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UNIVERSITY OF PLYMOUTH

Effect of phytoestrogens on breast and prostate cancer cell lines – potential implications for bone metastasis

By

Safaa Salman Mezban AL-Thamiree

A thesis submitted to Plymouth University in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

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Dedication

To everyone stand with me along my learning journey

To everyone wishes me the best in my life

To the soul of my father

To the kindness and prayers of my mother

To my uncle Dawood Al-Thamiree who showed me the meaning of learning and education

To my family who supported and encouraged me everyday

To my wife, my eyes Yousif and Nora

To all my friends

Safaa 2019

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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The effect of phytoestrogens on breast and prostate cancer cell lines – potential implications for bone preferential metastasis to bone

Safaa Salman Mezban AL-Thamiree

Abstract

Many epidemiological studies indicate that diets rich in phytoestrogens (PE), especially soy and grain products, may be associated with a lower risk of some steroid hormone-dependent cancers such as breast and prostate. In particular PE have been shown to reduce the incidence of skeletal metastasis which have a high degree of morbidity and mortality. However, the most effective combinations of PEs and the mechanism through which they may reduce bone metastasis remain unclear. Therefore, this study aims to establish the most effective combinations of common dietary PE on breast and prostate cancer line proliferation, motility, and expression of genes implicated in disease progression and preferential metastasis to the skeleton. The potential modifying effect of cytokines that tumour cells are exposed to in the bone micro environment will also be studied including TGF-β, BMP7 and IL-33.

Results showed that phytoestrogens genistein and daidzein significantly reduced prostate cancer cell viability (PC3 cells) with concentration 10^{-7} M (15 %, P = 0.01) and 10^{-6} M (11%, P = 0.04) for genistein and for daidzein the decrease in cell number was (18%, P value = 0.04) for 10^{-8} M and (22 %, P = 0.01) for 10^{-7} M. In breast cancer cell line (MCF7) genistein and coumestrol showed a significant decrease in cell number while daidzein did not. The decrease in (MCF7) cell number with genistein was (15 %, P = 0.04) and (25 %, P = 0.04) for 10^{-6} M and 10^{-5} M respectively, while in coumestrol concentrations

10^{-7} , 10^{-6} and 10^{-5} M showed the most significant decrease in cell number and were (29 %, P = 0.03), (34 %, P= 0.01) and (37%, value = 0.007) respectively.

Motility results showed no significant reduction in the closure time of the scratch in both cell lines and there was an acceleration in the healing time of the scratch in both cell lines but was significant only in breast cell line (MCF7) with coumestrol after 6 ,12 and 24 hours at concentrations between (10^{-9} - 10^{-5} M).

Non-selective oestrogen receptor modulator (ICI 182,780) abolishes the effect of genistein (10^{-7} and 10^{-6} M) and daidzein (10^{-8} and 10^{-7} M) reduction in cell viability and increased PC3 cell numbers significantly. In MCF7 cells, (ICI 182,780) also abolished the effect of coumestrol and genistein and increased the cell numbers but not to a significant levels.

While genistein, daidzein and coumestrol reduced breast and prostate cell viability individually in an oestrogen receptor dependent manner and this beneficial effect is lost when the effective concentrations are combined.

Although, transforming growth factor β (TGFβ), shows antagonist effect on phytoestrogens induced changes when combined with (daidzein and genistein) and blocks any effect of PEs and increase collagen type I gene expression. In MCF7 cells, the non-inhibitory effect of individual genistein on PTHrP is lost in the presence of TGFβ but continues with significant decrease with snail. The non-inhibitory effect of coumestrol on PTHrP and snail genes expression altered to reduced significantly which suggest a strong effect of TGFβ on coumestrol and genistein action by interfering as a bone microenvironment cytokine.

While bone morphogenic protein 7 (BMP7) had an inhibitory effect for the PEs (daidzein and genistein) and increased the expression of Runx2 in prostate

cancer cell line. In MCF7 cells, BMP7 inhibits the effect of (genistein and coumestrol) and increased the expression of snail to a high level.

Interleukin-33 (IL-33), reverse the inhibitory individual effect of phytoestrogens on Runx2, CXCR4, snail and Integrin α5 gene expression in prostate and breast cancer cell lines.

In conclusion, phytoestrogens are effective when administrated individually but lose their effect in combination with other phytoestrogens. Clinicians must consider the overall profile of phytoestrogens before administration. Epidemiologically, what applied to an area regarding the effect of phytoestrogens must not apply to other areas. Hence, administrating phytoestrogens at early ages might be of beneficial effect than in elder ages.

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Abbreviations

ABC	ATP-Binding Cassette
ALDH	Aldehyde dehydrogenase
AP-1	Activator protein
ATCC	American Type Culture Collection
BM	Basement membrane
BMP	Bone morphogenetic protein
BPA	Bisphenol A
BRCA 1 & 2	Breast Cancer type 1&2 susceptibility protein
CCA	Cholangiocarcinoma
CCD	Cleidocranial dysplasia
cDNA	Complementary deoxyribonucleic acid
CR2-Tag	Cryptdin-2 gene
CRC	Colorectal cancer
CTCs	Circulating tumour cells
CTGF	Connective tissue growth factor
DEC1	Antiapoptotic transcription factor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular matrix
EDTA	Trypsin-ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinases
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ETS1	Protein C-ets1
FCS	Fetal Calf Serum
FGF	Fibroblast growth factor
GFR	Growth factor receptor
HB-EGF	Heparin-binding Epidermal growth factor-like growth factor
HCA	Heterocyclic amines

