of breast cancer risk. However, it should be noted that the French E3N-EPIC cohort study investigating the risk of breast cancer associated with HT use by postmenopausal women, has shown that NoMAC, combined with estrogen, is in fact associated with increased breast cancer risk (Fournier *et al.*, 2008). It is important to remember that the development and progression of breast cancer is dependent on a number of regulatory processes. Thus, the observed effects of the progestins on proliferation should be considered in the light of the fact that there may be a balance between these processes.

Results from the Caspase-Glo 3/7 apoptosis assay used in this study, showed that none of the hormones induced apoptosis of either the T47D or the MCF-7 BUS breast cancer cell lines (Figure 3.9A and B, and summarised in Table 4.2). The results showing that Tamoxifen, a known inducer of apoptosis, induced apoptosis in the T47D cells, while TNFα induced apoptosis in the MCF-7 BUS cells (Addendum B, Figure B5), indicated that the apoptosis assay was functional in both these cell lines. Our results showing no effect with that E_2 and the progestogens on apoptosis are contradictory to previous studies that have shown that E_2 (Gompel *et al.*, 2000; Helguero *et al.*, 2005; Lewis-Wambi and Jordan, 2009), MPA as well as NET-A can inhibit apoptosis in the T47D and MCF-7 cells (Ory *et al.* 2001; Sweeney *et al.* 2014). Similarly, studies have shown that P_4 can either induce (Formby and Wiley, 1998, 1999) or inhibit (Moore *et al.*, 2006) apoptosis of T47D breast cancer cells. The difference between our results and these studies could possibly be due to the use of different assays. For example, Formby and Wiley, measured apoptosis by assays such as fluorescence activated cell sorting (FACS), semi-quantitative reverse transcriptase (RT)-PCR and immunoblotting for caspase 3 expression (Formby and Wiley, 1998). Differences in apoptotic effects when using different assays are also reflected in our own results. We showed that both E_2 and MPA increased the mRNA

expression of the pro-apoptotic Bax gene in the T47D cell line (Figure 3.8A), while having no effect on the Bcl-2 gene (Figure 3.8B). Neither E_2 nor MPA had an effect on the mRNA expression of either Bax or Bcl-2 in the MCF-7 BUS cell line (Figure 3.8C and D), suggesting that effects on apoptosis are also cell-specific. Taken together, the overall increase observed in proliferation of both the T47D and MCF-7 BUS cells in response E_2, P_4, MPA, NET-A, and GES, as well as LNG in the T47D cells, and the likely lack of induction of apoptosis, suggests that these hormones may lead to the survival of these breast cancer cells.

Table 4.2 Summary of the effects of E_2, the progestogens and the inflammatory mediators on the proliferation and apoptosis of T47D and MCF-7 BUS breast cancer cells.

	Proliferation				Apoptosis					
Test Compound	MTT assay		mRNA Ki-67		Caspase 3/7 activity		mRNA Bax		mRNA Bcl-2	
	T47D	MCF-7 BUS	T47D	MCF-7 BUS	T47D	MCF-7 BUS	T47D	MCF-7 BUS	T47D	MCF-7 BUS
E_2	↑	↑	↑	↑	-	-	↑	-	-	-
P_4	↑	↑	ND	ND	-	-	ND	ND	ND	ND
MPA	↑	↑	↑	↑	-	-	↑	-	-	-
NET-A	↑	↑	ND	ND	-	-	ND	ND	ND	ND
LNG	↑	-	ND	ND	-	-	ND	ND	ND	ND
GES	↑	↑	ND	ND	-	-	ND	ND	ND	ND
DRSP	-	-	ND	ND	-	-	ND	ND	ND	ND
NoMAC	-	-	ND	ND	-	-	ND	ND	ND	ND
IL-6*	-	-	-	-	-	-	↑	-	↑	-
TNFα*	-	↓	-	↓	-	↑	↑	-	↑	-

↑ Increase ↓ Decrease - No effect ND Not determined

*Neither IL-6 nor TNFα modulated the effects of the hormones on proliferation (MTT assay) or apoptosis (Caspase 3/7 activity)

4.5. Unlike IL-6, TNFα significantly decreased the cell viability and induced apoptosis of the MCF-7 BUS cell line, while having no effect on the growth of the T47D breast cancer cell line.

Evidence in the literature regarding the effects of IL-6 and TNFα on the proliferation and apoptosis of breast cancer cells are contradictory. Some studies have reported that both IL-6 and TNFα increase breast cancer cell proliferation, whereas others state that these cytokines inhibit cell proliferation. (Rubio *et al.*, 2006; Sasser *et al.*, 2007; Studebaker *et al.*, 2008). Results from this study showed that IL-6 had no effect on the proliferation of either the T47D or MCF-7 BUS cells (Figure 3.5A and B; Table 4.2), neither did it modulate the hormone-induced effects on proliferation of either cell line (Figure 3.5A and B). At least one other study has also shown that IL-6 has no effect on the proliferation of MCF-7 breast cancer cells (Jiang *et al.*, 2011), while two other studies have shown that IL-6 increases proliferation of this cell line (Sasser *et al.*, 2007; Studebaker *et al.*, 2008). In terms

of TNFα, it has previously been shown that it promotes apoptosis (Simstein *et al.*, 2003) in MCF-7 cells, while stimulating proliferation of T47D breast cancer cells (Nojek *et al.*, 2006). While we show that TNFα has no effect on the proliferation of T47D breast cancer cells, we do in fact observe a significant decrease in the viability of MCF-7 BUS cells (Figure 3.5C and D, Table 4.2). The fact that neither IL-6 nor TNFα increased proliferation of the T47D cells correlates with our results showing that neither of these cytokines had an effect on the mRNA expression of the Ki-67 proliferation marker in T47D cells, while its expression was significantly decreased in the MCF-7 BUS cells (Figure 3.6). In contrast, two other studies have shown that TNFα is associated with elevated levels of Ki-67 in tumour cells as well as in breast tumour tissues from mice (Baisch, 2002; Cai *et al.*, 2017). Although MCF-7 BUS cell proliferation was decreased in the presence of both TNFα and the hormones, these effects were likely due to TNFα decreasing the cell viability, and not because TNFα modulated the hormone-induced effects on cell proliferation (Figure 3.5D).

Results from the apoptosis assay confirmed that TNFα induced apoptosis in the MCF-7 BUS cells (Simstein *et al.*, 2003), but not in the T47D cells (Figure 3.7, Table 4.2). Surprisingly, IL-6 and TNFα both increased the mRNA expression of Bax and Bcl-2 in the T47D cells, while having no effect on these genes in the MCF-7 BUS cells (Figure 3.8, Table 4.2). The increase in both pro-and anti-apoptotic genes in response to the cytokines (Figure 3.8A and B), may explain the lack of an overall effect on apoptosis in the T47D breast cancer cells using the Caspase-Glo 3/7 assay (Figure 3.7A), as the prediction of cell survival is determined by the function of both these proteins. Furthermore, the combination of IL-6 or TNFα with E_2 or the progestogens, also had no effect on the apoptosis of T47D breast cancer cells (Figure 3.9C). The increased apoptosis in MCF-7 BUS cells in the presence of TNFα and the hormones, was likely due to TNFα inducing apoptosis and not as a result of the combination of TNFα and the hormones (Figure 3.9D). Taken together, and in agreement with previous studies (Simstein *et al.*, 2003; Martínez-reza *et al.*, 2017), our results show that TNFα induced apoptosis in the MCF-7 BUS cells, while having no effect on the T47D cells. Moreover, these cytokines did not modulate the hormone-mediated effects on either proliferation or apoptosis

4.6. Both IL-6 and TNFα, as well as E_2 and the progestins, increased the migration of the T47D breast cancer cell line, while the effects of the hormones could not be modulated by these inflammatory mediators.

Cancer cells can also migrate and invade, and 90% of cancer related deaths are due to these processes (Hanahan *et al.*, 2000; Wolczyk *et al.*, 2016). To date, studies investigating the effects of E_2 and the progestogens on breast cancer cell migration and invasion are limited. Some, however, have shown that E_2 causes an increase in migration of T47D (Zheng *et al.*, 2011) and MCF-7 breast cancer cells

(Shang *et al.*, 2015). Similarly, P_4, MPA, and DRSP have been shown to differentially increase the migration of T47D breast cancer cells, with MPA causing the most migration (Fu *et al.*, 2008). It is well recognised that IL-6 and TNFα increase the migration of MCF-7, T47D and MDA-MB-231 breast cancer cells (Badache and Hynes, 2001; Wolczyk *et al.*, 2016; Gallo *et al.*, 2018). In this study, effects on migration were evaluated only in the T47D breast cancer cell line, as we had previously shown that TNFα decreased the cell viability of MCF-7 BUS cells (Figure 3.5D), by the induction of apoptosis (Figure 3.7B). We showed that E_2 and all the progestins increased the migration of T47D breast cancer cells to a similar extent (Figure 3.12, and summarised in Table 4.3). Even though it appeared that P_4 increased migration, this increase was not statistically different compared to the vehicle control. Consistent with the literature, we show that both IL-6 and TNFα increased the migration of the T47D breast cancer cell line (Figure 3.10 and Figure 3.11, Table 4.3) (Badache and Hynes, 2001; Wolczyk *et al.*, 2016). Surprisingly, neither IL-6 (Figure 3.13B) nor TNFα (Figure 3.14B) influenced the effects of E_2 and the progestins on the migration of T47D breast cancer cells. The fact that IL-6 and TNFα increased the migration of T47D breast cancer cells, while having no effect on the proliferation of these cells, may be ascribed to the fact that these processes can occur independently of each other. For example, it has previously been shown that IL-6 inhibits the growth of T47D cells by activating the Janus kinase/signal transducers and activators of transcription (Jak/STAT) signalling pathway, while it simultaneously activates the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways to enhance migration (Badache and Hynes, 2001). Taken together, although E_2, all the progestins and both inflammatory mediators individually increased the migration of T47D breast cancer cells, the effects were not exacerbated when both hormone and inflammatory mediators were present. These results may suggest that women using these hormones in HT, as well as women with obesity-related inflammation, may be at risk for developing metastatic breast cancer.

4.7. Neither the inflammatory mediators, nor the hormones altered MMP-2 expression in the T47D breast cancer cell line.

Studies have shown that E_2 increases the invasion of MCF-7 (Albini *et al.*, 1986; Thompson *et al.*, 1988; Jiang *et al.*, 2013; Shang *et al.*, 2015) and T47D breast cancer cells (van den Brûle *et al.*, 1992). P_4, MPA, and DRSP have also been shown to enhance invasion in these cells (Fu *et al.*, 2008, 2010; Diaz *et al.*, 2012). However, studies investigating the effects of the other progestins such as NET-A, LNG, GES and NoMAC on the invasion of breast cancer cells are lacking. Interestingly, IL-6 and TNFα have also been shown to increase invasion of MCF-7 and MDA-MB-231 breast cancer cells (Wolczyk *et al.*, 2016; Gallo *et al.*, 2018). A few studies have suggested that these cytokines may

increase the invasiveness of cancer cells via a mechanism involving increased MMP-2 and MMP-9 secretion (Hagemann *et al.*, 2005; Mohamed *et al.*, 2010; Ibrahim *et al.*, 2016). These specific enzymes are gelatinases that can degrade extracellular matrix components and adhesion molecules such as E-cadherin (Murphy and Crabbe, 1995; Toth and Fridman, 2001; Ibrahim *et al.*, 2016). The presence of these MMP's within biological samples are often detected using the semi-quantitative gelatin zymography assay (Heusseneugene, 1980; Toth and Fridman, 2001). The gelatinases secreted from the breast cancer cells are able to exert their gelatinolytic activity in an SDS-PAGE gel containing gelatin, and detected as bands of clearance (Toth and Fridman, 2001; Hu and Beeton, 2010). Results from this study showed that neither E_2 or the progestogens (Figure 3.15D), IL-6 or TNFα (Figure 3.15C), or the combination thereof (Figure 3.15E), influenced MMP-2 levels secreted by T47D breast cancer cells. Wolczyk and co-workers have previously shown that TNFα has no significant effect on MMP-2 secretion in the MDA-MB-231 and MCF-7 breast cancer cell lines (Wolczyk *et al.*, 2016). Although we could not quantify effects on MMP-9 in the T47D cells, these authors have also shown that TNFα dose-dependently increased the secretion of MMP-9 in MDA-MB-231 and MCF-7 cells (Wolczyk *et al.*, 2016).

4.8. Preliminary results show that IL-6, MPA and DRSP appear to increase the invasion of the MDA-MB-231 breast cancer cell line, and that hormone-induced effects are decreased by TNFα.

Effects on invasion of MDA-MB-231 breast cancer cells was next investigated using the Boyden-chamber transwell assay, as attempts of this assay in both the T47D and MCF-7 BUS cells were unsuccessful. The MDA-MB-231 cell line was used as it is highly invasive compared to the T47D and MCF-7 cells (Gordon *et al.*, 2003; Sarnataro *et al.*, 2006; Sun *et al.*, 2016). Results showed that both the older first-generation progestin, MPA, and the newer fourth-generation progestin, DRSP, appeared to increase the migration of the MDA-MB-231 cells to the same extent (Figure 3.16C and summarised in Table 4.3). These results are in agreement with at least one study reporting that both MPA and DRSP increase invasion of T47D breast cancer cells (Fu *et al.*, 2008). In addition, our result showing that IL-6 appears to increase invasion of MDA-MB-231 cells (Figure 3.16C; Table 4.3), is consistent with a previous study (Gallo *et al.*, 2018). In contrast, we show that TNFα appears to decrease the invasion of MDA-MB-231 cells (Figure 3.16; Table 4.3), while others have indicated that it increases the invasion of MDA-MB-231 cells (Sangmin *et al.*, 2008; Wolczyk *et al.*, 2016; Martínez-reza *et al.*, 2017). Moreover, TNFα appears to decrease the progestin-induced invasion of the MDA-MB-231 breast cancer cells (Figure 3.16C). Interestingly, IL-6 also appeared to decrease the DRSP-induced increase in the invasion of the MDA-MB-231 cell line, while IL-6 had no effect

on MPA-induced invasion (Figure 3.16C). Taken together, these results suggest that TNFα may decrease the invasion of MDA-MB-231 breast cancer cells, while MPA and DRSP as well as IL-6 may increase invasion. Moreover, co-treatment with MPA or DRSP, in the presence of either of these proinflammatory cytokines, may decrease breast cancer cell invasion. However, as these results were from a single experiment, no definitive conclusions can be drawn on the effects of MPA, DRSP, IL-6 and TNFα on the invasion of the MDA-MB-231 breast cancer cell line.

Table 4.3 Summary of the effects of E_2, selected progestogens and the inflammatory mediators on the migration and invasion of breast cancer cell lines.

	E_2	P_4	MPA	NET-A	LNG	GES	DRSP	NoMAC	IL-6*	TNFα*
Migration T47D cells	↑	-	↑	↑	↑	↑	↑	↑	↑	↑
Invasion MDA-MB-231	ND	ND	↑	ND	ND	ND	↑	ND	↑	↓

↑ Increase ↓ Decrease - No effect ND Not determined

*Neither IL-6 nor TNFα modulated the effects of the hormones on the migration of T47D breast cancer cells

4.9. Conclusions and future work

Multiple progestins with different structures and biological activities are used in contraception and menopausal HT (Africander *et al.*, 2011; Stanczyk *et al.*, 2013). Alarmingly, a number of these progestins have been associated with increased risk of invasive breast cancer (Rossouw *et al.*, 2002; Million Women Study Collaborators, 2003; Fournier *et al.*, 2008). It is noteworthy that not all progestins have been evaluated in terms of breast cancer risk, and that the exact mechanism whereby some progestins contribute to increased breast cancer risk is not clear. Although these hormones were designed to mimic the activity of P_4 via the PR, it can also act via other steroid receptors such as the GR, AR and ER (Koubovec *et al.*, 2005; Guerra *et al.*, 2013; Stanczyk *et al.*, 2013; Africander *et al.*, 2014; Louw-du Toit *et al.*, 2017). Considering the important roles that these steroid receptors play in breast cancer (Ali and Coombes, 2000; Hopp *et al.*, 2004; Conzen, 2008; Robinson *et al.*, 2011; Rizza *et al.*, 2014; Karamouzis *et al.*, 2015; Thomas and Gustafsson, 2015), we evaluated effects of selected progestins on steroid receptor expression. We provide evidence that all progestins evaluated downregulated the expression of the PR-isoforms, ER-subtypes and the AR, while having no effect on the expression of the GR. In ER^+ breast cancer, the decreased expression of the PR and AR may be associated with a poor prognosis, as it is known that activated PR and AR decrease ERα-mediated signalling (Lanzino *et al.*, 2005; Peters *et al.*, 2009; Rizza *et al.*, 2014; Mohammed *et al.*, 2015). In contrast, the decrease in both ER subtypes, suggests an overall decrease in ER signalling, which may suggest a favourable outcome in terms of breast cancer. However, steroid receptor signalling in breast

cancer is quite complex, and further investigation would be needed to evaluate the precise physiological impact of these results. While we showed that some progestogens increased cell survival, all progestins, unlike P_4, increased migration of T47D breast cancer cells, suggesting that progestins may be associated with an increased risk for developing metastatic breast cancer. Even though we showed that DRSP and NoMAC have no effect on the proliferation of T47D and MCF-7 BUS breast cancer cells, both these hormones enhanced the migration of T47D breast cancer cells. The pro-inflammatory cytokines, IL-6 and TNFα, have also previously been linked to breast cancer risk (MacCiò *et al.*, 2009; MacCiò and Madeddu, 2011; Iyengar *et al.*, 2013). Our hypothesis that breast cancer would be increased in the presence of both progestin and either IL-6 or TNFα was disproved. To the best of our knowledge, we are the first to show that neither IL-6 nor TNFα modulated the effects of the progestins on steroid receptor expression levels in T47D breast cancer cells. We also provide evidence that these cytokines do not modulate the effects of the progestins on proliferation, apoptosis and migration of breast cancer cells. Our results do however confirm that both IL-6 and TNFα increased the migration of T47D breast cancer cells, which may contribute to an aggressive form of breast cancer. In summary, none of the results obtained in this study indicate that the effects of the progestins were exacerbated by the addition of the inflammatory mediators.

The present study has a few shortcomings that should be addressed in future studies. For example, this study investigated a single concentration of the progestins and the proinflammatory cytokines. The serum concentration levels of injectable contraceptives ranges up to ± 60 nM a few days post injection (Focan *et al.*, 2001; Africander *et al.*, 2011; Blode *et al.*, 2012; Gerrits *et al.*, 2013; Louw-du Toit, Perkins, *et al.*, 2016; Louw-du Toit *et al.*, 2017), while serum concentrations of progestins used in HT are not easily found in the literature. However, it has been reported that a typical HT regimen of NET can result in peak plasma concentrations ranging between 3.64-17.7 nM, while oral administration of 0.25 mg LNG displayed a peak level of ± 15 nM within 1-3 h of administration (reviewed in Africander *et al.* 2011). The specific concentrations of IL-6 and TNFα used in this study, were previously used in studies investigating the effects of these cytokines on breast cancer cell proliferation (Chiu *et al.*, 1996; Rubio *et al.*, 2006; Sasser *et al.*, 2007). However, it would be ideal to mimic serum concentrations of these cytokines found in obese women. While we used 5 000 pg/ml IL-6 and 20 000 pg/ml TNFα, serum levels of approximately 76.38 ± 45 pg/ml for IL-6 and 42.02 ± 11.91 pg/ml for TNFα have previously been reported in obese women (Winkler *et al.*, 2003; Park *et al.*, 2005; Feitosa *et al.*, 2013). Therefore, performing dose response analysis may provide better insight into the effects of physiological concentrations of progestins and cytokines on breast cancer biology. This is important as varying concentrations will affect receptor occupancy and lead to different physiological effects (Africander *et al.* 2011).

Apoptosis was measured by evaluating the mRNA expression of pro- and anti-apoptotic genes, as well as the use of a commercial kit to evaluate the activity of caspase-3 and caspase-7. The investigation on the effects on the markers of apoptosis, Bcl-2 and Bax, should be extended to include all progestins in the absence and presence of the inflammatory mediators. A cell undergoing apoptosis displays many morphological and biochemical characteristics, which can differ depending on the inducer of apoptosis, the cell type and when the process of apoptosis is observed. The majority of apoptotic hallmarks can be observed by flow cytometry. Thus, future studies can assess the effects of the progestins and/or the inflammatory mediators using FACS, which will allow the sorting of live and intact cells, cells in early and late apoptosis, as well as necrotic cells (Herzenberg and Sweet, 1976; Schellenberger *et al.*, 2004). A major limitation of the study is that only a single experiment was performed to evaluate the effects of MPA, DRSP, IL-6 and TNFα on invasion of the MDA-MB-231 breast cancer cell line. This assay should thus be repeated for all the progestins in the absence and presence of IL-6 and TNFα. Furthermore, this assay was only performed in the ER^- MDA-MB-231 breast cancer cell line and not in the ER^+ T47D or MCF-7 cell lines. As it is known that conditions such as matrigel concentration and cell density can influence the invasive capability of cell lines (Hall and Brooks, 2014), future studies should include the optimisation of these conditions for the ER^+ breast cancer cell lines.

To improve our understanding of the effects of progestins and the inflammatory mediators in breast cancer, this study can be extended to a model for ER^- breast cancer. For example, effects on the different hallmarks of cancer can be repeated in the MDA-MB-231 cell line. As mentioned, this study already investigated the effects of two of the hormones and both inflammatory mediators on invasion of this cell line, with preliminary results suggesting that both MPA and DRSP as well as IL-6 increase invasion, while TNFα decreases invasion. Moreover, considering that progestins are administered in combination with estrogen in menopausal HT, and some contraceptives, future studies should investigate the progestin responses on the different hallmarks of breast cancer in the presence of E_2.

Finally, it has been suggested that obesity is associated with an increased production of pro-inflammatory cytokines such as IL-6 and TNFα which may contribute to the development and progression of breast cancer (Cole, 2009; MacCiò and Madeddu, 2011). Therefore, the focus of this study included the investigation of the effects of these two cytokines, also called adipokines when secreted by adipose cells, on breast cancer biology. However, it is also known that numerous other adipokines such as leptin and adiponectin, as well as IL-1β, also play important roles in breast cancer development and progression (MacCiò and Madeddu, 2011; Iyengar *et al.*, 2013). Elevated levels of leptin and IL-1β is suggested to induce proliferation of breast cancer cells and are associated with increased breast cancer risk (MacCiò and Madeddu, 2011; Iyengar *et al.*, 2013; Zahid *et al.*, 2018).

Thus, the role of these and other adipokines, combined with progestins, should also be investigated. Finally, to directly evaluate the effects of progestins, adiposity and its associated inflammation on breast cancer, adipose cells treated with progestins can be co-cultured with breast cancer cells.

REFERENCES

Abduljabbar, R., Negm, O. H., Lai, C., Jerjees, D. A., Al-Kaabi, M., Hamed, M. R., Tighe, P. J., Buluwela, L., Mukherjee, A., Green, A. R., Ali, S., Rakha, E. A. and Ellis, I. O. (2015) 'Clinical and biological significance of glucocorticoid receptor (GR) expression in breast cancer', *Breast Cancer Research and Treatment*. Springer US, 150(2), pp. 335–346.

Africander, D., Louw, R. and Hapgood, J. P. (2013) 'Investigating the anti-mineralocorticoid properties of synthetic progestins used in hormone therapy', *Biochemical and Biophysical Research Communications*, 433(3), pp. 305–310.

Africander, D. and Storbeck, K. H. (2018) 'Steroid metabolism in breast cancer: Where are we and what are we missing?', *Molecular and Cellular Endocrinology*. Elsevier Ireland Ltd, 466, pp. 86–97.

Africander, D., Storbeck, K. and Hapgood, J. (2014) 'A comparative study of the androgenic properties of progesterone and the progestins, medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A)', *Journal of Steroid Biochemistry and Molecular Biology*. Elsevier Ltd, 143, pp. 404–415.

Africander, D., Verhoog, N. and Hapgood, J. (2011) 'Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception', *Steroids*. Elsevier Inc., 76(7), pp. 636–652.

Agoff, S. N., Swanson, P. E., Linden, H., Hawes, S. E. and Lawton, T. J. (2003) 'Androgen Receptor Expression in Estrogen Receptor – Negative Breast Cancer Immunohistochemical , Clinical , and Prognostic Associations', *American Society for Clinical Pathology*, pp. 725–731.

Albini, A., Graf, J., Kitten, G. T., Kleinman, H. K., Martin, G. R., Veillettet, A. and Lippmant, M. E. (1986) 'Estradiol regulates and v-Ha-ras transfection constitutively enhances MCF7 breast cancer cell interactions with basement membrane', *Proc. Natl. Acad. Sci*, 83, pp. 8182–8186.

Ali, S. and Coombes, R. C. (2000) 'Estrogen receptor alpha in human breast cancer: occurrence and significance', *Journal of Mammary Gand Biology and Neoplasia*, 5(3), pp. 271–281.

Andò, S., De Amicis, F., Rago, V., Carpino, A., Maggiolini, M., Panno, M. . and Lanzino, M. (2002) 'Breast cancer: from estrogen to androgen receptor', *Molecular and Cellular Endocrinology*. Elsevier, 193(1–2), pp. 121–128.

Aranda, P. S., LaJoie, D. M. and Jorcyk, C. L. (2012) 'Bleach Gel: A Simple Agarose Gel for Analyzing RNA Quality', *Electrophoresis*, 33(2), pp. 366–369.

Ariazi, E. A., Leitão, A., Oprea, T. I., Chen, B., Louis, T., Bertucci, A. M., Sharma, C. G. N., Gill, S. D., Kim, H. R., Shupp, H. A., Pyle, J. R., Madrack, A., Donato, A. L., Cheng, D., Paige, J. R. and Jordan, V. C. (2007) 'Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen.', *Molecular cancer therapeutics*. American Association for Cancer Research, 6(11), pp. 2817–2827.

Aspinall, S. R., Stamp, S., Davison, A., Shenton, B. K. and Lennard, T. W. J. (2004) 'The proliferative effects of 5-androstene-3β,17β-diol and 5α-dihydrotestosterone on cell cycle analysis and cell proliferation in MCF7, T47D and MDAMB231 breast cancer cell lines', *The Journal of Steroid Biochemistry and Molecular Biology*. Pergamon, 88(1), pp. 37–51.

Avenant, C., Ronacher, K., Stubsrud, E., Louw, A. and Hapgood, J. P. (2010) 'Role of ligand-dependent GR phosphorylation and half-life in determination of ligand-specific transcriptional activity', *Molecular and Cellular Endocrinology*. Elsevier, 327(1–2), pp. 72–88.

Badache, A. and Hynes, N. E. (2001) 'Interleukin 6 Inhibits Proliferation and, in Cooperation with an Epidermal Growth Factor Receptor Autocrine Loop, Increases Migration of T47D Breast Cancer Cells', *Cancer Research*, 61(1), pp. 383–391.

Baisch, H. (2002) 'Elevated Ki-67 expression is correlated with TNFα- and IFNγ-induced apoptosis in tumour cells', *Cell Proliferation*. Wiley/Blackwell (10.1111), 35(6), pp. 333–342.

Balkwill, F. and Mantovani, A. (2001) 'Inflammation and cancer: back to Virchow?', *The Lancet*, 357, pp. 539–545.

Barkhem, T., Carlsson, B. O., Nilsson, Y., Enmark, E. V. A. and Gustafsson, J.-åke (1998) 'Differential Response of Estrogen Receptor α and Estrogen Receptor β to Partial Estrogen Agonists / Antagonists', *Molecular Pharmacology*, 54, pp. 105–112.

Barnes, P. J. (1998) 'Anti-inflammatory actions of glucocorticoids: molecular mechanisms.', *Clinical science (London, England : 1979)*. Portland Press Limited, 94(6), pp. 557–72.

Beatson, G. T. (1896) 'On the Treatment of Inoperable Cases of Carcinoma of the Mamma: Suggestions for a New Method of Treatment, with Illustrative Cases.', *Transactions. Medico-Chirurgical Society of Edinburgh*. Biomedical Journal Digitization Project, 15, pp. 153–179.

Birrell, S. N., Bentel, J. M., Hickey, T. E., Ricciardelli, C., Weger, M. A., Horsfall, D. J. and Tilley, W. D. (1995) 'Androgens induce divergent proliferative responses in human breast cancer cell lines', *The Journal of Steroid Biochemistry and Molecular Biology*. Pergamon, 52(5), pp. 459–467.

Birrell, S. N., Hall, R. E. and Tilley, W. D. (1998) 'Role of the Androgen Receptor in Human Breast Cancer', *Journal of Mammary Gland Biology and Neoplasia*, 3(1), pp. 95–103.

Blode, H., Kowal, K., Roth, K. and Reif, S. (2012) 'Pharmacokinetics of drospirenone and ethinylestradiol in Caucasian and Japanese women.', *The European journal of contraception & reproductive health care*. Taylor & Francis, 17(4), pp. 284–97.

Botella, J., Duranti, E., Duck, I., Cognet, A. M., Delansorne, R. and Paris, J. (1994) 'Inhibition by nomegestrol acetate and other synthetic progestins on proliferation and progesterone receptor content of T47-D human breast cancer cells', *Journal of Steroid Biochemistry and Molecular Biology*, 50(1), pp. 41–47.

Bourguet, W., Germain, P. and Gronemeyer, H. (2000) 'Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications.', *Trends in pharmacological sciences*, 21(10), pp. 381–388.

van den Brûle, F. A., Engel, J., Stetler-Stevenson, W. G., Liu, F. T., Sobel, M. E. and Castronovo, V. (1992) 'Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progestin in human breast-cancer cells.', *International journal of cancer*. Wiley-Liss Inc., 52(4), pp. 653–7.

Van den Burg, B., Kalkhoven, E., Isbrücker, L. and de Laat, S. (1992) 'Effects of progestins on the proliferation of estrogen-dependent human breast cancer cells under growth factor-defined conditions', *The Journal of Steroid Biochemistry and Molecular Biology*, 42(5), pp. 457–465.

Burnstein&, K. L., Jewell, C. M. and Cidlowski, J. A. (1990) 'Human Glucocorticoid Receptor cDNA Contains Sequences Sufficient for Receptor Down-regulation', *The Journal of Biological Chemistry*, 265(13), pp. 7264–7291.

Burnstein, K. L., Jewell, C. M., Sar, M. and Cidlowski, J. A. (1994) 'lntragenic Sequences of the Human Glucocorticoid Receptor Complementary DNA Mediate Hormone-Inducible Receptor Messenger RNA Down-Regulation through Multiple Mechanisms', *Molecular Endocrinology*, 8(12), pp. 1764–1773.

Busillo, J. M., Azzam, K. M. and Cidlowski, J. A. (2011) 'Glucocorticoids sensitize the innate immune system through regulation of the NLRP3 inflammasome.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 286(44), pp. 38703–38713.

Buxant, F., Engohan-aloghe, C. and Noël, J. (2010) 'Estrogen Receptor, Progesterone Receptor, and Glucocorticoid Receptor Expression in Normal Breast Tissue, Breast In Situ Carcinoma, and Invasive Breast Cancer', *Applied Immunohistochemistry &amp*. Applied Immunohistochemistry & Molecular Morphology, 18(3), pp. 254–257.

Cabal-hierro, L. and Lazo, P. S. (2012) 'Signal transduction by tumor necrosis factor receptors', *Cellular Signalling*. Elsevier Inc., 24(6), pp. 1297–1305.

Cai, X., Cao, C., Li, J., Chen, F., Zhang, S., Liu, B., Zhang, W., Zhang, X. and Ye, L. (2017) 'Inflammatory factor TNF-α promotes the growth of breast cancer via the positive feedback loop of TNFR1/NF-κB (and/or p38)/p-STAT3/HBXIP/TNFR1.', *Oncotarget*. Impact Journals, LLC, 8(35), pp. 58338–58352.

Cailleau, R., Young, R., Olivé, M. and Reeves, W. J. (1974) 'Breast Tumor Cell Lines From Pleural Effusions', *Journal of the National Cancer Institute*, 53(3).

Catherino, W. H., Jeng, M. H. and Jordan, V. C. (1993) 'Norgestrel and gestodene stimulate breast cancer cell growth through an oestrogen receptor mediated mechanism', *Br. J. Cancer*, 67, pp. 945–952.

Cauley, J. A., Lucas, F. L., Kuller, L. H., Stone, K., Browner, W. and Cummings, S. R. (1999) 'Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Study of Osteoporotic Fractures Research Group.', *Annals of internal medicine*, 130(4 Pt 1), pp. 270–7.

Chang, C., Lee, S. O., Wang, R.-S., Yeh, S. and Chang, T.-M. (2013) 'Androgen receptor (AR) physiological roles in male and female reproductive systems: lessons learned from AR-knockout mice lacking AR in selective cells', *Biology of reproduction*. Oxford University Press, 89(1), p. 21.

Chang, E. C., Frasor, J., Komm, B. and Katzenellenbogen, B. S. (2006) 'Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells.', *Endocrinology*, 147(10), pp. 4831–4842.

Cheang, M. C. U., Chia, S. K., Voduc, D., Gao, D., Leung, S., Snider, J., Watson, M., Davies, S., Bernard, P. S., Parker, J. S., Perou, C. M., Ellis, M. J. and Nielsen, T. O. (2009) 'Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer', *Articles | JNCI*, 101(10), pp. 736–750.

Chen, F. P., Chien, M. H., Chen, H. Y., Huang, T. S. and Ng, Y. T. (2013) 'Effects of estradiol and progestogens on human breast cells: Regulation of sex steroid receptors', *Taiwanese Journal of Obstetrics and Gynecology*. Elsevier Taiwan LLC, 52(3), pp. 365–373.