hER β	Human oestrogen receptor β
HER2	Human epidermal growth factor receptor 2
Hh	Hedgehog
HRT	Hormone replacement therapy
IGF-1	Insulin-like growth factor
IL-33	Interleukine-33
JNK	c-Jun NH2-terminal kinase
LNCap	lymph node carcinoma of the prostate
LNE	*larrea nitida* extract
MAPK	Mitogen-activated protein kinases
MaSCs	Mammary stem cells
MCP-1	Monocyte chemoattractant protein-1
MDA-MB 231	Oestrogen receptor- negative invasive ductal breast carcinoma
MET	Mesenchymal to epithelial transition
MHC I/II	Major histocompatibility complex class I/II
MMPs	Matrix metalloproteases
mRNA	messenger Ribonucleic acid
NCI	National cancer institute
NFκB	Nuclear factor-keppa light-chain-enhancer of activated B cells
NK	Natural killer cells
NKT	Natural killer T cells
NSCLC	Nonsmall cell lung cancer
O-Dma	O-Desmethylangolensin
Oct4	Transcription factor for somatic cell reprograming
OPG	Osteoprotegerin
Osx	Osterix
PAHs	polycyclic aromatic hydrocarbons
PAP	Prostatic acid phosphatase
PCa	prostate cancer
PCR	polymerase chain reaction
PDGF	Platelet-derived growth factor
PEs	Phytoestrogens
PKCδ	protein kinase δ
PR	Progesterone receptor
PSA	Prostate specific antigens
PTH-1R	Parathyroid hormone receptor

PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RTK	Receptor tyrosine kinase
RGD	Arginylglycylaspartic acid
RNA	Ribonucleic acid
RNAase	Ribonucleic acid enzyme
ROS	Reactive oxygen species
RQ	Relative quantification
RUNX2	Runt-related transcription factor 2
SDF-1	Stromal cell-derived factor 1 (SDF1)
SERMs	Selective oestrogen receptor modulators
SHBG	Sex hormone binding globulin
SIF	Soy isoflavones
SIP 1	Smad interacting protein 1
SMAD	The mothers against decapentaplegic
SNAIL	Zinc finger protein SNAI
SOX2	Sex determining region Y-box 2
sRANK	soluble Receptor activator of nuclear factor-κB
sST2	soluble Interleukin-33 receptor
ST2	Interleukin-33 receptor
TAG	Tris-acetate-EDTA
TAM	Tumour associated macrophage
TGF-β	Transforming growth factor-β
Th1	T-helper1 lymphocyte
TIMP	tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis actor-α
TP	Thymidine phosphate
TGFβR I/II	Transforming growth factor-β receptor type I/II
uPA	urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
Wnt	Wingless-type MMTV integration site family member
ZEB 1 & 2	Zinc finger E-box-binding homeobox 1&2

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Chapter one: General introduction

1.1 General Introduction

Cancer can be defined as an unregulated growth arising from one cell. The scientific or medical term for cancer is a malignant neoplasm, which is defined as an independent growth of tissue not subject to the basic regulation of normal growth and apoptosis that is able to invade local tissues and metastasise to distant sites.

Cancer accounts for about 30% of all deaths in developed Western countries and one person out of three will be treated for cancer in their lifetime. As the incidence of most cancers rises with age, the number is expected to grow, if life expectancy continues to increase. If one considers the incidence and mortality by tissue type most are carcinomas which arise from epithelial tissue (Schulz, 2005). Breast cancer in women and prostate cancer in men account for a significant proportion of carcinomas alongside those of the lung and large intestine. Less prevalent but still posing a substantial burden are carcinomas of the bladder, stomach, liver, kidney, pancreas, oesophagus, cervix and ovary. Each of these like breast and prostate display geographical differences in incidence (Schulz, 2005).

1.2 Breast cancer

Breast cancer is a complicated disease that displays distinct clinical, morphological and molecular subtypes (Eroles et al., 2012). Four basic types of breast cancer have been recognised (Luminal A, Luminal B, HER2-enriched, and basal-like) and a normal breast-like group (Prat & Perou, 2011; Sørlie et al., 2001). The basal subtype is defined by the absence of ER, PR, and HER2 expression and studies suggest that the triple negative and basal types may be synonymous(Vallejos et al., 2010) .

During the last decade, technological advances, in particular high throughput genomic analysis, suggest that the four subtype classification system is too simplistic (Eroles *et al.*, 2012). Furthermore, even if a tumour initially displays a specific mutation that could be targeted, subsequent changes can lead to drug resistance as the disease progress (Eroles *et al.*, 2012), generating additional molecular subtypes of breast cancer. A greater understanding of these molecular variants could enable the development of new therapeutics to target these resistant forms (Ashworth, 2008; Farmer et al., 2005).

The concept that breast cancers are heterogeneous has led to the potential for a more personalized approach to prognosis and therapy to enable the best outcome (Eroles et al., 2012). Personalized therapy is already available for ER and HER2neu positivity, but additional specific therapeutic/screening plans have the potential to improve death rates (Rahib et al., 2014). For instance, a new breast cancer intrinsic subtype, known as Claudin-low, has been identified in human and, mouse tumours (Herschkowitz et al., 2007), and a panel of breast cancer cell lines (Prat et al., 2010). Clinically, the majority of Claudin-low tumours are poor prognosis ER-negative (ER-), PR-negative (PR-), and HER2-negative (HER2-) (triple-negative) invasive ductal carcinomas with a high frequency of metaplastic and medullary differentiation. Data shows that they have a response rate to standard neoadjuvant chemotherapy (a cheomotherapy with other treatments given before breast cancer surgery) that is intermediate between Basal-like and Luminal tumours. Also, claudin-low tumours are enriched with unique biologic features linked to mammary stem cells (MaSCs), a main EMT signature, and show properties of tumour initiating cells (known as Cancer Stem Cells, CSCs) (Prat & Perou, 2011), the study of which is leading to the formulation of new hypothesis regarding the ‘cell of origin’ of the different

subtypes of breast. Furthermore, genes that are highly expressed in tumours with a poor prognosis are a possible target for the logical development of new drugs. Interestingly, ras^h gene (an early player in many signal transduction pathways to regulate cell division in response to growth factor stimulation) in both transfected and control transfected MCF7 cells lines will have a higher incidence of metastasis than the wild type MCF7 cells when supplemented with oestradiol but there was no association of ras^h expression with *in vivo* metastatic capacity of human mammary carcinoma cell line (Gelmann, Thompson & Sommers, 1992).

Thus, applying of screening/prevention strategy and novel treatment planning is decreasing breast cancer mortality. However, approximately 120,000 deaths due to breast cancer are predicted annually in the US and Europe (Jemal et al., 2009; La Vecchia et al., 2009). As genomic studies develop, more sub-classification of breast tumours into new molecular structures is expected to take place.

1.3 Prostate cancer

The prostate is located in the pelvis, surrounded by the rectum posteriorly and the bladder superiorly. It is formed from branching glands, with ducts that are padded with secretory epithelial and basal cells. Scattered neuroendocrine cells are also present within the tissue and are believed to serve a paracrine function for the differentiation of male specific characteristics. Epithelial cells represent the major cell type in the gland and are androgen-dependent for growth. The epithelium consists of two histologically distinct layers. The secretory luminal layer formed from tall columnar cells that produce Prostate Specific Antigen (PSA), Prostatic Acid Phosphatase (PAP) and human kallikrein-2 that are

secreted as part of seminal fluid. Traditionally, basal and luminal cells were considered two distinct cell types; however, differentiating transit amplifying cells give rise to heterogeneous subpopulations of cells as they migrate from the basal to the luminal layer (Hudson et al., 2001). The basal layer is not androgen dependent for growth and is believed to contain epithelial stem cells. Surrounding the gland is a stroma that includes fibroblasts, smooth muscle, nerves, and lymphatics. Stromal-epithelial interactions remain poorly understood, but insights suggest that stroma produces multiple factors important for development and growth of a healthy prostate as well as potentially contributing to the pathogenesis of prostate cancer Like activated stromal cell phenotypes, modified extracellular matrix (ECM) composition, and increased microvessel density (Krušlin, Ulamec & Tomas, 2015; Oh et al., 2003).

Prostate cancer is the most common non-skin cancer in elderly males (> 70 years of age) in Europe. It is an important health concern, especially in developed countries with a greater proportion of elderly men. The incidence is highest in Northern and Western Europe (> 200 per 100,000 men), while rates in Eastern and Southern Europe are lower but have shown a continuous rise. The incidence of prostate cancer has significantly increased in the past two decades due to screening, advanced biopsy techniques for diagnosis and greater public awareness. Changes in mortality however have not increased concordantly and in some countries mortality has decreased. During the last twenty years, the 5-year relative survival percentages for PCa steadily increased from 73.4% in 1999-2001 to 83.4% in 2005-2007 (Mottet et al., 2016). The difference between reported incidence and mortality rates leads to the potential conclusion that only a small proportion of low-risk prostate cancers will

proceed to life-threatening disease or alternatively new treatments have improved prognosis (Damber, 2008).

One of the major risk factors for prostate cancer, in addition to age and inherited susceptibility, is diet. Diets rich in fat and red meat are suggested to be associated with increased prostate cancer incidence whereas vitamin E, carotenoids, and selenium are protective (DeMarzo et al., 2003). In contrast, a comprehensive follow-up of Selenium and Vitamin E Cancer Prevention Trial (SELECT) participants showed that healthy men with average risk of prostate cancer exposed to contemporary community standards of screening who took a common dose and formulation of vitamin E have a significantly increased risk of prostate cancer. Thus, it is inconclusive that selenium and Vitamin E are of beneficial effect against prostate cancer progression (Klein et al., 2011).

The formation of carcinogens during cooking such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) has been suggested to contribute to tumour formation (Chiang & Quek, 2017). Saturated fats may lead to increased circulating insulin-like growth factor-1 (IGF-1), which in turn leads to PCa progression (Masko, Allott & Freedland, 2013). Most protective dietary factors such as vitamin E are potent antioxidants suggesting that oxidative stress could promote prostate carcinogenesis. Possible sources of oxidant stress are endogenous metabolism and inflammation (DeMarzo *et al.*, 2003)

Clinically, prostate cancer is diagnosed as local or advanced and treatments vary from monitoring to radical local treatment or androgen-deprivation. Androgen-deprivation decreases symptoms in about 70-80% of patients with advanced prostate cancer, however, most tumours relapse in two years to an incurable androgen-independent state (Amin et al., 2005).

Prognosis depends on disease burden at diagnosis (Damber, 2008). In general, patients with a high tumour burden do poorly, whereas patients with a low tumour burden will do much better, irrespective of treatment regimen. In other words currently the disease determines outcomes more than the choice of treatment. Stage, grade, and PSA are potent predictors and other markers seem to, so far, add little prognostic information (Graefen et al., 2004). Tissue markers of prostate needle-biopsy samples have failed to supply useful further prognostic information, even though many of the markers provide independent prognostic proof on post-prostatectomy specimens (i.e., too late to be of real value). Thus there is the need to identify other markers that could improve prognostics and outcomes. However, identifying molecular pathways that control the development and progression of prostate cancer is a real challenge, because of the heterogeneity and multifocality of tumours. The rise of new investigative tools such as DNA microarray technology, next generation sequencing and the use of proteomics may improve the knowledge concerning the initiation, development and progression of prostate cancer. These strategies allow detection of nucleotide substitutions, insertions, deletions, copy number variations, and chromosomal rearrangements. These have identified biomarkers and novel targets for drug developments such as v-erb-b2 avian erythroblastic leukaemia viral oncogene homolog 2 (ERBB2/ Her-2/neu) status (Hassan & Gomez, 2015). It may also become possible to distinguish between less-aggressive and aggressive types reserving radical treatment for the latter.