Chen, T. R. (1977) 'In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain', *Experimental cell research*, 104(2), pp. 255–262.

Chervenak, J. (2009) 'Bioidentical hormones for maturing women', *Maturitas*, 64, pp. 86–89.

Chiu, J. J., Sgagias, M. K. and Cowan, K. H. (1996) 'Interleukin 6 Acts as a Paracrine Growth Factor in Human Mammary Carcinoma Cell Lines', *Clinical Cancer Research*, 2, pp. 215–221.

Chlebowski, R., Cuzick, J., Amakye, D., Bauerfeind, I., Buzdar, A., Chia, S., Cutuli, B., Linforth, R., Maass, N., Noguchi, S., Robidoux, A., Verma, S. and Peyman, H. (2009) 'Clinical perspectives on the utility of aromatase inhibitors for the adjuvant treatment of breast cancer', *The Breast*, 18, pp. 1–11.

Chottanapund, S., Van Duursen, M. B. M., Navasumrit, P., Hunsonti, P., Timtavorn, S., Ruchirawat, M. and Van Den Berg, M. (2013) 'Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts', *Journal of Steroid Biochemistry and Molecular Biology*,

138, pp. 54–62.

Ciruelos, E., Pascual, T., Luisa, M., Vozmediano, A., Blanco, M., Manso, L., Parrilla, L., Muñoz, C., Vega, E., Calderón, M. J., Sancho, B. and Cortes-Funes, H. (2014) 'The therapeutic role of fulvestrant in the management of patients with hormone receptor-positive breast cancer', *The Breast*, 23, pp. 201–208.

Cleary, M. P. and Grossmann, M. E. (2009) 'Minireview: Obesity and Breast Cancer: The Estrogen Connection', *The Endocrine Society*, 150, pp. 2537–2542.

Cole, S. W. (2009) 'Chronic inflammation and breast cancer recurrence', *Journal of Clinical Oncology*, 27(21), pp. 3418–3419.

Collaborative Group EBCTC (1998) 'Tamoxifen for early breast cancer: an overview of the randomised trials.', *The Lancet*, 351(9114), pp. 1451–1467.

Conzen, S. D. (2008) 'Nuclear receptors and breast cancer', *Molecular endocrinology*, 22(10), pp. 2215–2228.

Cops, E. J., Bianco-Miotto, T., Moore, N. L., Clarke, C. L., Birrell, S. N., Butler, L. M. and Tilley, W. D. (2008) 'Antiproliferative actions of the synthetic androgen, mibolerone, in breast cancer cells are mediated by both androgen and progesterone receptors', *Journal of Steroid Biochemistry and Molecular Biology*, 110(3–5), pp. 236–243.

Cordina-Duverger, E., Truong, T., Anger, A., Sanchez, M., Arveux, P., Kerbrat, P. and Guénel, P. (2013) 'Risk of Breast Cancer by Type of Menopausal Hormone Therapy: a Case-Control Study among Post-Menopausal Women in France', *PLoS ONE*. Edited by W. B. Coleman, 8(11), p. e78016.

Courtin, A., Communal, L., Vilasco, M., Cimino, D., Mourra, N., de Bortoli, M., Taverna, D., Faussat, A. M., Chaouat, M., Forgez, P. and Gompel, A. (2012) 'Glucocorticoid receptor activity discriminates between progesterone and medroxyprogesterone acetate effects in breast cells', *Breast cancer Res Treat*, 131(1), pp. 49–63.

Cox, D. G., Blanché, H., Pearce, C. L., Calle, E. E., Colditz, G. A., Pike, M. C., Albanes, D., Allen, N. E., Amiano, P., Berglund, G., Boeing, H., Buring, J., Burtt, N., Canzian, F., Chanock, S., Clavel-Chapelon, F., Feigelson, H. S., *et al.* (2006) 'A comprehensive analysis of the androgen receptor gene and risk of breast cancer: results from the National Cancer Institute Breast and Prostate Cancer Cohort Consortium (BPC3).', *Breast cancer research : BCR*. BioMed Central, 8(5), p. R54.

Cruz-topete, D. and Cidlowski, J. A. (2016) 'One Hormone Two Actions: Anti- and Pro-inflammatory Effects of Glucocorticoids', *Neuroimmunomodulation*, 22, pp. 20–32.

Cuzick, J. (2008) 'Hormone replacement therapy and the risk of breast cancer', *European Journal of Cancer*, 44(16), pp. 2344–2349.

Dai, X., Li, T., Bai, Z., Yang, Y., Liu, X., Zhan, J. and Shi, B. (2015) 'Breast cancer intrinsic subtype classification, clinical use and future trends', *Am J Cancer Res*, 5(10), pp. 2929–2943.

Dai, X., Xiang, L., Li, T. and Bai, Z. (2016) 'Cancer Hallmarks, Biomarkers and Breast Cancer Molecular Subtypes', *Journal of cancer*, 7(10), pp. 1281–1294.

Daniel, A. R., Gaviglio, A. L., Knutson, T. P., Ostrander, J. H., D'Assoro, A. B., Ravindranathan, P., Peng, Y., Raj, G. V, Yee, D. and Lange, C. A. (2015) 'Progesterone receptor-B enhances estrogen responsiveness of breast cancer cells via scaffolding PELP1- and estrogen receptor-containing transcription complexes', *Oncogene*. Nature Publishing Group, 34(4), pp. 506–515.

Deinzer, R., Granrath, N., Stuhl, H., Twork, L., Idel, H., Waschul, B. and Herforth, A. (2004) 'Acute stress effects on local Il-1β responses to pathogens in a human in vivo model', *Brain, Behavior, and*

Immunity. Academic Press, 18(5), pp. 458–467.

DeMayo, F. J., Zhao, B., Takamoto, N. and Tsai, S. Y. (2002) 'Mechanisms of action of estrogen and progesterone', *Ann N Y Acad Sci*, 955, p. 48,396-406.

Dhabhar, F. S. (2002) 'Stress-induced augmentation of immune function—The role of stress hormones, leukocyte trafficking, and cytokines', *Brain, Behavior, and Immunity*. Academic Press, 16(6), pp. 785–798.

Diaz, J., Aranda, E., Henriquez, S., Quezada, M., Espinoza, E., Bravo, M. L., Olivia, B., Lange, S., Villalon, M., Jones, M., Brosens, J. J., Kato, S., Cuello, M. A., Knutson, T. P., Lange, C. A., Leyton, L. and Owen, G. I. (2012) 'Progesterone promotes focal adhesion formation and migration in breast cancer cells through induction of protease-activated receptor-1', *Journal of Endocrinolody*, 214(2), pp. 165–175.

Diep, C. H., Daniel, A. R., Mauro, L. J., Knutson, T. P. and Lange, C. A. (2015) 'Progesterone action in breast, uterine, and ovarian cancers', *J Mol Endocrinol*, 54(2), pp. 31–53.

Diest, P. J. Van, Wall, E. Van Der and Baak, J. P. A. (2004) 'Prognostic value of proliferation in invasive breast cancer: a review', *J Clin Pathol*, 57, pp. 675–681.

Dimitrakakis, C. and Bondy, C. (2009) 'Androgens and the breast.', *Breast cancer research : BCR*. BioMed Central, 11(5), p. 212.

Dirat, B., Bochet, L., Dabek, M., Ele Daviaud, D., Ephanie Dauvillier, S., Majed, B., Wang, Y. Y., Meulle, A., Salles, B., Le Gonidec, S., Garrido, I., Escourrou, G., Valet, P. and Muller, C. (2011) 'Cancer-Associated Adipocytes Exhibit an Activated Phenotype and Contribute to Breast Cancer Invasion', *American Association for Cancer Research*, 71(7), pp. 2455–2465.

Doan, T. B., Graham, J. D. and Clarke, C. L. (2017) 'Emerging functional roles of nuclear receptors in breast cancer', *Journal of Molecular Endocrinology*, 58(3), pp. 169–190.

Doane, A. S., Danso, M., Lal, P., Donaton, M., Zhang, L., Hudis, C. and Gerald, W. L. (2006) 'An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen', *Oncogene*. Nature Publishing Group, 25(28), pp. 3994–4008.

Dong, Y., Poellinger, L., Gustafsson, J. and Okret, S. (1988) 'Regulation of Glucocorticoid Receptor Expression: Evidence for Transcriptional and Posttranslational Mechanisms', *Molecular Endocrinology*, 2(12), pp. 1256–1264.

Esquivel-vela, M., Ostoa-saloma, P., Palacios-arreola, M. I., Nava-castro, K. E., Castro, J. I. and Morales-montor, J. (2015) 'The Role of Cytokines in Breast Cancer Development and progression', *Journal of interferon and cytokine research*, 35(1), pp. 1–16.

Evan, G. I. and Vousden, K. H. (2001) 'Proliferation, cell cycle and apoptosis in cancer', *Nature*, 411, pp. 342–348.

Fan, W., Chang, J. and Fu, P. (2015) 'Endocrine therapy resistance in breast cancer: current status, possible mechanisms and overcoming strategies.', *Future Medicinal Chemistry*. Future Science Group, 7(12), pp. 1511–1519.

Feghali, C. A., Ph, D. and Wright, T. M. (1997) 'Cytokines in acute and chronic inflammation', *Frontiers in Bioscience*, 2, pp. 12–26.

Feitosa, M. C. P., Lima, V. B. S., Neto, J. M. M. and Marreiro, D. do N. (2013) 'Plasma concentration of IL-6 and TNF-α and its relationship with zincemia in obese women', *Rev. Assoc. Med. Bras.*, 59(5), pp. 429–434.

Fernandez-Real, J. M. and Ricart, W. (2003) 'Insulin Resistance and Chronic Cardiovascular Inflammatory Syndrome', *Endocrine Reviews*, 24(3), pp. 278–301.

Fernando, R. I. and Wimalasena, J. (2004) 'Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt.', *Molecular biology of the cell*. American Society for Cell Biology, 15(7), pp. 3266–3284.

Focan, C., Beauduin, M., Salamon, E., de Greve, J., de Wasch, G., Lobelle, J. P., Majois, F., Tagnon, A., Tytgat, J., van Belle, S., Vandervellen, R. and Vindevoghel, A. (2001) 'Adjuvant high-dose medroxyprogesterone acetate for early breast cancer: 13 years update in a multicentre randomized trial.', *British journal of cancer*. Nature Publishing Group, 85(1), pp. 1–8.

Formby, B. and Wiley, T. S. (1998) 'Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53.', *Annals of clinical and laboratory science*, 28(6), pp. 360–9.

Formby, B. and Wiley, T. S. (1999) 'Bcl-2, survivin and variant CD44 v7-v10 are downregulated and p53 is upregulated in breast cancer cells by progesterone: Inhibition of cell growth and induction of apoptosis', *Molecular and Cellular Biochemistry*, 202, pp. 53–61.

Fournier, A., Berrino, F., Riboli, E., Avenel, V. and Clavel-Chapelon, F. (2005) 'Breast cancer risk in relation to different types of hormone replacement therapy in the E3N-EPIC cohort', *Int. J. Cancer*, 114, pp. 448–454.

Fournier, A., Berrino, F. and Clavel-Chapelon, F. (2008) 'Unequal risks for breast cancer associated with different hormone replacement therapies: results from the E3N cohort study.', *Breast cancer research and treatment*. Inserm, 107(1), pp. 103–111.

Franke, H. R. and Vermes, I. (2003) 'Differential effects of progestogens on breast cancer cell lines', *MAturitas*, 46(1), pp. 55–58.

Fu, X., Giretti, M. S., Goglia, L., Flamini, M. I., Sanchez, A. M., Baldacci, C., Garibaldi, S., Sitruk-Ware, R., Genazzani, A. R. and Simoncini, T. (2008) 'Comparative actions of progesterone, medroxyprogesterone acetate, drospirenone and nestorone on breast cancer cell migration and invasion', *BMC cancer*. BioMed Central, 8, p. 166.

Fu, X., Goglia, L., Sanchez, A. M., Flamini, M., Giretti, M. S., Tosi, V., Genazzani, A. R. and Simoncini, T. (2010) 'Progesterone receptor enhances breast cancer cell motility and invasion via extranuclear activation of focal adhesion kinase', *Endocrine-Related Cancer*, 17(2), pp. 431–443.

Furuya, Y., Kohno, N., Fujiwara, Y. and Saitoh, Y. (1989) 'Mechanisms of Estrogen Action on the Proliferation of MCF-7 Human Breast Cancer Cells in an Improved Culture Medium', *Cancer Research*, 49, pp. 6670–6674.

Gach, K., Szemraj, J., Wyrebska, A. and Janecka, A. (2011) 'The influence of opioids on matrix metalloproteinase-2 and -9 secretion and mRNA levels in MCF-7 breast cancer cell line', *Mol Biol Rep*, 38, pp. 1231–1236.

Gallo, M., Frezzetti, D., Roma, C., Chicchinelli, N., Barbieri, A., Arra, C., Scognamiglio, G., Botti, G., De Luca, A. and Normanno, N. (2018) 'RANTES and IL-6 cooperate in inducing a more aggressive phenotype in breast cancer cells', *Oncotarget*, 9(25), pp. 17543–17553.

Gerrits, M. G. F., Schnabel, P. G., Post, T. M. and Peeters, P. A. M. (2013) 'Pharmacokinetic profile of nomegestrol acetate and 17β-estradiol after multiple and single dosing in healthy women', *Contraception*, 87(2), pp. 193–200.

Giovannelli, P., Di Donato, M., Galasso, G., Di Zazzo, E., Bilancio, A. and Migliaccio, A. (2018) 'The Androgen Receptor in Breast Cancer.', *Frontiers in endocrinology*. Frontiers Media SA, 9, p.

492.

Giulianelli, S., Vaqué, J. P., Soldati, R., Wargon, V., Vanzulli, S. I., Martins, R., Zeitlin, E., Molinolo, A. A., Helguero, L. A., Lamb, C. A., Gutkind, J. S. and Lanari, C. (2012) 'Estrogen receptor alpha mediates progestin-induced mammary tumor growth by interacting with progesterone receptors at the Cyclin D1/MYC promoters', *American Association for cancer*, 72, pp. 2416–2427.

Glaser, R. L. and Dimitrakakis, C. (2013) 'Reduced breast cancer incidence in women treated with subcutaneous testosterone, or testosterone with anastrozole: A prospective, observational study', *Maturitas*, 76(4), pp. 342–349.

Gompel, A., Somaï, S., Chaouat, M., Kazem, A., Kloosterboer, H. J., Beusman, I., Forgez, P., Mimoun, M. and Rostène, W. (2000) 'Hormonal regulation of apoptosis in breast cells and tissues', *Steroids*. Elsevier, 65(10–11), pp. 593–598.

Gordon, L., Mulligan, K., Maxwell-Jones, H., Adams, M., Walker, R. and Jones, J. (2003) 'Breast cell invasive potential relates to the myoepithelial phenotype.', *Int J Cancer*, 106(1), pp. 8–16.

Greendale, G. A., Lee, N. P. and Arriola, E. R. (1999) 'The menopause', *The Lancet*, 353, pp. 571–580.

Griekspoor, A., Zwart, W., Neefjes, J. and Michalides, R. (2007) 'Visualizing the action of steroid hormone receptors in living cells', *Nuclear Receptor Signaling*, 5, pp. 1–9.

Grivennikov, S. I., Greten, F. R. and Karin, M. (2011) 'Immunity, Inflammation, and Cancer', *Cell*, 140(6), pp. 883–899.

Groshong, S. D., Owen, G. I., Grimison, B., Schauer, I. E., Todd, M. C., Langan, T. A., Sclafani, R. A., Lange, C. A. and Horwitz, K. B. (1997) 'Biphasic Regulation of Breast Cancer Cell Growth by Progesterone: Role of the Cyclin-Dependent Kinase Inhibitors, p21 and p27Kip1', *Molecular Endocrinology*, 11(11), pp. 1593–1607.

Grott, M., Karakaya, S., Mayer, F., Baertling, F., Beyer, C., Kipp, M. and Kopp, H.-G. (2013) 'Progesterone and estrogen prevent cisplatin-induced apoptosis of lung cancer cells.', *Anticancer research*. International Institute of Anticancer Research, 33(3), pp. 791–800.

Gucalp, A., Tolaney, S., Isakoff, S. J., Ingle, J. N., Liu, M. C., Carey, L. A., Blackwell, K., Rugo, H., Nabell, L., Forero, A., Stearns, V., Doane, A. S., Danso, M., Ellen Moynahan, M., Momen, L. F., Gonzalez, J. M., Akhtar, A., *et al.* (2013) 'Phase II Trial of Bicalutamide in Patients with Androgen Receptor Positive, Hormone Receptor Negative Metastatic Breast Cancer', *American Association of Cancer*.

Guerra, J. A., Lopez-Munoz, F. and Alamo, C. (2013) 'Progestins in combined contraceptives', *Journal of Experimental and Clinical Medicine(Taiwan)*. Elsevier Taiwan LLC, 5(2), pp. 51–55.