1.4 Breast and prostate cancer metastatic process

Tumour cells are capable of invading adjacent and distant tissues to form secondary tumours through a process known as metastasis (Hanahan & Weinberg, 2000), which is a key characteristic of malignancy (Voulgari &

Pintzas, 2009). Approximately 90% of all cancer-related deaths are associated with metastasis (Spano et al., 2012). Most tumours metastasise through the bloodstream and this involves a series of cellular adaptations, including invasion and migration from the primary tumour; intravasation into the vasculature; arrest and extravasation from the vasculature into the distant organ; and proliferation and survival in the new tissue microenvironment The acquisition of these biological traits by tumour cells involves the coordination of both intrinsic and extrinsic signals.

The first step of metastasis is invasion into the surrounding tissue. Cells must undergo modification in their cell-cell and cell-matrix adhesion interactions to disassociate themselves from the tumour (Cavallaro & Christofori, 2004). Acquisition of an invasive phenotype require alterations in expression of genes that regulate cell-cell adhesion, as well as proteolytic degradation of the extracellular matrix (ECM) (Friedl & Wolf, 2003). The acquisition of this phenotype alongside an increase in cell mobility is fundamental to epithelial to mesenchymal transition (EMT) (Fig 1.1). Tumour cells undergoing EMT show unique phenotypes express higher levels of cell motility proteins and have enhanced migratory and invasive potential. This involves changes in cell adhesion molecule expression in particular switching of E-cadherin expression to N-cadherin expression. During EMT E-cadherin expression is typically downregulated whereas N-cadherin expression is up-regulated, referred to as a “cadherin switch”. TGF-beta originates from many tissues, but is most abundant in bone, kidney, lung, and placental tissue. TGF-beta is synthesied by many but not all parenchymal cell types, and is also produced or released by infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets (Branton & Kopp, 1999). E/N-cadherin switch promotes cancer progression via TGF-β-

induced EMT in extrahepatic cholangiocarcinoma. During EMT, epithelial cells are transformed into mesenchymal cells changing their morphology from a cobblestone-like monolayer with apical-basal polarity to flat and spindle-shaped mesenchymal cells to gain the ability to move (Lee, Hwang & Choi, 2016).

Cells undergoing EMT develop interactions with the extracellular environment in localized areas of the carcinoma, where they lose intercellular coherence, modify extracellular matrix (ECM) and cytoskeletal structure to enable invasion into the extracellular space (Yang & Weinberg, 2008). EMT is associated with the expression of extracellular matrix proteases, such as urokinase-type plasminogen activator (uPA) and matrix metalloproteases (MMPs) Such as MMP-2, MMP-13 and MMP-12 in breast cancer and MMP-2 in prostate cancer , which degrade the ECM linked to the plasma membrane and localized to invadopodia during metastasis (Friedl & Wolf, 2003; Gilles et al., 2005; Son & Moon, 2010). EMT is a reversible and an inverse process, called mesenchymal-epithelial transition (MET) can occur at metastatic sties. Cells undergoing MET show increase cell-cell adhesion and gain back an epithelial phenotype (Davies, 1996; Foroni et al., 2012).

It is well known that tumour expansion requires a combination of increased proliferation and decreased apoptosis. One of the most well-studied regulators of cellular proliferation and apoptosis is TGFβ, which can inhibit cell cycle progression in non-malignant cells and early malignant tumour cells, but can also stimulate proliferation through poorly understood processes in more progressed cancer cells; TGFβ can also inhibit apoptosis in a variety of cell types (Massagué, 2012). TGF-β is a multifunctional cytokine, which triggers diverse cellular processes including growth arrest, tissue fibrosis, and EMT. To activate SMAD and MAPK signalling pathways TGF-β binds to TGF-β type II

(TβRII) and type I (TβRI) serine/threonine kinase receptors, respectively. Recent studies have shown a crucial role for TGF- β signalling pathways that induce EMT through Smad-dependent by phosphorylation of the cytoplasmic signaling molecules Smad2 and Smad3 and Smad-independent pathways (Meulmeester & Ten Dijke, 2011; Son & Moon, 2010). Transcriptional activities of Snail, ZEB and basic helix-loop-helix (bHLH) families which are important factors as inducers of epithelial–mesenchymal transition (EMT) and potent repressors of E-cadherin expression regulated by TGF-β to activate mesenchymal markers and degrade epithelial markers in a Smad-dependent fashion(Nantajit, Lin & Li, 2015; Peinado, Olmeda & Cano, 2007a), leading to changes in cytoskeleton organization, cell survival, migration and invasion. These effects are mediated through a range of zinc finger transcription factors including snail, twist and ZEB family members, which directly bind the E-box of the E-cadherin promoter to suppress E-cadherin expression. Furthermore, high level N-cadherin expression is often associated with poor prognosis. N-cadherin is also expressed in endothelial cells and plays an important role in the maturation and maintenance of normal vessels and tumour-associated angiogenic vessels (Batlle et al., 2000; Mariotti et al., 2007).

Cadherins are important mediators of EMT. They are type 1 membrane glycoproteins and function as active membrane-spanning macromolecular complexes. The extracellular regions are responsible for adhesive recognition, binding to the ectodomains of other cadherins presented on neighbouring cells. Cadherin cytoplasmic elements interact with proteins that connect the cadherin receptor to key intracellular processes including the actin cytoskeleton, cell signalling and trafficking (Jeanes, Gottardi & Yap, 2008). Cell-cell adhesions are mediated primarily by cadherins expressed at adherent junctions. E-cadherin,

as a typical molecule in epithelial adherent junctions, has been found to inactivate and repress tumour progression by maintaining intact cell-cell interactions and inhibiting cell mobility, invasion and metastasis (Perl et al., 1998; Thiery & Sleeman, 2006). Decreased E-cadherin expression is usually observed in aggressive cancers within epigenetic silencing, proteolytic cleavage, proteosomal degradation or mutation (Niederhuber et al., 2013). This helps to initiate the progression of invasive carcinoma by promoting subsequent aspects of EMT, as the loss of many epithelial markers including E-cadherin, occludin, claudins and β-catenin. This induces the expression of mesenchymal markers including N-cadherin, Snail, vimentin, R-cadherin and cadherin-11 and acquisition of mesenchymal phenotype, such as invasion and cell motility (Bailey, Singh & Hollingsworth, 2007; Zeisberg & Neilson, 2009). As a consequence, primary cancer cells lose cell-cell adhesion through E-cadherin repression, break through the basement membrane and enter the bloodstream through intravasation. Later, the circulating tumour cells exit the bloodstream to migrate to specific metastatic sites where they undergo mesenchymal to epithelial transition (MET) for clonal outgrowth. Factors directly secreted by osteoblast or released from the bone matrix during resorption are potential chemoattractive agents for bone colonizing breast and melanoma tumours.

Collagen I as a chemoattractant for human breast cancer cells (MB-MDA-231) demonstrated that tumour cells can secret enzymes to release collagen I from bone, because the bone matrix is generally composed of type I collagen, which is enzymatically degraded during the process of bone resorption and tumour infiltration, these collagen fragments could play a biological role in bone directed metastasis *in vivo*. Many cytokines normally present in bone were evaluated as possible chemotactic agents for human prostate tumours, including nterleukin-1

alpha, interleukin-2, interleukin-6, tumour necrosis factor-beta, transforming growth factor-beta, interferon alpha 2-a, EGF and granulocyte macrophage colony-stimulating factor (Chaffer & Weinberg, 2011; Cooper & Pienta, 2000).

E-cadherin influence tumourigenesis by modulation of mitogenic signalling. This notion first suggested by observations that cadherin adhesion could promote cell proliferation in the PC9 lung carcinoma cell line (Jeanes, Gottardi & Yap, 2008). Although these cells possess E-cadherin and β-catenin, they adhere poorly to one another due to lack of α-catenin. Restoration of α-catenin supports cell–cell cohesion and hinders proliferation, suggesting E-cadherin adhesion might associate in contact inhibition of growth. Growth factor stimulation is a major driver of proliferation and is often upregulated in tumours. Notably, many epithelial cancers display high levels of epidermal growth factor receptor (EGF-R, ErbB1), which is implicated in cell proliferation, invasion and metastasis (Bublil & Yarden, 2007). Support for this idea comes from the following lines of evidence. First, E-cadherin co-accumulates with EGF-R at cell–cell contacts and can physically interact with the EGF receptor and also with other members of the ErbB receptor tyrosine kinase family. EGF-R did not, however, interact with N-cadherin, suggesting a degree of selectivity among the classical cadherins. Second, E-cadherin can inhibit cell responsiveness to EGF stimulation. This was first suggested by (Qian et al., 2004) who observed that mitogenic responsiveness to EGF (measured cell proliferation and activation of Ras signalling) decreased as cells grew to confluence. In addition, activation of the Src family of kinases or of the Ras/MAPK pathway can, however, be initiated by a kinase-impaired EGFR and is linked to survival.(Jeanes, Gottardi & Yap, 2008; Walker et al., 1998)

N-cadherin overexpression has been observed in several carcinomas. Expression of N-cadherin is related to metastasis in prostate cancer and increases metastases in patients with castration-resistant tumours (Zhuo et al., 2013). N-cadherin expression is regulated by a wide range of factors leading to changes in cell-cell adhesion, differentiation, embryogenesis, migration, invasion and signal transduction. The aberrant expression of N-cadherin attributes a more fibroblastic phenotype to cancer cells, and they become more motile and invasive. One of the transcription factors responsible for upregulation is twist (Derycke & Bracke, 2004). The up-regulation of N-cadherin in aggressive carcinomas is crucial for invasion. Studies have shown that N-cadherin mediates transendothelial migration of cancer cells an essential step in metastasis (Drake et al., 2009; Qi et al., 2005) and N-cadherin aimed therapeutic strategies minimize N-cadherin positive tumour growth and metastasis (Shih & Yamada, 2012).

Kim et. al. (2012) explained that N-cadherin expression appears to be more critical for tumour malignancy than E-cadherin. N-cadherin promotes cell motility and invasion via interactions with growth factor receptors such as FGF receptors and PDGF receptor. N-cadherin further promotes cell growth and survival by suppressing apoptotic signals and numerous clinical studies have shown that aggressive human tumours express N-cadherin in situ, indicating a critical role for cadherin switch in human tumourigenesis. Therefore, both EMT and resistance to apoptosis in the absence of attachment to extracellular matrix (ECM) or upon cell adhesion to inappropriate location are important processes for metastasis and they share common regulators, such as Twist, Snail, Zeb1, E-cadherin, and N-cadherin (Kim et al., 2012; Paoli, Giannoni & Chiarugi, 2013).