Guo, J., Jiang, C., Wang, Z., Lee, H., Hu, H., Malewicz, B., Lee, H., Lee, J., Baek, N., Jeong, J., Kim, D., Kang, K., Kim, S. and Lu, J. (2007) 'A novel class of pyranocoumarin anti-androgen receptor signaling compounds', *Molecular Cancer Therapeutics*, 6(3), pp. 907–917.

Gustafsson, J. . (1999) 'Estrogen receptor β– a new dimension in estrogen mechanism of action', *Journal of Endocrinology*, 163, pp. 379–383.

Gyamfi, J., Eom, M., Koo, J. S. and Choi, J. (2018) 'Multifaceted Roles of Interleukin-6 in Adipocyte–Breast Cancer Cell Interaction', *Translational Oncology*. Oncoinvent AS, 11(2), pp. 275–285.

Hagemann, T., Wilson, J., Kulbe, H., Li, N. F., Leinster, D. A., Charles, K., Klemm, F., Pukrop, T., Binder, C. and Balkwill, F. R. (2005) 'Macrophages Induce Invasiveness of Epithelial Cancer Cells

Via NF-κB and JNK', *The Journal of Immunology*, 175(2), pp. 1197–1205.

Hall, D. M. S. and Brooks, S. A. (2014) 'In Vitro Invasion Assay Using Matrigel™: A Reconstituted Basement Membrane Preparation', in *Metastasis Research Protocols*. Humana Press, New York, NY, pp. 1–11.

Hanahan, D. and Weinberg, R. A. (2011) 'Hallmarks of Cancer: The Next Generation', *Cell*, 144(March), pp. 646–674.

Hanahan, D., Weinberg, R. A. and Francisco, S. (2000) 'The Hallmarks of Cancer', *Cell*, 100, pp. 57–70.

Hao, Y., Montiel, R., Li, B., Huang, E., Zeng, L. and Huang, Y. (2010) 'Association between androgen receptor gene CAG repeat polymorphism and breast cancer risk: a meta-analysis', *Breast Cancer Research and Treatment*. Springer US, 124(3), pp. 815–820.

Hapgood, J. P., Koubovec, D., Louw, A. and Africander, D. (2004) 'Not all progestins are the same: implications for usage', *Pharmacological sciences*, 25(11), pp. 554–557.

Hapgood, J. P., Africander, D., Louw, R., Ray, R. M. and Rohwer, J. M. (2013) 'Potency of progestogens used in hormonal therapy: Toward understanding differential actions', *Journal of Steroid Biochemistry and Molecular Biology*. Elsevier Ltd, pp. 1–9.

Helguero, L. A., Faulds, M. H., Gustafsson, J.-Å. and Haldosén, L.-A. (2005) 'Estrogen receptors alfa (ERα) and beta (ERβ) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11', *Oncogene*. Nature Publishing Group, 24(44), pp. 6605–6616.

Henderson, B. E., Ross, R. and Bernstein, L. (1988) 'Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture', *Cancer research*. American Association for Cancer Research, 48(2), pp. 246–53.

Herzenberg, L. A. and Sweet, R. (1976) 'Fluorescence-activated Cell Sorting', *Scientific American*, 234(3), pp. 108–118.

Heusseneugene, C. (1980) 'Electrophoretic Analysis of Plasminogen Activators in Polyacrylamide Gels Containing Sodium Dodecyl Sulfate and Copolymerized Substrates', *Analytical Biochemistry*, 202, pp. 196–202.

Holton, S. E., Bergamaschi, A., Katzenellenbogen, B. S. and Bhargava, R. (2014) 'Integration of Molecular Profiling and Chemical Imaging to Elucidate Fibroblast-Microenvironment Impact on Cancer Cell Phenotype and Endocrine Resistance in Breast Cancer', *PLoS ONE*. Edited by A. Aboussekhra, 9(5), p. e96878.

Honma, N., Horii, R., Iwase, T., Saji, S., Younes, M., Takubo, K., Matsuura, M., Ito, Y., Akiyama, F. and Sakamoto, G. (2008) 'Clinical Importance of Estrogen Receptor-β Evaluation in Breast Cancer Patients Treated With Adjuvant Tamoxifen Therapy', *Journal of Clinical Oncology*, 26(22), pp. 3727–3734.

Hopp, T. A., Weiss, H. L., Hilsenbeck, S. G., Cui, Y., Allred, D. C., Horwitz, K. B. and Fuqua, S. A. W. (2004) 'Breast Cancer Patients with Progesterone Receptor PR-A-Rich Tumors Have Poorer Disease-Free Survival Rates', *Clinical Cancer Research* , 10, pp. 2751–2760.

Horwitz, K. B., Zava, D. T., Thilagar, A. K., Jensen, E. M. and Mcguire, W. L. (1978) 'Steroid Receptor Analyses of Nine Human Breast Cancer Cell Lines', *Cancer Research*, 38(August), pp. 2434–2437.

Horwitz, K. B., Costlow, M. E. and McGuire, W. L. (1975) 'MCF-7: A human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors', *Steroids*, 26(6), pp. 785–795.

Horwitz, K. B. and Freidenberg, G. R. (1985) 'Growth Inhibition and Increase of Insulin Receptors in Antiestrogen-resistant T47DCO Human Breast Cancer Cells by Progestins: Implications for Endocrine Therapies', *Cancer Research*, 45, pp. 167–173.

Horwitz, K. B. and Mcguire, W. L. (1978) 'Estrogen Control of Progesterone Receptor in Human Breast Cancer', *Journal of Biological Chemistry*, 253(7), pp. 2223–2228.

Horwitz, K. B. and Mcguire, W. L. (1978) 'Nuclear Mechanisms of Estrogen Action', *Journal of Biological Chemistry*, 253(22), pp. 8185–8191.

Hruby, A. and Hu, F. B. (2015) 'The Epidemiology of Obesity: A Big Picture', *Pharmacoeconomics*, 33(7), pp. 673–689.

Hsiao, Y., Chou, M., Fowler, C., Mason, J. T. and Man, Y. (2010) 'Breast cancer heterogeneity: mechanisms, proofs, and implications', *Journal of Cancer*, 1, pp. 6–13.

Hsieh, C. and Huang, Y. (2016) 'Aspirin Breaks the Crosstalk between 3T3-L1 Adipocytes and 4T1 Breast Cancer Cells by Regulating Cytokine Production', *PLoS ONE*, pp. 1–17.

Hu, R., Dawood, S., Holmes, M. D., Collins, L. C., Stuart, J., Cole, K., Marotti, J. D., Hankinson, S. E., Graham, A. and Tamimi, R. M. (2011) 'Androgen Receptor Expression and Breast Cancer Survival in Postmenopausal Women', *American Association of Cancer*, 10, pp. 1–23.

Hu, X. and Beeton, C. (2010) 'Detection of Functional Matrix Metalloproteinases by Zymography', *Journal of Visualized Experiments*, 45, pp. 1–5.

Ibrahim, S. A., El-Ghonaimy, E. A., Hassan, H., Mahana, N., Mahmoud, M. A., El-Mamlouk, T., El-Shinawi, M. and Mohamed, M. M. (2016) 'Hormonal-receptor positive breast cancer: IL-6 augments invasion and lymph node metastasis via stimulating cathepsin B expression.', *Journal of advanced research*. Elsevier, 7(5), pp. 661–670.

Irahara, N., Miyoshi, Y., Taguchi, T., Tamaki, Y. and Noguchi, S. (2006) 'Quantitative analysis of aromatase mRNA expression derived from various promoters (I.4, I.3 , PII and I.7) and its association with expression of TNF-alpha , IL-6 and COX-2 mRNAs in human breast cancer', *Int. J. Cancer*, 118, pp. 1915–1921.

Ishibashi, H., Suzuki, T., Suzuki, S., Moriya, T., Kaneko, C. and Sasano, H. (2003) 'Sex Steroid Hormone Receptors in Human Thymoma', *Journal of Clinical Endocrinology and Metabolism*, 88(5), pp. 2309–2317.

Iyengar, N. M., Hudis, C. A. and Dannenberg, A. J. D. (2013) 'Obesity and Inflammation: New Insights into Breast Cancer Development and Progression', *Am Soc Clin Oncol Educ Book*, 33, pp. 46–51.

Iyengar, P., Combs, T. P., Shah, S. J., Gouon-Evans, V., Pollard, J. W., Albanese, C., Flanagan, L., Tenniswood, M. P., Guha, C., Lisanti, M. P., Pestell, R. G. and Scherer, P. E. (2003) 'Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization', *Oncogene*, 22, pp. 6408–6423.

Izzo, F., Mercogliano, F., Venturutti, L., Tkach, M., Inurrigarro, G., Schillaci, R., Cerchietti, L., Elizalde, P. V and Proietti, C. J. (2014) 'Progesterone receptor activation downregulates GATA3 by transcriptional repression and increased protein turnover promoting breast tumor growth', *Breast Cancer Research*, 16(6), p. 491.

Jacobsen, B. M. and Horwitz, K. B. (2012) 'Progesterone Receptors, their Isoforms and Progesterone Regulated Transcription', *Mol Cell Endocrinol*, 357(1), pp. 18–29.

Jiang, Q., Wu, T., Yang, J., Dong, C., Wang, N., Liu, X. and Liu, Z. (2013) '17β-Estradiol promotes

the invasion and migration of nuclear estrogen receptor-negative breast cancer cells through cross-talk between GPER1 and CXCR1', *The Journal of Steroid Biochemistry and Molecular Biology*. Pergamon, 138, pp. 314–324.

Jiang, X.-P., Yang, D. C., Elliott, R. L. and Head, J. F. (2011) 'Down-regulation of expression of interleukin-6 and its receptor results in growth inhibition of MCF-7 breast cancer cells.', *Anticancer research*. International Institute of Anticancer Research, 31(9), pp. 2899–2906.

Johnson, S. R. (1998) 'Menopause and hormone replacement therapy', *Medical Clinics of North America*, 82(2), pp. 297–320.

Jordan, V. C. (2007) 'SERMs: Meeting the promise of multifunctional medicines', *Journal of the National Cancer Institute*, 99(5), pp. 350–356.

Kalkhoven, E., Kwakkenbos-Isbrücker, L., de Laat, S. W., van der Saag, P. T. and van der Burg, B. (1994) 'Synthetic progestins induce proliferation of breast tumor cell lines via the progesterone or estrogen receptor', *Molecular and Cellular Endocrinology*, 102(1), pp. 45–52.

Kandouz, M., Lombet, A., Perrot, J.-Y., Jacob, D., Carvajal, S., Kazem, A., Rostene, W., Therwath, A. and Gompel, A. (1999) 'Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells', *Journal of Steroid Biochemistry and Molecular Biology*, 69, pp. 463–471.

Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordó N-Cardo, C., Guise, T. A. and Massagué, J. (2003) 'A multigenic program mediating breast cancer metastasis to bone', *Cancer cell*, 3, pp. 537–549.

Karamouzis, M. V, Papavassiliou, K. A., Adamopoulos, C. and Papavassiliou, A. G. (2015) 'Targeting Androgen/Estrogen Receptors Crosstalk in Cancer.', *Trends in cancer*. Elsevier, 2(1), pp. 35–48.

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. and Chambon, P. (1990) 'Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B.', *The EMBO journal*. European Molecular Biology Organization, 9(5), pp. 1603–1614.

Katerinaki, E., Evans, G. S., Lorigan, P. C. and Macneil, S. (2003) 'TNF- α increases human melanoma cell invasion and migration in vitro: the role of proteolytic enzymes', *British Journal of Cancer*, 89, pp. 1123–1129.

Kelsey, J. L. and Bernstein, L. (1996) 'Epidemiology and the prevention of breast cancer', *Annual Reviews of Public Health*, 17(61), pp. 47–67.

Kenna, N. J. M. C., Lanz, R. B. and O'Malley, B. W. (1999) 'Nuclear Receptor Coregulators: Cellular and Molecular Biology', *Endocrine Reviews*, 20(3), pp. 321–344.

Kershaw, E. E. and Flier, J. S. (2004) 'Adipose Tissue as an Endocrine Organ', *The Journal of Clinical Endocrinology & Metabolism*, 89(6), pp. 2548–2556.

Kinyamu, H. K. and Archer, T. K. (2003) 'Estrogen receptor-dependent proteasomal degradation of the glucocorticoid receptor is coupled to an increase in mdm2 protein expression.', *Molecular and cellular biology*. American Society for Microbiology Journals, 23(16), pp. 5867–5881.

Klinge, C. M. (2001) 'Estrogen receptor interaction with estrogen response elements', *Nucleic Acids Research*, 29(14), pp. 2905–2919.

Klinge, C. M., Jernigan, S. C., Mattingly, K. A., Risinger, K. E. and Zhang, J. (2004) 'Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors α and β by coactivators and corepressors', *Journal of Molecular Endocrinology*, 33, pp. 387–410.

Klinkhammer-schalke, E. C. I. M. (2013) 'Ki-67 is a prognostic parameter in breast cancer patients : results of a large population-based cohort of a cancer registry', pp. 539–552.

Knüpfer, H. and Preiss, R. (2007) 'Significance of interleukin-6 (IL-6) in breast cancer (review)', *Breast cancer Res Treat*, 102(2), pp. 129–135.

Koubovec, D., Ronacher, K., Elisabeth, S., Ann, L. and Patricia, H. J. (2005) 'Synthetic progestins used in HRT have different glucocorticoid agonist properties', *Molecular and Cellular Endocrinology*, 242(1–2), pp. 23–32.

Krämer, E. A., Seeger, H., Krämer, B., Wallwiener, D. and Mueck, A. O. (2006) 'The effect of progesterone, testosterone and synthetic progestogens on growth factor-and estradiol-treated human cancerous and benign breast cells', *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 129, pp. 77–83.

Kraus, W. L., Weis, K. E. and Katzenellenbogen, B. S. (1995) 'Inhibitory cross-talk between steroid hormone receptors: differential targeting of estrogen receptor in the repression of its transcriptional activity by agonist- and antagonist-occupied progestin receptors.', *Molecular and cellular biology*, 15(4), pp. 1847–57.

Krishnan, A. V, Swami, S. and Feldman, D. (2001) 'Estradiol inhibits glucocorticoid receptor expression and induces glucocorticoid resistance in MCF-7 human breast cancer cells', *The Journal of Steroid Biochemistry and Molecular Biology*. Pergamon, 77(1), pp. 29–37.

Krongrad, A., Wilson, C. M., Wilson, J. D., Allman, D. R. and McPhaul, M. J. (1991) 'Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells', *Molecular and Cellular Endocrinology*, 76(1–3), pp. 79–88.

Kuiper, G. G. J. M., Enmark, E. V. A., Pelto-huikkot, M., Nilssont, S. and Gustafsson, J. (1996) 'Cloning of a novel estrogen receptor expressed in rat prostate and ovary', *Proc. Natl. Acad. Sci*, 93, pp. 5925–5930.

Lanari, C., Lamb, C. A., Fabris, V. T., Helguero, L. A., Cerliani, J. P., Wargon, V. and Molinolo, A. (2009) 'The MPA mouse breast cancer model: evidence for a role of progesterone receptors in breast cancer', *Endocrine-Related Cancer*, 16, pp. 333–350.

Lanari, C., Wargon, V., Rojas, P. and Molinolo, A. A. (2012) 'Antiprogestins in breast cancer treatment: are we ready?', *Endocrine-Related Cancer*, 13(3), pp. 35–50.

Lanzino, M., De Amicis, F., Mcphaul, M. J., Marsico, S., Panno, M. L. and Andò, S. (2005) 'Endogenous coactivator ARA70 interacts with ERα and modulates the functional ERα/AR interplay in MCF-7 cells', *J. Biol. Chem*, pp. 1–25.

Lanzino, M., Maris, P., Sirianni, R., Barone, I., Casaburi, I., Chimento, A., Giordano, C., Morelli, C., Sisci, D., Rizza, P., Bonofiglio, D., Catalano, S. and Andò, S. (2013) 'DAX-1, as an androgen-target gene, inhibits aromatase expression: a novel mechanism blocking estrogen-dependent breast cancer cell proliferation.', *Cell death & disease*. Nature Publishing Group, 4(7), p. e724.

Lauber, S. N., Ali, S. and Gooderham, N. J. (2004) 'The cooked food derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine is a potent oestrogen: a mechanistic basis for its tissue-specific carcinogenicity', *Carcinogenesis*. Oxford University Press, 25(12), pp. 2509–2517.

Lavery, D. N. and McEwan, I. J. (2005) 'Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations', *Biochem. J*, 391, pp. 449–464.

Lazennec, G., Bresson, D., Lucas, A., Chauveau, C. and Vignon, F. (2001) 'ERβ Inhibits Proliferation and Invasion of Breast Cancer Cells', *Endocrinology*, 142(9), pp. 4120–4130.

Lee, A. V, Oesterreich, S. and Davidson, N. E. (2015) 'MCF-7 Cells — Changing the Course of Breast Cancer Research and Care for 45 Years', *JNCI J Natl Cancer Inst*, 107(7), pp. 1–4.

Lee, D. K. and Chang, C. (2003) 'Expression and Degradation of Androgen Receptor: Mechanism and Clinical Implication', *The Journal of Clinical Endocrinology & Metabolism*. Oxford University Press, 88(9), pp. 4043–4054.

Levin, E. R. (1999) 'Cellular Functions of the Plasma Membrane Estrogen Receptor.', *Trends in endocrinology and metabolism: TEM*, 10(9), pp. 374–377.

Lewis-Wambi, J. S. and Jordan, V. C. (2009) 'Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit?', *Breast cancer research*. BioMed Central, 11(3), p. 206.

Leygue, E. and Murphy, L. C. (2013) 'A bi-faceted role of estrogen receptor β in breast cancer', *Endocrine-Related Cancer*, 20(3), pp. 127–139.

Li, Z., Carrier, L. and Rowan, B. G. (2008) 'Methylseleninic acid synergizes with tamoxifen to induce caspase-mediated apoptosis in breast cancer cells', *Mol Cancer Ther*, 7(9), pp. 3056–63.

Liang, Y., Brekken, R. A. and Hyder, S. M. (2006) 'Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones', *Endocrine-Related Cancer*, 13(3), pp. 905–919.

Liao, X. H., Lu, D. L., Wang, N., Liu, L. Y., Wang, Y., Li, Y. Q., Yan, T. B., Sun, X. G., Hu, P. and Zhang, T. C. (2014) 'Estrogen receptor α mediates proliferation of breast cancer MCF-7 cells via a p21/PCNA/E2F1-dependent pathway', *FEBS Journal*, 281(3), pp. 927–942.

Lim, E., Tarulli, G., Portman, N., Hickery, T., Tilley, W. D. and Palmierie, C. (2016) 'Pushing estrogen receptor around in breast cancer', *Endocrine-Related Cancer*, 23(12), pp. 227–241.

Lim, K. T., Cosgrave, N., Hill, A. D. and Young, L. S. (2006) 'Nongenomic oestrogen signalling in oestrogen receptor negative breast cancer cells: a role for the angiotensin II receptor AT1.', *Breast cancer research : BCR*. BioMed Central, 8(3), p. R33.

Lin, K.-T. and Wang, L.-H. (2016) 'New dimension of glucocorticoids in cancer treatment', *Steroids*. Elsevier, 111, pp. 84–88.

Lin, V. C., Ng, E. H., Aw, S. E., Tan, M. G., Ng, E. H., Chan, V. S. and Ho, G. H. (1999) 'Progestins inhibit the growth of MDA-MB-231 cells transfected with progesterone receptor complementary DNA.', *Clinical cancer research*, 5(2), pp. 395–403.

Lince-Deroche, N., Rayne, S., van Rensburg, C., Benn, C., Masuku, S. and Holele, P. (2017) *Breast cancer in South Africa: developing an affordable and achievable plan to improve detection and survival*, *SAHR*.

Lippman, M., Bolan, G. and Huff, K. (1976) 'The Effects of Glucocorticoids and Progesterone on Hormone responsive Human Breast Cancer in Long-Term Tissue Culture', *Cancer Research*, 36, pp. 4602–4609.

Liu, L., Liu, Y., Zhang, X., Chen, M., Wu, H., Lin, M., Zhan, Y., Zhuang, C., Lin, J., Li, J., Xu, W., Fu, X., Zhang, Q., Sun, X., Zhao, G. and Huang, W. (2016) 'Inhibiting cell migration and cell invasion by silencing the transcription factor ETS-1 in human bladder cancer.', *Oncotarget*. Impact Journals, LLC, 7(18), pp. 25125–34.

Louw-du Toit, R., Perkins, M. S., Snoep, J. L., Storbeck, K. and Africander, D. (2016) 'Fourth-Generation Progestins Inhibit 3β -Hydroxysteroid Dehydrogenase Type 2 and Modulate the Biosynthesis of Endogenous Steroids', *PLoS ONE*, 10, pp. 1–23.

Louw-du Toit, R., Storbeck, K.-H., Cartwrite, M., Cabral, A. and Africander, D. (2016) 'Progestins

used in endocrine therapy and the implications for the biosynthesis and metabolism of endogenous steroid hormones', *Molecular and Cellular Endocrinology*.

Louw-du Toit, R., Perkins, M. S., Hapgood, J. P. and Africander, D. (2017) 'Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy', *Biochemical and Biophysical Research Communications*. Elsevier Ltd, pp. 1–7.

Louw-du Toit, R., Hapgood, J. P. and Africander, D. (2014) 'Medroxyprogesterone acetate differentially regulates interleukin (IL)-12 and IL-10 in a human ectocervical epithelial cell line in a glucocorticoid receptor (GR)-dependent manner', *Journal of Biological Chemistry*, 289(45), pp. 31136–31149.

Lundin, K. B., Henningson, M., Hietala, M., Ingvar, C., Rose, C. and Jernström, H. (2011) 'Androgen receptor genotypes predict response to endocrine treatment in breast cancer patients', *British Journal of Cancer*. Nature Publishing Group, 105(11), pp. 1676–1683.

Mabuchi, S., Ohmichi, M., Kimura, A., Nishio, Y., Arimoto-Ishida, E., Yada-Hashimoto, N., Tasaka, K. and Murata, Y. (2004) 'Estrogen Inhibits Paclitaxel-Induced Apoptosis via the Phosphorylation of Apoptosis Signal-Regulating Kinase 1 in Human Ovarian Cancer Cell Lines', *Endocrinology*. Oxford University Press, 145(1), pp. 49–58.

MacCiò, A. and Madeddu, C. (2011) 'Obesity, Inflammation, and Postmenopausal Breast Cancer: Therapeutic Implications', *The Scientific World Journal*, 11, pp. 2020–2036.

MacCiò, A., Madeddu, C. and Mantovani, G. (2009) 'Adipose tissue as target organ in the treatment of hormone-dependent breast cancer: New therapeutic perspectives', *Obesity Reviews*, 10(6), pp. 660–670.

Majumder, A., Singh, M. and Tyagi, S. C. (2017) 'Post-menopausal breast cancer: from estrogen to androgen receptor.', *Oncotarget*. Impact Journals, LLC, 8(60), pp. 102739–102758.

Mannell, A. (2016) 'The role of Ki-67 in breast cancer', *S Afr J Surg*, 54(2), pp. 10–13.

Marino, M., Galluzzo, P. and Ascenzi, P. (2006) 'Estrogen signaling multiple pathways to impact gene transcription.', *Current genomics*. Bentham Science Publishers, 7(8), pp. 497–508.

Marsden, J. (2003) 'The menopause, hormone replacement therapy and breast cancer', *Journal of Steroid Biochemistry and Molecular Biology*, 83, pp. 123–132.

Martin, T. A., Sanders, A. J., Lane, J. and Jiang, W. G. (2013) 'Cancer Invasion and Metastasis: Molecular and Cellular Perspective', *Madame Curie Bioscience Database*.

Martínez-reza, I., Díaz, L. and García-becerra, R. (2017) 'Preclinical and clinical aspects of TNF-α and its receptors TNFR1 and TNFR2 in breast cancer', *Journal of Biomedical Science*. Journal of Biomedical Science, 24(90), pp. 1–8.

McEwan, I. J., Lavery, D., Fischer, K. and Watt, K. (2007) 'Natural disordered sequences in the amino terminal domain of nuclear receptors: lessons from the androgen and glucocorticoid receptors', *Nuclear Receptor Signaling*, 5, pp. 1–6.

Mcfall, T., Patki, M., Rosati, R. and Ratnam, M. (2015) 'Role of the short isoform of the progesterone receptor in breast cancer cell invasiveness at estrogen and progesterone levels in the pre- and post-menopausal ranges', *Oncotarget*, 6(32), pp. 33146–33164.

McFall, T., McKnight, B., Rosati, R., Kim, S., Huang, Y., Viola-Villegas, N. and Ratnam, M. (2018) 'Progesterone receptor A promotes invasiveness and metastasis of luminal breast cancer by suppressing regulation of critical microRNAs by estrogen', *Journal of Biological Chemistry*, 293(4), pp. 1163–1177.

Million Women Study Collaborators (2003) 'Breast cancer and hormone-replacement therapy in the Million Women Study', *The Lancet*, 362, pp. 419–427.

Mohamed, M. M., Cavallo-Medved, D., Rudy, D., Anbalagan, A., Moin, K. and Sloane, B. F. (2010) 'Interleukin-6 Increases Expression and Secretion of Cathepsin B by Breast Tumor-Associated Monocytes', *Cell Physiol Biochem*, 25, pp. 315–324.

Mohammed, H., Russell, I. A., Stark, R., Rueda, O. M., Theresa, E., Tarulli, G. A., Serandour, A. A. A., Birrell, S. N., Saadi, A., Menon, S., Hadfield, J., Pugh, M., Raj, G. V, Brown, G. D., Santos, C. D., Robinson, J. L. L., Silva, G., *et al.* (2015) 'Progesterone receptor modulates estrogen receptor- α action in breast cancer', *Nature*, 523(7560), pp. 313–317.

Mooney, L. M., Al-sakkaf, K. A., Brown, B. L. and Dobson, P. R. M. (2002) 'Apoptotic mechanisms in T47D and MCF-7 human breast cancer cells', *British Journal of Cancer*, 87, pp. 909–917.

Moore, M. R., Spence, J. B., Kiningham, K. K. and Dillon, J. L. (2006) 'Progestin inhibition of cell death in human breast cancer cell lines', *The Journal of Steroid Biochemistry and Molecular Biology*. Pergamon, 98(4–5), pp. 218–227.

Moore, N. L., Buchanan, G., Harris, J. M., Selth, L. A., Bianco-Miotto, T., Hanson, A. R., Birrell, S. N., Butler, L. M., Hickey, T. E. and Tilley, W. D. (2012) 'An androgen receptor mutation in the MDA-MB-453 cell line model of molecular apocrine breast cancer compromises receptor activity', *Endocrine-Related Cancer*, 19(4), pp. 599–613.

Morley, J. E. and Baumgartner, R. N. (2004) 'Cytokine-Related Aging Process', *Journal of Gerontology*, 59(9), pp. 924–929.

Morris, P. G., Hudis, C. A., Giri, D., Morrow, M., Falcone, D. J., Zhou, X. K., Du, B., Brogi, E., Crawford, C. B., Kopelovich, L. and Dannenberg, A. J. (2012) 'Inflammation and increased aromatase expression occur in the breast tissue of obese women with breast cancer', *Cancer Prev Res*, 4(7), pp. 1021–1029.

Mosmann, T. (1983) 'Rapid Colorimetric Assay for Cellular Growth and Survival : Application to Proliferation and Cytotoxicity Assays', *Journal of lmmunological Methods*, 65, pp. 55–63.

Mote, P. A., Bartow, S., Tran, N. and Clarke, C. L. (2002) 'Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis', *Breast Cancer Research and Treatment*, 72, pp. 163–172.

Murphy, G. and Crabbe, T. (1995) 'Gelatinases A and B.', *Methods in enzymology*, 248, pp. 470–84.

Musgrove, E. A., Lee, C. S. and Sutherland, R. L. (1991) 'Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor alpha, epidermal growth factor receptor, c-fos, and c-myc genes', *Molecular and Cellular Biology*, 11(10), pp. 5032–5043.

Nardulli, A. M. and katzenelenbogen, B. S. (1988) 'Progesterone Receptor Regulation in T47D Human Breast Cancer Cells: Analysis by Density Labeling of Progesterone Receptor Synthesis and Degradation and Their Modulation by Progestin*', *Endocrinology*. Oxford University Press, 122(4), pp. 1532–1540.

Ndlovu, E. (2016) *Crosstalk between the androgen and estrogen receptors in breast cancer*. Stellenbosch : Stellenbosch University.

Nojek, I. M., Kordon, E. C., Rubio, M. F., Werbajh, S., Cafferata, E. G. A., Quaglino, A. and Colo, G. P. (2006) 'TNF- alpha enhances estrogen-induced cell proliferation of estrogen-dependent breast tumor cells through a complex containing nuclear factor-kappa B', *Oncogene*, 25(9), pp. 1367–1377.

O'Connor, K. A., Johnson, J. D., Hansen, M. K., Wieseler Frank, J. L., Maksimova, E., Watkins, L. R. and Maier, S. F. (2003) 'Peripheral and central proinflammatory cytokine response to a severe acute stressor', *Brain Research*. Elsevier, 991(1–2), pp. 123–132.

Ochnik, A. M., Moore, N. L., Jankovic-Karasoulos, T., Bianco-Miotto, T., Ryan, N. K., Thomas, M. R., Birrell, S. N., Butler, L. M., Tilley, W. D. and Hickey, T. E. (2014) 'Antiandrogenic actions of medroxyprogesterone acetate on epithelial cells within normal human breast tissues cultured ex vivo', *Menopause*, 21(1), pp. 79–88.

Ogawa, Y., Hai, E., Matsumoto, K., Ikeda, K., Tokunaga, S., Nagahara, H., Sakurai, K., Inoue, T. and Nishiguchi, Y. (2008) 'Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers', *Clinical Oncology*, 13(5), pp. 431–435.

Ory, K., Lebeau, J., Levalois, C., Bishay, K., Fouchet, P., Allemand, I., Therwath, A. and Chevillard, S. (2001) 'Apoptosis inhibition mediated by medroxyprogesterone acetate treatment of breast cancer cell lines', *Breast Cancer Research and Treatment*, 68, pp. 187–198.

Ouchi, N., Parker, J. L., Lungus, J. J. and Walsh, K. (2011) 'Adipokines in inflammation and metabolic disease', *Nat.Rev.Immunol.*, 11(2), pp. 85–97.

Overmoyer, B., Sanz-Altamira, P., Taylor, R., Michael, L., Hancock, M. and Dalton, J. (2014) 'Enobosarm: A targeted therapy for metastatic, androgen receptor positive, breast cancer.', *J Clin Oncol*, 32, p. 568.

Overmoyer, B. A. and Cancers, S. S. C. for W. (2015) *Efficacy and Safety of GTx-024 in Patients With ER+/AR+ Breast Cancer - Full Text View - ClinicalTrials.gov, Last update posted:June 5, 2018*.

Paech, K., Webb, P., Kuiper, G. G. J. M., Nilsson, S., Gustafsson, J.-åke, Kushner, P. J. and Scanlan, T. S. (1997) 'Differential Ligand Activation of Estrogen Receptors ERα and ERβ at AP1 Sites', *Science*, 277, pp. 1995–1998.

Pan, D., Kocherginsky, M. and Conzen, S. D. (2011) 'Activation of the Glucocorticoid Receptor Is Associated with Poor Prognosis in Estrogen Receptor-Negative Breast Cancer', *Cancer Research*. American Association for Cancer Research, 71(20), pp. 6360–6370.

Park, H. S., Park, J. Y. and Yu, R. (2005) 'Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-α and IL-6', *Diabetes Research and Clinical Practice*. Elsevier, 69(1), pp. 29–35.

Perkins, M. S., Louw-du Toit, R. and Africander, D. (2018) 'Hormone Therapy and Breast Cancer: Emerging Steroid Receptor Mechanisms', *Society for Endoctinology*, pp. 1–71.

Perkins, M. S., Toit, R. L. and Africander, D. (2017) 'A comparative characterization of estrogens used in hormone therapy via estrogen receptor (ER)-α and -β', *Journal of Steroid Biochemistry and Molecular Biology*, pp. 1–14.

Peters, A. A., Buchanan, G., Ricciardelli, C., Bianco-Miotto, T., Centenera, M. M., Harris, J. M., Jindal, S., Segara, D., Jia, L., Moore, N. L., Henshall, S. M., Birrell, S. N., Coetzee, G. A., Sutherland, R. L., Butler, L. M. and Tilley, W. D. (2009) 'Androgen Receptor Inhibits Estrogen Receptor-alpha Activity and Is Prognostic in Breast Cancer', *Cancer Res*, 69(15), pp. 6131–6171.

Peters, K. M., Edwards, S. L., Nair, S. S., French, J. D., Bailey, P. J., Salkield, K., Stein, S., Wagner, S., Francis, G. D., Clark, S. J. and Brown, M. A. (2012) 'Androgen receptor expression predicts breast cancer survival: the role of genetic and epigenetic events', *BMC Cancer*. BioMed Central, 12(1), p. 132.

Pfaffl, M. W. (2001) 'A new mathematical model for relative quantification in real-time RT-PCR.', *Nucleic Acids Research*, 29(9), pp. 2002–2007.

Pierce, B. L., Ballard-Barbash, R., Bernstein, L., Baumgartner, R. N., Neuhouser, M. L., Wener, M. H., Baumgartner, K. B., Gilliland, F. D., Sorensen, B. E., Mctiernan, A. and Ulrich, C. M. (2009) 'Elevated Biomarkers of Inflammation Are Associated With Reduced Survival Among Breast Cancer Patients', *J Clin Oncol*, 27(21), pp. 3437–3444.