Transcriptional regulation of E-cadherin differs in cultured cells versus xenografts, which more realistically reflect E-cadherin regulation in cancers in humans. Moreover, the aggressive nature of xenografts positive for E-cadherin and the frequency of metastases positive for E-cadherin suggest that high E-cadherin expression in metastatic prostate cancer is associated with aggressive tumour growth (Putzke et al., 2011). Genetic susceptibility factors may influence loss of E-cadherin expression in breast cancer, might provide new insights on pathways of E-cadherin loss against obtaining N-cadherin expression consistent with histology analysis (Horne et al., 2018).

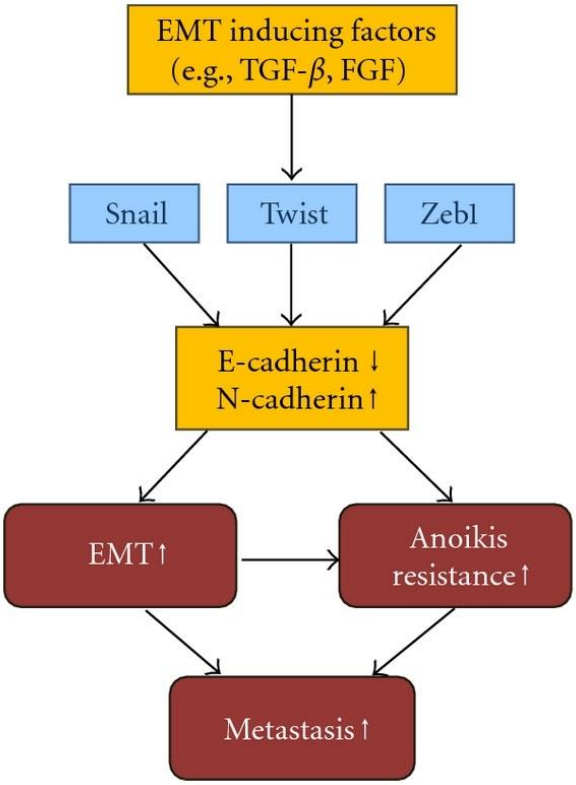

Figure 1.1 Schematic representation of EMT and anoikis resistance. EMT-inducing factors, such as TGF-β and FGF activate transcriptional factor, Twist, Snail and Zeb1. Activated these transcriptional factors repress E-cadherin (encoded CDH1 gene) expression and induce N-cadherin expression (Cadherin switch). Cadherin switch induces EMT and anoikis resistance, which are associated with tumour metastasis (Kim *et al.*, 2012).

1.5 Transcriptional regulators of EMT: Snail, slug, ZEB1, ZEB2 and Twist

Snail family members are the most studied effectors of EMT. These include scratch (SNAIL1), Slug (SNAIL2) and the less characterized SMUC (SNAIL3) (Nieto, 2002). All are zinc-finger type transcription factors and share a highly conserved C-terminal domain, containing four to six C2H2 type zinc fingers and bind to the E-box 5′-CACCTG-3′(Nieto, 2002). They have differential binding to

E box with snail having the greatest affinity (Bolós et al., 2003). Snail inhibits expression of epithelium-specific genes such as PTEN, Muc1, claudin, and occludin as well as some nuclear factor receptors (Vitamin D receptor, HNF-1α) (Peinado, Olmeda & Cano, 2007b). PTEN as an important tumour suppressor for many types of cancer and can inhibit cellular proliferation, survival and growth by inactivating PI 3-kinase-dependent signalling (Leslie & Downes, 2004).

TGFβ and Wnt family proteins and growth factors that work through Receptor Tyrosine Kinases (RTKs), all promote SNAIL1 expression. SNAIL1 and SNAIL2 co-operate with other transcription regulators to control gene expression. For example, SNAIL1 co-operates with ETS1, which is activated by MAPK, to activate MMP expression. Matrix metalloproteinase-3 (MMP3) can stimulate EMT of cultured mouse mammary epithelial cells through a process that involves increased expression of Rac1b, a protein that stimulates alterations in cytoskeletal structure and cause cells to spread and cover large surfaces. Thus, MMP-3 causes EMT associated with malignant transformation through a pathway dependent upon production of reactive oxygen species (ROS). Snail, a ROS-dependent mediator of MMP-3-induced changes, is regulated by NF-kB in response to MMP-3. Furthermore, MMP-3 gives rise to binding of p65 and cRel NF-kB subunits to the Snail promoter, leading to its transcription. It also co-operates with the SMAD3-SMAD4 complex to cause TGFβ-mediated repression of E-cadherin and occludin expression (Gao et al., 2016; Nelson et al., 2008). Many studies have described the presence and role of MMPs in many types of cancers including breast cancer such as MMPs (MMP-2, -7, -9, -10, -11, -13, -14 and -15) have been involved in BC progression and metastasis. Specifically

MMP-1, -2, -3, -7, -9 and -14 are implicated as key factors in tumour invasion, metastasis and angiogenesis (Merdad et al., 2014).

The ZEB family of transcription factors consists of two members: ZEB1(TCF8 and δ EF1) and ZEB2 (ZFXH1B and SIP1) (Vandewalle, Van Roy & Berx, 2009). The family's structure is characterised by the presence of two zinc finger domains and a homeodomain. The zinc finger domains are located at both ends of the protein and contain three to four zinc fingers of the C2H2 and C3H type. The homeodomain is found in the middle part of the protein. ZEB proteins interact with DNA through the simultaneous binding of the two zinc finger domains to E-boxes (Vandewalle, Van Roy & Berx, 2009). Both are potent repressors of E-cadherin, but the relative impact of ZEB and Snail members in EMT is contested. Some studies suggesting that Snail may be more influential (Vega et al., 2004), whereas other studies found that silencing of ZEB1 has a greater impact on E-cadherin expression than Snail (Eger et al., 2005; Vandewalle et al., 2005; Vandewalle, Van Roy & Berx, 2009).