Pike, M. C., Spicer, D. V, Dahmoush, L. and Press, M. F. (1993) 'Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk', *Epidemiologic reviews*, 15(1), pp. 17–35.

Platet, N., Cathiard, A. M., M, G. and Garcia, M. (2004) 'Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion', *Crit. Rev. Oncol. Hematol.*, 51, pp. 55–67.

Poggio, F., Lambertini, M., Blondeaux, E., Vaglica, M., Levaggi, A., Pronzato, P. and Del Mastro, L. (2016) 'Role of fulvestrant in the treatment of postmenopausal metastatic breast cancer patients', *Expert Review of Clinical Pharmacology*. Taylor & Francis, 9(9), pp. 1153–1161.

Proietti, C., Scientific, N., Hospital, S. S. and Experimental, M. (2008) 'TNF alpha acting on TNFR1 promotes breast cancer growth via p42/P44 MAPK, JNK, Akt and NF-kappa B-dependent pathways', *Experimental Cell Research*, 314(3), pp. 509–529.

Purohit, A., Ghilchik, M. W., Duncan, L., Wang, D. Y., Singh, A., Walker, M. M. and Reed, M. J. (1995) 'Aromatase activity and interleukin-6 production by normal and malignant breast tissues', *The Journal of Clinical Endocrinology & Metabolism*. Oxford University Press, 80(10), pp. 3052–3058.

Purohit, A., Newman, S. P. and Reed, M. J. (2002) 'The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer', *Breast Cancer Res*, 4, pp. 65–69.

Raghu, H., Sodadasu, P. K., Malla, R. R., Gondi, C. S., Estes, N. and Rao, J. S. (2010) 'Localization of uPAR and MMP-9 in lipid rafts is critical for migration, invasion and angiogenesis in human breast cancer cells', *BMC Cancer*. BioMed Central, 10(1), p. 647.

Rathkopf, D. and Scher, H. I. (2013) 'Androgen receptor antagonists in castration-resistant prostate cancer.', *Cancer journal*. NIH Public Access, 19(1), pp. 43–49.

Rays, A., Prefontaine, K. E. and Ray, P. (1994) 'Down-modulation of Interleukin-6 Gene Expression by l7β-Estradiol in the Absence of High Affinity DNA Binding by the Estrogen Receptor', *Journal of Biological Chemistry*, 269(17), pp. 12940–12946.

Read, L. D., Snider, C. E., Miller, J. S., Greene, G. L., Katzeneilenbogen, B. S. and Biophysics, L. D. R. (1988) 'Ligand-Modulated Regulation of Progesterone Receptor Messenger Ribonucleic Acid and Protein in Human Breast Cancer Cell Lines', *Molecular and Cellular Endocrinology*, 2(3), pp. 263–271.

Rizza, P., Barone, I., Zito, D., Giordano, F., Lanzino, M., Amicis, F. De, Mauro, L., Sisci, D., Catalano, S., Wright, K. D., Gustafsson, J. and Andò, S. (2014) 'Estrogen receptor beta as a novel target of androgen receptor action in breast cancer cell lines', *Breast Cancer Research*, 16(1), pp. 1–13.

Robinson, J. L. L., Macarthur, S., Ross-Innes, C. S., Tilley, W. D., Neal, D. E., Mills, I. G. and Carroll, J. S. (2011) 'Androgen receptor driven transcription in molecular apocrine breast cancer is mediated by FoxA1.', *The EMBO journal*. European Molecular Biology Organization, 30(15), pp. 3019–3027.

Rojas, P. A., May, M., Sequeira, G. R., Elia, A., Alvarez, M., Martínez, P., Gonzalez, P., Hewitt, S., He, X., Perou, C. M., Molinolo, A., Gibbons, L., Abba, M. C., Gass, H. and Lanari, C. (2017) 'Progesterone Receptor Isoform Ratio: A Breast Cancer Prognostic and Predictive Factor for Antiprogestin Responsiveness.', *Journal of the National Cancer Institute*. Oxford University Press, 109(7).

Ronacher, K., Hadley, K., Avenant, C., Stubsrud, E., Stoney Simons Jr, S., Louw, A. and Hapgood, J. P. (2009) 'Ligand-selective transactivation and transrepression via the glucocorticoid receptor: Role of cofactor interaction', *Molecular and Cellular Endocrinology*, 299, pp. 219–231.

Rose, D. P., Komninou, D. and Stephenson, G. D. (2004) 'Obesity, adipocytokines, and insulin resistance in breast cancer', *Obesity Reviews*. Wiley/Blackwell (10.1111), 5(3), pp. 153–165.

Ross, R. K. ., Paganini-Hill, A., Wan, P. C. . and Pike, M. C. (2000) 'Effect of Hormone Replacement Therapy on Breast Cancer Risk: Estrogen Versus Estrogen Plus Progestin', *J Natl Cancer Inst*, 92, pp. 328–332.

Rossouw, J., Anderson, G., Prentice, R., LaCroix, A., Kooperberg, C., Stefanick, M., Jackson, R., Beresford, S., Howard, B., Johnson, K., Kotchen, J. and Ockene, J. (2002) 'Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women', *JAMA*, 288(3), pp. 321–333.

Ruan, X., Schneck, H., Schultz, S., Fehm, T., Cahill, M. A., Seeger, H., Chen, R., Yu, Q., Muech, A. O. and Neubauer, H. (2012) 'Nomegestrol acetate sequentially or continuously combined to estradiol did not negatively affect membrane-receptor associated progestogenic effects in human breast cancer cells', *Gynecol Endocrinol*, 28(11), pp. 863–866.

Ruan, X., Neubauer, H., Schneck, H., Schultz, S., Fehm, T., Cahill, M. A., Seeger, H. and Muech, A. O. (2012) 'Progestogens and membrane-initiated effects on the proliferation of human breast cancer cells', *Climacteric*, 15(5), pp. 467–472.

Rubio, M. F., Werbajh, S., Cafferata, E. G. A., Quaglino, A., Coló, G. P., Nojek, I. M., Kordon, E. C., Nahmod, V. E. and Costas, M. A. (2006) 'TNF-α enhances estrogen-induced cell proliferation of estrogen-dependent breast tumor cells through a complex containing nuclear factor-kappa B', *Oncogene*, 25(9), pp. 1367–1377.

Russo, J., Ao, X., Grill, C. and Russo, I. H. (1999) 'Pattern of distribution of cells positive for estrogen receptor and progesterone receptor in relation to proliferating cells in the mammary gland', *Breast Cancer Research and Treatment*. New Jersey, 53, pp. 217–227.

Russo, J. and Russo, I. H. (2006) 'The role of estrogen in the initiation of breast cancer', *The Journal of steroid biochemistry and molecular biology*. NIH Public Access, 102(1–5), pp. 89–96.

Ryde, Carolyn, M., Nicholls, J. E. and Dowsett, M. (1992) 'Steroid and Growth Factor Modulation of Aromatase Activity in MCF7 and T47D Breast Carcinoma Cell Lines', *Cancer Research*, 52, pp. 1411–1415.

Sangmin, K., Choi, J. H., Kim, J. Bin, Nam, S. J., Yang, J.-H., Kim, J.-H. and Lee, J. E. (2008) 'Berberine Suppresses TNF-α-induced MMP-9 and Cell Invasion through Inhibition of AP-1 Activity in MDA-MB-231 Human Breast Cancer Cells', *Molecules*, 13, pp. 2975–2985.

Sarnataro, D., Pisanti, S., Santoro, A., Bifulco, M., Malfitano, A. M., Laezza, C. and Gazzerro, P. (2006) 'The Cannabinoid CB1 Receptor Antagonist Rimonabant (SR141716) Inhibits Human Breast Cancer Cell Proliferation through a Lipid Raft-Mediated Mechanism', *Molecular Pharmacology*, 70(4), pp. 1298–1306.

Sartorius, C. A., Melville, M. Y., Hovland, A. R., Tung, L., Takimoto, G. S. and Horwitz, K. B. (1994) 'A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform', *Molecular Endocrinology*, 8(10), pp. 1347–1360.

Sasser, A. K., Sullivan, N. J., Studebaker, A. W., Hendey, L. F., Axel, A. E. and Hall, B. M. (2007) 'Interleukin-6 is a potent growth factor for ER- alpha -positive human breast cancer', *The FASEB Journal*, 21(13), pp. 3763–3770.

Satrorius, C. A., Groshong, S. D., Miller, L. A., Powell, R. L., Tung, L., Takimoto, G. S. and Horwitz,

K. B. (1994) ‘New T47D Breast Cancer Cell Lines for the Independent Study of Progesterone B- and A-Receptors: Only Antiprogestin-occupied B-Receptors Are Switched to Transcriptional Agonists by cAMP’, *Cancer Research*, 54, pp. 3868–3877.

Schellenberger, E. A., Reynolds, F., Weissleder, R. and Josephson, L. (2004) ‘Surface-Functionalized Nanoparticle Library Yields Probes for Apoptotic Cells’, *ChemBioChem*, 5, pp. 275–279.

Schindler, A. E., Campagnoli, C., Druckmann, R., Huber, J., Pasqualini, J. R., Schweppe, K. W. and Thijssen, J. H. H. (2003) ‘Classification and pharmacology of progestins’, *Matu*, 1, pp. 7–16.

Schindler, A. E. (2014) ‘The “newer” progestogens and postmenopausal hormone therapy (HRT)’, *The Journal of Steroid Biochemistry and Molecular Biology*, 142, pp. 48–51.

Schoonen, W. G. E. J., Joosten, J. W. H. and Kloosterboer, H. J. (1995) ‘Effects of two classes of progestagens, pregnane and 19-nortestosterone derivatives, on cell growth of human breast tumor cells: I. MCF-7 cell lines’, *The Journal of Steroid Biochemistry and Molecular Biology*. Pergamon, 55(3–4), pp. 423–437.

Shang, D., Li, Z., Zhu, Z., Chen, H., Zhao, L., Wang, X. and Chen, Y. (2015) ‘Baicalein suppresses 17-β-estradiol-induced migration, adhesion and invasion of breast cancer cells via the G protein-coupled receptor 30 signaling pathway’, *Oncology Reports*. Spandidos Publications, 33(4), pp. 2077–2085.

Simon, Wolfgang, E., Albrecht, M., Trams, G., Dietel, M. and Holzel, F. (1984) ‘In Vitro Growth Promotion of Human Mammary Carcinoma Cells by Steroid Hormones, Tamoxifen, and Prolactin’, *JNCI*, 73, pp. 313–321.

Simstein, R., Burow, M., Parker, A., Weldon, C. and Beckman, B. (2003) ‘Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system.’, *Exp Biol Med*, 228(9), pp. 995–1003.

Singer, C. F., Kronsteiner, N., Hudelist, G., Marton, E., Walter, I., Kubista, M., Czerwenka, K., Schreiber, M., Seifert, M. and Kubista, E. (2003) ‘Interleukin 1 System and Sex Steroid Receptor Expression in Human Breast Cancer: Interleukin 1 Protein Secretion Is Correlated with Malignant Phenotype’, *Clinical Cancer Research* , 9, pp. 4877–4883.

Sitruk-Ware, R. (2004) ‘Pharmacological profile of progestins’, *Maturitas*. Elsevier, 47(4), pp. 277–283.

Sitruk-Ware, R. and Nath, A. (2010) ‘The use of newer progestins for contraception’, *Contraception*, 82(5), pp. 410–417.

Slattery, M. L., Curtin, K., Sweeney, C., Wolff, R. K., Baumgartner, R. N., Baumgartner, K. B., Giuliano, A. R. and Byers, T. (2008) ‘Modifying Effects of IL-6 Polymorphisms on Body Size-Associated Breast Cancer Risk’, *Obesity*, 16, pp. 339–347.

Smith, I. E. and Dowsett, M. (2003) ‘Aromatase Inhibitors in Breast Cancer’, *New England Journal of Medicine*. Edited by A. J. J. Wood. Massachusetts Medical Society, 348(24), pp. 2431–2442.

Somboonporn, W. and Davis, S. R. (2004) ‘Testosterone Effects on the Breast: Implications for Testosterone Therapy for Women’, *Endocrine Reviews*, 25(3), pp. 374–388.

Song, R. X., Mor, G., Naftolin, F., McPherson, R. A., Song, J., Zhang, Z., Yue, W., Wang, J. and Santen, R. J. (2001) ‘Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol.’, *Journal of the National Cancer Institute*, 93(22), pp. 1714–23.

Sorrells, S. F. and Sapolsky, R. M. (2007) ‘An inflammatory review of glucocorticoid actions in the CNS.’, *Brain, behavior, and immunity*. NIH Public Access, 21(3), pp. 259–272.

South Africa Demographic and Health Survey (SADHS) (2016) *Key Indicators Report, Statistics South Africa*.

Speroff, L. and Darney, P. D. (1996) *Injectable contraception. In: A clinical guide for contraception. 2nd Edition Baltimore, MD: Williams and Wilkins*.

Stanczyk, F. Z., Hapgood, J. P., Winer, S. and Mishell, D. R. (2013) 'Progestogens Used in Postmenopausal Hormone Therapy: Differences in Their Pharmacological Properties, Intracellular Actions, and Clinical Effects', *Endocrine Reviews*, 34(2), pp. 171–208.

Straub, R. H. and Cutolo, M. (2016) 'Glucocorticoids and chronic inflammation', *Rheumatology*. Oxford University Press, 55(suppl 2), pp. 6–14.

Ström, A., Hartman, J., Foster, J. S., Kietz, S., Wimalasena, J. and Gustafsson, J.-Å. (2004) 'Estrogen receptor β inhibits 17-estradiol-stimulated proliferation of the breast cancer cell line T47D', *PNAS*, 101(6), pp. 1566–1571.

Studebaker, A. W., Storci, G., Werbeck, J. L., Sansone, P., Sasser, A. K., Tavolari, S., Huang, T., Chan, M. W. Y., Marini, F. C., Rosol, T. J., Bonafe, M. and Hall, B. M. (2008) 'Fibroblasts Isolated from Common Sites of Breast Cancer Metastasis Enhance Cancer Cell Growth Rates and Invasiveness in an Interleukin-6 – Dependent Manner', *Cancer Res*, 68(21), pp. 9087–9096.

Stute, P., Wildt, L. and Neulen, J. (2018) 'The impact of micronized progesterone on breast cancer risk: a systematic review', *Climacteric*, 21(2), pp. 111–122.

Suarez-Cuervo, C., Harris, K. W., Kallman, L., Kalervo Väänänen, H. and Selander, K. S. (2003) 'Tumor necrosis factor-α induces interleukin-6 production via extracellular-regulated kinase 1 activation in breast cancer cells', *Breast Cancer Research and Treatment*, 80, pp. 71–78.

Sun, N., Xu, H. N., Luo, Q. and Li, L. Z. (2016) 'Potential Indexing of the Invasiveness of Breast Cancer Cells by Mitochondrial Redox Ratios.', *Advances in experimental medicine and biology*. NIH Public Access, 923, pp. 121–127.

Surakasula, A., Nagarjunapu, G. C. and Raghavaiah, K. V (2014) 'A comparative study of pre- and post-menopausal breast cancer: Risk factors, presentation, characteristics and management', *Journal of research in pharmacy practice*. Wolters Kluwer -- Medknow Publications, 3(1), pp. 12–18.

Sweeney, E., Fan, P. and Jordan, V. C. (2014) 'Molecular modulation of estrogen-induced apoptosis by synthetic progestins in hormone replacement therapy: An insight into the Women's Health Initiative study', *Cancer Research*, 74(23), pp. 7060–7068.

Tan, H., Zhong, Y. and Pan, Z. (2009) 'Autocrine regulation of cell proliferation by estrogen receptor-alpha in estrogen receptor-alpha-positive breast cancer cell lines', *BMC Cancer*, 9(31), pp. 1–12.

Taplin, M.-E., Bubley, G. J., Shuster, T. D., Frantz, M. E., Spooner, A. E., Ogata, G. K., Keer, H. N. and Balk, S. P. (1995) 'Mutation of the Androgen-Receptor Gene in Metastatic Androgen-Independent Prostate Cancer', *New England Journal of Medicine*, 332(21), pp. 1393–1398.

Tchafa, A. M., Zhong, Z., Meng, R., Quong, J. N. and Quong, A. A. (2013) 'Increased Cellular Invasion and Proliferation via Estrogen Receptor after 17-β-Estradiol Treatment in Breast Cancer Cells Using Stable Isotopic Labeling with Amino Acids in Cell Culture (SILAC)', *Advances in Breast Cancer Research*, (2), pp. 32–43.

The American Cancer Society (2015) *Normal weight ranges: Body mass index (BMI)*.

Thomas, C. and Gustafsson, J. Å. (2015) 'Progesterone receptor-estrogen receptor crosstalk: A novel insight', *Trends in Endocrinology and Metabolism*, 26(9), pp. 453–454.

Thompson, E. W., Reich, R., Shima, T. B., Albini, A., Graf, J., Martin, G. R., Dickson, R. B. and

Lippman, M. E. (1988) 'Differential regulation of growth and invasiveness of MCF-7 breast cancer cells by antiestrogens', *Cancer Research*. Waverly Press, 48(23), pp. 6764–6768.

Tian, J.-M., Ran, B., Zhang, C.-L., Yan, D.-M. and Li, X.-H. (2018) 'Estrogen and progesterone promote breast cancer cell proliferation by inducing cyclin G1 expression', *Brazillian Journal of Medical and Biological Research*, 51(3), pp. 1–7.

Tomasz, M. (1995) 'Mitomycin C: small, fast and deadly (but very selective)', *Chemistry and Biology*, 2(9), pp. 575–579.

Toth, M. and Fridman, R. (2001) 'Assessment of Gelatinases (MMP-2 and MMP-9) by Gelatin Zymography', *Methods Mol Med*, 57, pp. 1–10.

Tung, L., Abdel-Hafiz, H., Shen, T., Harvell, D. M. E., Nitao, L. K., Richer, J. K., Sartorius, C. A., Takimoto, G. S. and Horwitz, K. B. (2006) 'Progesterone Receptors (PR)-B and -A Regulate Transcription by Different Mechanisms: AF-3 Exerts Regulatory Control over Coactivator Binding to PR-B', *Molecular Endocrinology*. Oxford University Press, 20(11), pp. 2656–2670.

Turashvili, G. and Brogi, E. (2017) 'Tumor Heterogeneity in Breast Cancer', *Frontiers in Medicine*, 4, pp. 1–11.

Verhoog, N. J. D., Joubert, E. and Louw, A. (2007) 'Evaluation of the phytoestrogenic activity of Cyclopia genistoides (honeybush) methanol extracts and relevant polyphenols', *Journal of Agricultural and Food Chemistry*, 55(11), pp. 4371–4381.

Vilasco, M., Communal, L., Mourra, N., Courtin, A., Forgez, P. and Gompel, A. (2011) 'Glucocorticoid receptor and breast cancer', *Breast Cancer Research and Treatment*, 130(1).

Villalobos, M., Olea, N., Brotons, J. A., Olea-Serrano, M. F., Ruiz de Almodovar, J. M. and Pedraza, V. (1995) 'The E-screen assay: a comparison of different MCF7 cell stocks.', *Environmental health perspectives*. National Institute of Environmental Health Science, 103(9), pp. 844–50.

De Visser, K. E., Eichten, A. and Coussens, L. M. (2006) 'Paradoxical roles of the immune system during cancer development', *Nature Reviews Cancer*, 6(1), pp. 24–37.

Visser, K., Mortimer, M. and Louw, A. (2013) 'Cyclopia extracts act as ER-alpha antagonists and ER-beta agonists, in vitro and in vivo', *PLoS ONE*, 8(11), pp. 1–22.

Vivian, F., Waitzberg, Â. F. L., Madeira, M., de Toledo Osório, C. A. B., Soares, F. A. and Gebrim, L. H. G. (2011) 'Expression of BCL-2 and BAX proteins in breast cancer after in vivo tamoxifen administration', *Rev Bras Mastologia*, 21(1), pp. 3–8.

Vladusic, E. A., Hornby, A. E., Guerra-Vladusic, F. K., Lakins, J. and Lupu, R. (2000) 'Expression and regulation of estrogen receptor beta in human breast tumors and cell lines.', *Oncology Reports*. Spandidos Publications, 7(1), pp. 157–224.

Vona-Davis, L. and Rose, D. P. (2007) 'Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression', *Endocrine-Related Cancer*, 14, pp. 189–206.

Vozarova, B., Weyer, C., Hanson, K., Tataranni, P. a, Bogardus, C. and Pratley, R. E. (2001) 'Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion', *Obesity research*, 9(7), pp. 414–7.

Wajant, H., Pfizenmaier, K. and Scheurich, P. (2003) 'Tumor necrosis factor signaling', *Cell death and differentiation*, 10, pp. 45–65.

Wang, T. T. Y. and Phang, J. M. (1995) 'Effects of Estrogen on Apoptotic Pathways in Human Breast Cancer Cell Line MCF-7', *Cancer Research*, 55, pp. 2487–2489.

Wang, X. and Lin, Y. (2008) 'Tumor necrosis factor and cancer, buddies or foes?', *Acta Pharmacol Sin*, 29(11), pp. 1275–1288.

Wang, Y., Lehuédé, C., Laurent, V., Dirat, B., Dauvillier, S., Bochet, L., Le, S., Escourrou, G., Valet, P. and Muller, C. (2015) 'Adipose tissue and breast epithelial cells : A dangerous dynamic duo in breast cancer', *Cancer Letters*. Elsevier Ireland Ltd, 324(2), pp. 142–151.

Wang, Y. and Rao, H. (2014) 'Heat shock protein 90 β Stabilizes focal adhesion kinase and Enhances cell migration and invasion in breast Cancer Cells .', *Experimental Cell Research*. Elsevier, 326(1), pp. 78–89.

Wang, Z., Lee, H. J., Wang, L., Jiang, C., Baek, N. I., Kim, S. H. and Lü, J. (2009) 'Anti-androgen receptor signaling and prostate cancer inhibitory effects of sucrose- and benzophenone-compounds', *Pharmaceutical Research*, 26(5), pp. 1140–1148.

Wargon, V., Riggio, M., Giulianelli, S., Sequeira, G. R., Rojas, P., May, M., Polo, M. L., Gorostiaga, M. A., Jacobsen, B., Molinolo, A., Novaro, V. and Lanari, C. (2015) 'Progestin and antiprogestin responsiveness in breast cancer is driven by the PRA/PRB ratio via AIB1 or SMRT recruitment to the CCND1 and MYC promoters.', *International journal of cancer*. NIH Public Access, 136(11), pp. 2680–2692.

Waters, J. P., Pober, J. S. and Bradley, J. R. (2013) 'Tumour necrosis factor and cancer', *J Pathol*, 230, pp. 241–248.

Webster, J. C., Oakley, R. H., Jewell, C. M. and Cidlowski, J. A. (2001) 'Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative β isoform: A mechanism for the generation of glucocorticoid resistance', *PNAS*. National Academy of Sciences, 98(12), pp. 6865–6870.

Wellberg, E. A., Checkley, L. A., Giles, E. D., Johnson, S. J., Oljira, R., Wahdan-Alaswad, R., Foright, R. M., Dooley, G., Edgerton, S. M., Jindal, S., Johnson, G. C., Richer, J. K., Kabos, P., Thor, A. D., Schedin, P., MacLean, P. S. and Anderson, S. M. (2017) 'The Androgen Receptor Supports Tumor Progression After the Loss of Ovarian Function in a Preclinical Model of Obesity and Breast Cancer', *Hormones and Cancer*. Hormones and Cancer, 8(5–6), pp. 269–285.

Werner, H. M. J., Franke, H. R. and Vermes, I. (2005) 'Apoptosis and proliferation in breast cancer cells, cultured in vitro: effects of SERMs', *Climacteric*, 8(3), pp. 294–299.

Williams, C., Edvardsson, K., Lewandowski, S. a, Ström, a and Gustafsson, J. (2008) 'A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells.', *Oncogene*, 27(7), pp. 1019–1032.

Winkler, G., Kiss, S., Keszthelyi, L., Sapi, Z., Ory, I., Salamon, F., Kovacs, M., Vargha, P., Szekeres, O., Karadi, I., Sikter, M., Kaszas, E., Dworak, O., Gero, G. and Cseh, K. (2003) 'Expression of tumor necrosis factor (TNF)-alpha protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF-alpha, soluble serum TNF-receptor-2 concentrations and C-peptide level', *European Journal of Endocrinology*, 149(2), pp. 129–135.

Wolczyk, D., Zaremba-czogalla, M., Hryniewicz-jankowska, A., Tabola, R., Grabowski, K., Sikorski, A. F. and Augoff, K. (2016) 'TNF-α promotes breast cancer cell migration and enhances the concentration of membrane-associated proteases in lipid rafts', *Cellular Oncology*. Cellular Oncology, 39, pp. 353–363.

Wood, C. E., Branstetter, D., Jacob, A. P., Cline, J. M., Register, T. C., Rohrbach, K., Huang, L.-Y., Borgerink, H. and Dougall, W. C. (2013) 'Progestin effects on cell proliferation pathways in the postmenopausal mammary gland', *Breast Cancer Research*. BioMed Central, 15(4).

Wu, W., Chaudhuri, S., Brickley, D. R., Pang, D., Karrison, T. and Conzen, S. D. (2004) 'Microarray Analysis Reveals Glucocorticoid-Regulated Survival Genes That Are Associated With Inhibition of Apoptosis in Breast Epithelial Cells', *Cancer Research*, 64, pp. 1757–1764.

Wu, Y. and Vadgama, J. V (2017) 'Androgen Receptor as a Potential Target for Treatment of Breast Cancer.', *International journal of cancer research and molecular mechanisms*. NIH Public Access, 3(1).

Xu, J. and O'Malley, B. W. (2002) 'Molecular Mechanisms and Cellular Biology of the Steroid Receptor Coactivator (SRC) Family in Steroid Receptor Function', *Reviews in Endocrine and Metabolic Disorders*. Kluwer Academic Publishers, 3(3), pp. 185–192.

Xue, Q., Patterson, A. J., Xiao, D. and Zhang, L. (2014) 'Glucocorticoid modulates angiotensin II receptor expression patterns and protects the heart from ischemia and reperfusion injury.', *PloS one*. Public Library of Science, 9(9), p. e106827.

Yang, F., Zhao, Z. and Zhao, N. (2017) 'Clinical implications of tumor necrosis factor receptor 2 in breast cancer.', *Oncology letters*. Spandidos Publications, 14(2), pp. 2393–2398.

Yang, M. X. (1995) 'Repression of the Interleukin-6 Promoter by Estrogen Receptor Is Mediated by NFκB and C/EBPβ', *Molecular and Cellular Biology*, 15(9), pp. 4971–4979.

Yeap, B. B., Krueger, R. G. and Leedman, P. J. (1999) 'Differential Posttranscriptional Regulation of Androgen Receptor Gene Expression by Androgen in Prostate and Breast Cancer Cells', *Endocrinology*. Oxford University Press, 140(7), pp. 3282–3291.

Yeh, W.-L., Shioda, K., Coser, K. R., Rivizzigno, D., McSweeney, K. R. and Shioda, T. (2013) 'Fulvestrant-Induced Cell Death and Proteasomal Degradation of Estrogen Receptor α Protein in MCF-7 Cells Require the CSK c-Src Tyrosine Kinase', *PLoS ONE*. Edited by A. Migliaccio. Public Library of Science, 8(4), pp. 1–11.

Zahid, H., Subbaramaiah, K., Iyengar, N. M., Zhou, X. K., Chen, I., Bhardwaj, P., Gucalp, A., Morrow, M., Hudis, C. A., Andrew, J., Brown, K. A., Arabia, S., Sloan, M., Cancer, K., Sloan, M. and Cancer, K. (2018) 'Leptin regulation of the p53-HIF1α/PKM2-aromatase axis in breast adipose stromal cells – a novel mechanism for the obesity-breast cancer link', *Int J Obes*, 42(4), pp. 711–720.

Zhang, G. and Adachi, I. (1999) 'Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma', *Anticancer Research*, 19, pp. 1427–1432.

Zhang, Y., Zhao, H., Asztalos, S., Chisamore, M., Sitabkhan, Y. and Tonetti, D. A. (2009) 'Estradiol-Induced Regression in T47D:A18/PKCα Tumors Requires the Estrogen Receptor and Interaction with the Extracellular Matrix', *Molecular Cancer Research*, 7(4), pp. 498–510.

Zhang, Y., Leung, D. Y. M., Nordeen, S. K. and Goleva, E. (2009) 'Estrogen inhibits glucocorticoid action via protein phosphatase 5 (PP5) mediated glucocorticoid receptor dephosphorylation', *J. Biol. Chem*. JBC Papers in Press, pp. 1–29.

Zhao, Y., Yang, F., Li, W., Xu, C., Li, L., Chen, L., Liu, Y. and Sun, P. (2017) 'miR-29a suppresses MCF-7 cell growth by downregulating tumor necrosis factor receptor 1', *Tumor Biology*. SAGE PublicationsSage UK: London, England, 39(2), pp. 1–11.

Zheng, S., Huang, J., Zhou, K., Zhang, C., Xiang, Q., Tan, Z., Wang, T. and Fu, X. (2011) '17β-Estradiol enhances breast cancer cell motility and invasion via extra-nuclear activation of actin-binding protein ezrin.', *PloS one*. Public Library of Science, 6(7), p. e22439.

Zheng, Z.-Y., Bay, B.-H., Aw, S.-E. and Lin, V. C.-L. (2005) 'A novel antiestrogenic mechanism in progesterone receptor-transfected breast cancer cells', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 280(17), pp. 17480–17487.

Zhou, J. and Cidlowski, J. A. (2005) 'The human glucocorticoid receptor: One gene, multiple proteins and diverse responses', *Steroids*. Elsevier, 70(5–7), pp. 407–417.

Zhu, L.-J., Hardy, M. P., Inigo, I. V., Huhtaniemi, I., Bardin, C. W. and Moo-Young, A. J. (2000) 'Effects of Androgen on Androgen Receptor Expression in Rat Testicular and Epididymal Cells: A Quantitative Immunohistochemical Study', *Biology of Reproduction*. Oxford University Press, 63(2), pp. 368–376.

Van Zyl, S., Van Der Merwe, L. J., Van Rooyen, F. C., Joubert, G. and Walsh, C. M. (2017) 'The relationship between obesity, leptin, adiponectin and the components of metabolic syndrome in urban African women, Free State, South Africa', *South African Journal of Clinical Nutrition*, 30(3), pp. 68–73.

ADDENDUM A

BUFFERS AND SOLUTIONS

Cell culture and Mammalian tissue culture

Charcoal stripping buffer

1 M sucrose

1.5 mM $MgCl_2$ Hexahydrate

10 mM Hepes

0.25% w/v Norit-A charcoal

0.0025% w/v Dextran

Add all together and adjust final volume to 1 L with autoclaved MilliQ H_2O.

Cell viability assay

5 mg/mL MTT

5 mg MTT

1 mL sterile PBS

Dissolve and filter sterilise.

Electrophoresis and western blotting solutions

2X Laemmli SDS sample buffer

0.04 M Tris-HCl pH 6.8

2% (v/v) SDS

0.04% (w/v) bromophenol blue

8% (v/v) glycerol

2% (v/v) β-mercaptoethanol

Adjust to a final volume of 25 mL using RO water.

1X SDS PAGE running buffer

25 mM Tris-HCl

192 mM glycine

0.1% (v/v) SDS

Adjust to 1 L using RO water and store at room temperature.

1X Transfer buffer

25 mM Tris-HCl

192 mM glycine

10% (v/v) methanol

Adjust to 1 L using RO water and store at 4°C.

10X Tris-buffered saline (TBS)

50 mM TRIS

150 mM NaCl

Dissolve in 800 mL dH_2O and adjust pH to 7.5

Adjust volume to 1 L with dH_2O

Store at 4 °C for up to three months.

1X TBS-Tween (TBST)

100 mL 10X TBS and 900 mL dH_2O

Dissolve 1 ml Tween-20 in 1X TBS buffer.

Gelatin Zymography assay

4X Sample buffer

0.28 M Tris-HCl (pH 6.8)

44% glycerol

0.31 M SDS

0.01% (w/v) bromophenol blue

Dissolve and store at room temperature.

Coomassie Blue stain solution

5% (v/v) methanol

10% (v/v) acetic acid

1% (w/v) Coomassie Blue

Adjust to 100 mL with RO water and store at room temperature.

Destain solution

40% (v/v) methanol

7% (v/v) acetic acid

Adjust to 1 L with RO water and store at room temperature.

Renaturing buffer

2.5% (v/v) Triton X

Adjust volume to 1 L with RO water and store at room temperature.

Developing buffer

50 mM Tris-HCl (pH 7.4)

10 mM $CaCl_2$

0.02% (w/v) NaN_3

Adjust to 1 L with RO water and store at 4°C.