TWIST, a highly conserved basic helix-loop-helix transcriptional factor is transcriptionally controlled by EGFR/ STAT3 and NF-kB signalling. In cancer cells, TWIST1 represses E-cadherin and induces N-cadherin expression independent of SNAIL and probably by the association with other proteins. TWIST recruits the methyltransferase SET8, which interferes H4K20 monomethylation, a histone mark that is associated with suppression at E-cadherin promoters and with activation at N-cadherin promoters. Studies also showed that ectopic expression of TWIST results in both morphological and molecular modifications in the expression of particular proteins through downregulation of epithelial markers and upregulation of mesenchymal markers (Gao *et al.*, 2016).

1.6 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are proteolytic enzymes responsible for the hydrolysis of basic elements of the extracellular matrix (ECM) to facilitate tumour cell dissemination. MMPs have a multi-domain structure which includes a signal peptide domain, a pro-peptide domain and a catalytic domain (Gong, Chippada-Venkata & Oh, 2014). Evidence suggests that MMPs participate in several stages of cancer progression modifying six principal processes of cell physiology, including self-supporting growth signals, resistance to growth inhibitory signals, insensitivity to apoptosis, over replication, angiogenesis, and invasion to other tissues and metastasis (Egeblad & Werb, 2002; Hanahan & Weinberg, 2000). Furthermore, MMPs can directly facilitate cancer progression by degrading the basement membrane, allowing cancer cells to invade into the surrounding stroma (Fig 1.2), but MMPs can also act directly on the tumour cells, releasing factors that promote growth or suppress apoptosis (Gialeli, Theocharis & Karamanos, 2011). Imbalances in MMPs activate cellular processes that cause DNA damage and stimulate genomic instability(Radisky & Bissell, 2006)

Furthermore, MMPs have been implicated in tumour angiogenesis, the penetration of a tumour by new vessels sprouting from existing ones (Kessenbrock, Plaks & Werb, 2010; Weis & Cheresh, 2011). MMPs can also affect the tumour microenvironment by promoting the development of activated stromal cells. Fibrosis, the abundance deposition of collagen and fibroblast proliferation that is correlated with most types of cancer, is largely the product of myofibroblasts. These cells accumulate by activation of stromal fibroblasts or circulating fibrocytes, or directly from epithelial cells by EMT (Mehner & Radisky, 2013). Myofibroblasts are significant origins of breast cancer MMPs (Del Casar

et al., 2009; Heppner et al., 1996; Vizoso et al., 2007) and tumour progression and poor prognosis is associated with stromal expression of MMP-1, MMP-7, and MMP-12 (Finak et al., 2008) and with fibroblast-specific production of MMP-9, MMP-11, and MMP-14 (Del Casar *et al.*, 2009; Vizoso *et al.*, 2007). Cancer cells can also directly secrete variant isoforms of collagen that are resistant to cleavage by MMPs and that can function as tracks for guiding cancer cell invasion in MMP-rich microenvironments (Han et al., 2010; Makareeva et al., 2010).

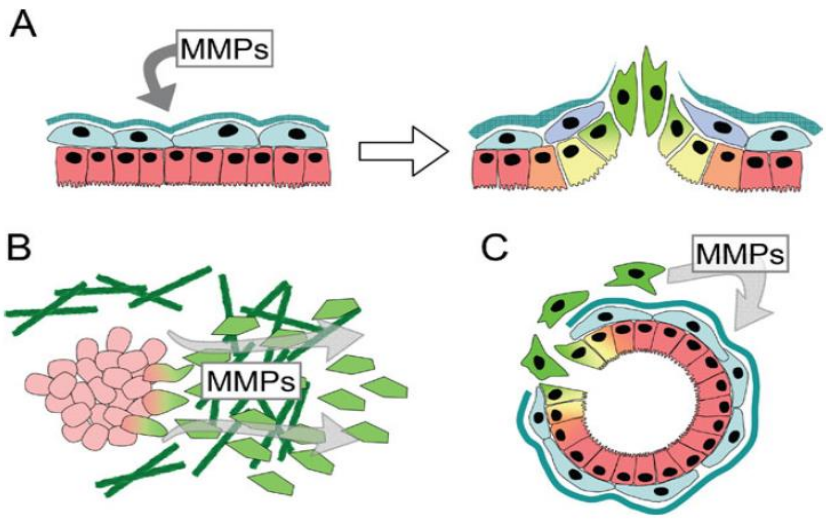

Figure 1.2 MMPs facilitate EMT-associated tumour progression. A. Exposure of epithelial cells to MMPs can directly induce EMT. B. Increased expression of MMPs in cells which have undergone EMT facilitates cancer cell invasion. C. EMT can produce non-malignant stromal cells which drive tumour initiation and progression through production of MMPs (Radisky & Radisky, 2010).