ADDENDUM B

ADDITIONAL DATA

B1. T47D, MCF-7 BUS and MDA-MB-231 breast cancer cells used in this study were mycoplasma negative

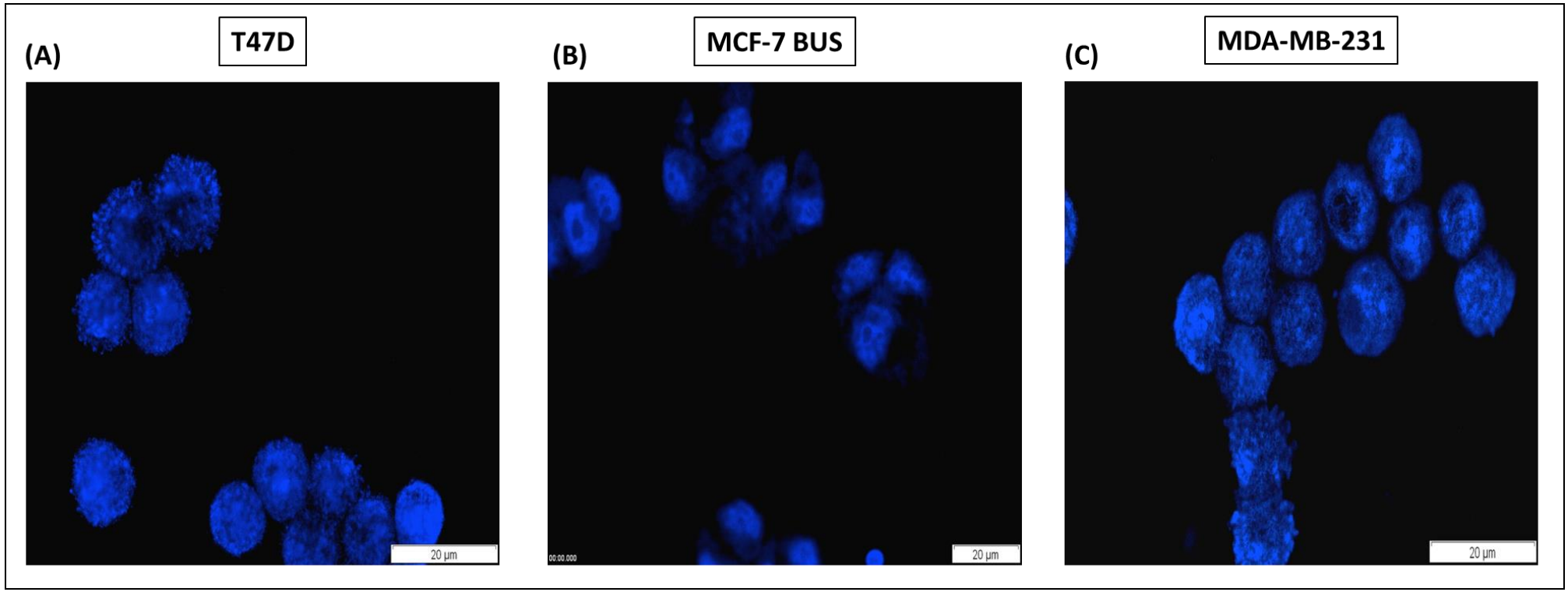


Figure B1. Mycoplasma negative (A) T47D, (B) MCF-7 BUS and (C) MDA-MB-231 breast cancer cells. Cells were fixed with a ratio of 3:1 methanol to glacial acetic acid before staining with the DNA Hoechst 33258 dye (Sigma-Aldrich, SA). The Hoechst dye only stains DNA-containing nuclei. Fluorescent images were captured using the Olympus IX81 inverted fluorescence microscope.

B2. RNA isolated from T47D and MCF-7 BUS breast cancer cells were intact

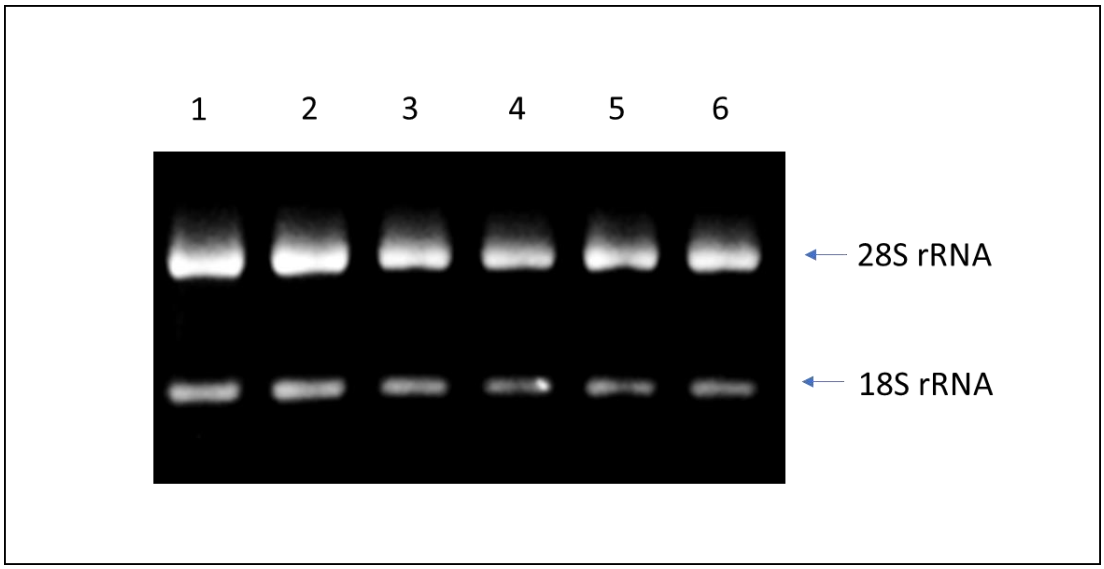


Figure B2. Representative 1% denaturing agarose gel indicating intact RNA isolated from T47D and MCF-7 BUS breast cancer cells. Total RNA was isolated from T47D (lanes 1-3) and MCF-7 BUS (lanes 4-5) breast cancer cell lines treated with either 0.1% (v/v) EtOH (lanes 1 and 4), 5 ng/ml IL-6 (lanes 2 and 5) or 20 ng/ml TNFα (lanes 3 and 6). RNA was isolated using Tri-reagent as described in Chapter 2, Section 2.8. A total of 1 µg RNA was loaded onto the agarose gel, and the RNA was visualised with Nancy-520 nucleic acid stain.

B3. A single amplicon was obtained for the Ki-67, Bax, Bcl-2 and GAPDH target genes

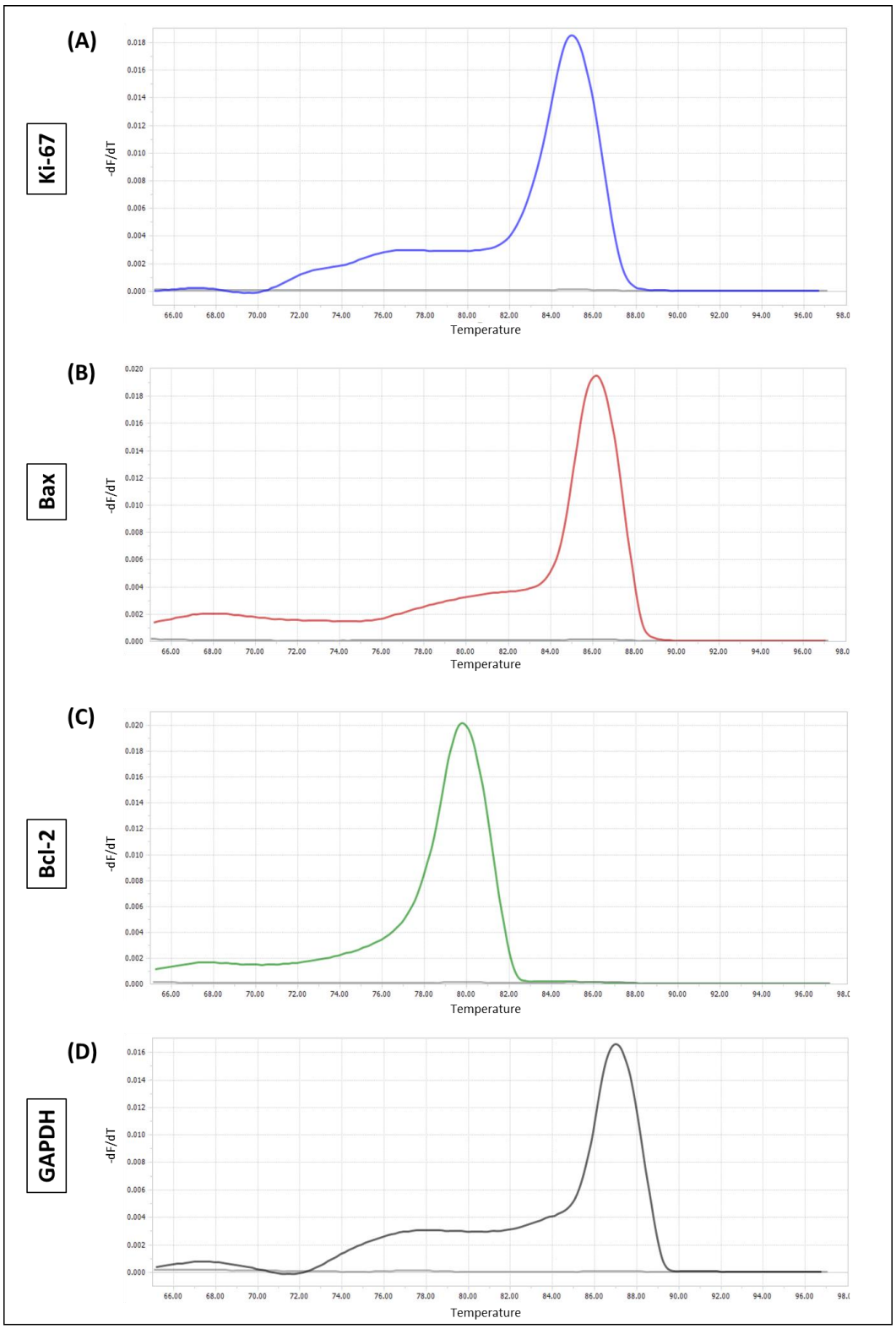


Figure B3. Representative melting curves obtained for the genes evaluated in this study. Single melting curves were generated for (A) Ki-67, (B) Bax, (C) Bcl-2 and (D) GAPDH using the LightCycler® 96 software. The grey lines represent the no template negative control.

B4. The correct qPCR products were confirmed with agarose gel electrophoresis

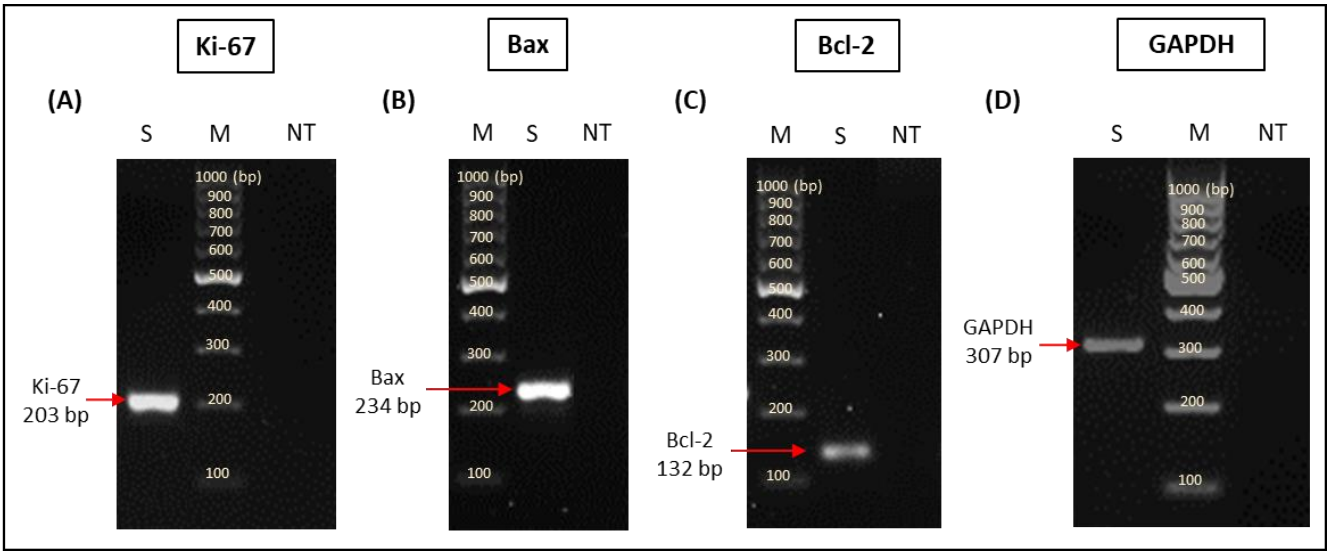


Figure B4. Representative agarose gels indicating qPCR products of (A) Ki-67, (B) Bax, (C) Bcl-2 and (D) GAPDH. PCR products were subjected to agarose gel electrophoresis (2% w/v), and visualised with Nancy-520 nucleic acid stain. M: GeneRuler 100 bp DNA Ladder (Thermo Scientific, USA), S: sample treated with 0.1% (v/v) EtOH, NT: no template negative control.

B5. The optimal cell density for the Caspase-Glo 3/7 apoptosis assay in both the T47D and MCF-7 BUS cells was 1 x 10^3 cells per well

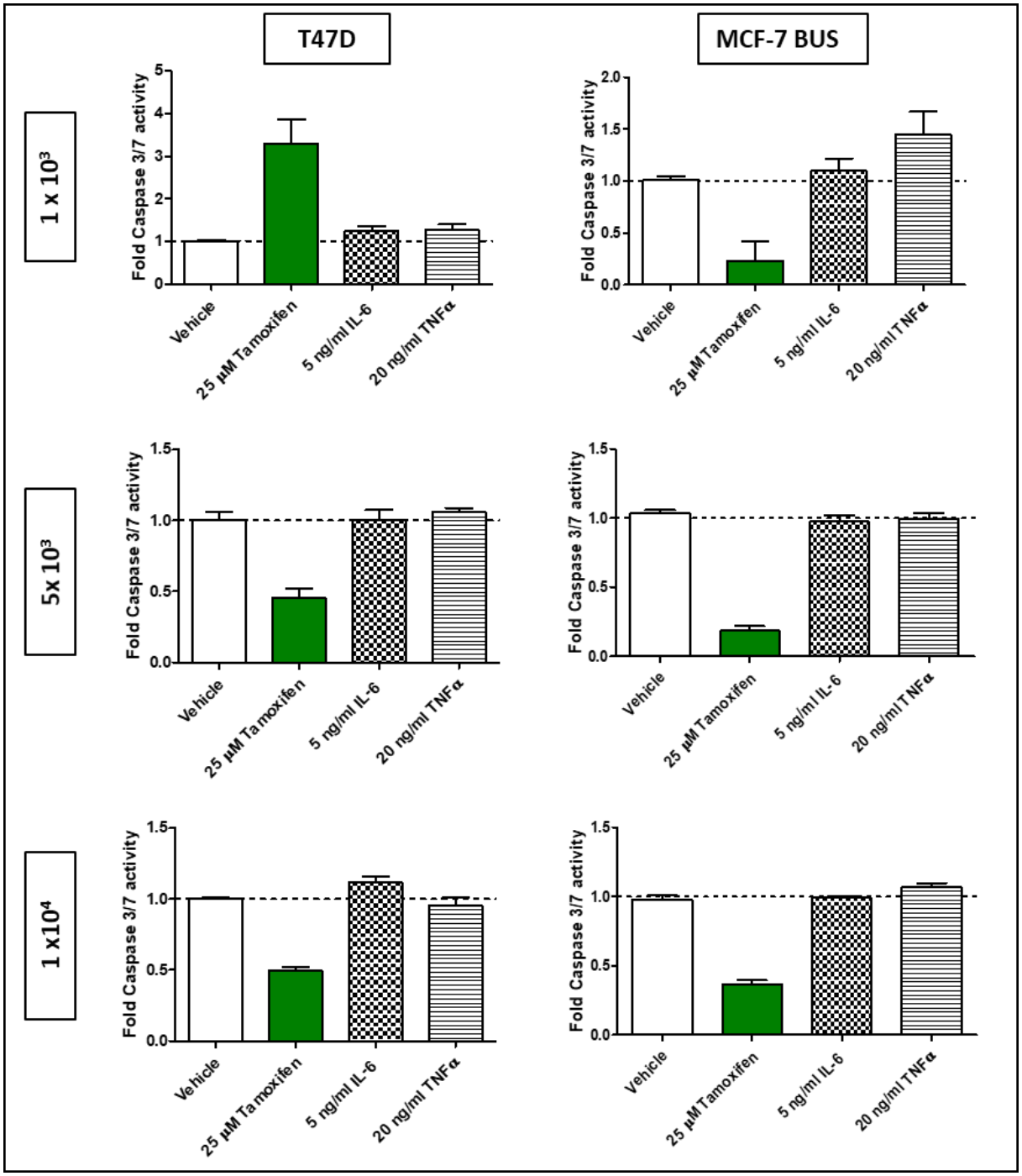


Figure B5. The optimal cell density for both the T47D and MCF-7 BUS cell lines was 1x10^3 cells per well. T47D and MCF-7 BUS breast cancer cells were seeded at either 1 x 10^3, 5 x 10^3 or 1 x 10^4 cells per well and allowed to settle. Cells were treated with either 0.1% (v/v) EtOH (vehicle), 25 µM Tamoxifen, 5 ng/ml IL-6 or 20 ng/ml TNFα for 24 and 6 hours, respectively. The Caspase-Glo 3/7 luminescent assay was used to determine caspase-3 and 7 activities, as a measure of apoptosis. Results are expressed as fold caspase 3/7 activity relative to EtOH (vehicle) set as 1, and are representative of a single experiment performed in triplicate.