In prostate cancer tissue, there is an imbalanced expression of MMPs and tissue inhibitor of metalloproteinase (TIMP), displayed as a general loss of TIMP-1 and upregulation of MMPs. As a result, it is generally understood that MMPs are more effective in advanced stages of prostate cancer, as shown by the fact that most MMPs display higher expression in cancers with high Gleason scores. Analysis of MMP mRNA and protein levels in the serum and tissue specimens from prostate cancer patients revealed that increased expression of MMP-2, -3, -7, -9, -13, -14, -15 and -26 is associated with advanced or metastatic disease, while MMP-1 expression is associated with lower grade tumours and incidence of invasion. The distinct roles these MMPs play in the hallmarks of cancer progression are illustrated in (Fig 1.3). Among the various members of the homologous MMP family, MMP-2, -7, -9 and MT1-MMP are the best investigated for their roles in prostate cancer progression. Overall, expression of these MMPs promote prostate cancer progression but with differences in their mode of expression, role and prognostic importance. For instance, in genetically-engineered mice, although the reduction of MMP-2, -7, or -9 in CR2-Tag mice all led to decreased tumour vascularity, the loss of MMP-2 decreased lung metastasis and improved survival, whereas the lack of MMP-9 led to increased perivascular invasion (Littlepage et al., 2010). As described previously by (Merdad *et al.*, 2014) MMPs play an important role in breast cancer progression , metastasis, invasion and angiogenesis and warrant further study as diagnostic markers and potential drug targets (Fig 1.4)

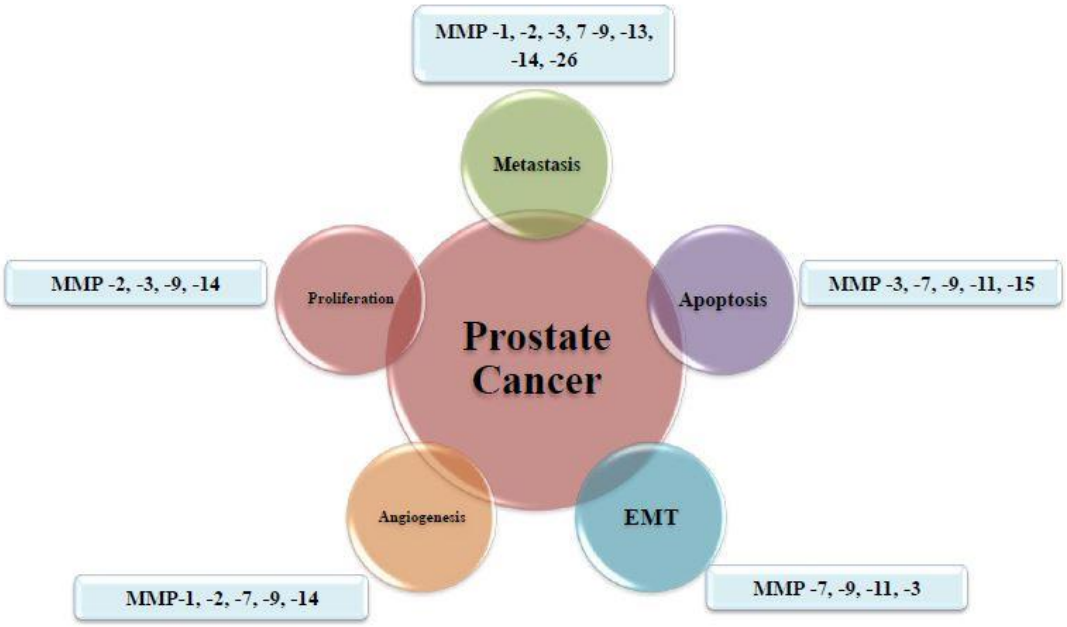

Figure 1.3 Roles of MMPs in several hallmarks of prostate cancer progression (Gong, Chippada-Venkata & Oh, 2014)

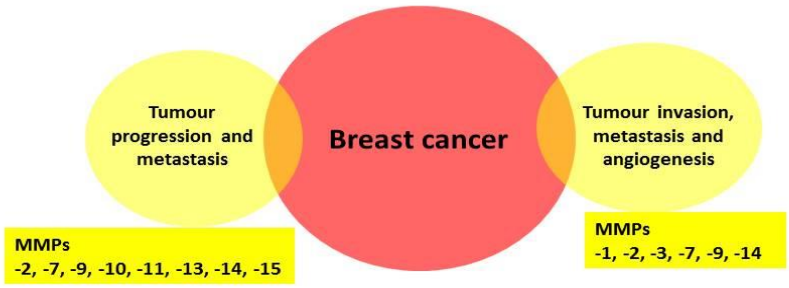

Figure 1.4 Role of MMPs in breast cancer progression

1.7 Preferential metastasis of prostate and breast cancer to bone

In 1889, Stephen Paget classically described the “seed and soil” hypothesis as the propensity of circulating tumour cells (CTCs) to home to specific organs, irrespective of the nearest anatomical location to the primary tumour (Paget, 1989). The diffusion of prostate and breast cancer metastatic cells particularly to the skeleton is defined as osteotropism and is defined by many markers expressed by the tumour cells and the bone microenvironment such as stromal cells. The interactions between cancer cells and the endothelium of the bone marrow vasculature are one of the core processes supporting extravasation from blood vessels and is proposed to underlie bone-specific dissemination (Fig 1.5). During this process, surface molecules expressed on cancer cells such as the chemokine (C-X-C motif) receptor CXCR4 which broadly expressed by both mononuclear cells and progenitor cells in the bone marrow, CCL2, αvβ3 integrin, CD44 and RANK are directly engaged in homing to the bone. The connection between CXCR4 and its ligand CXCL12 also known as stromal derived factor 1 (SFD1), expressed at high levels in tissues invaded by metastasis, is very important in prostate and breast cancer preferential metastasis to the skeleton (Zoni & van der Pluijm, 2016). CXCL-12 is produced in large amounts by bone marrow. Metastatic cells from breast or prostate cancers that express CXCR-4, the CXCL-12 receptor, migrate preferentially to the marrow due to the chemotactic properties of CXCL-12. RANKL and RANK are required for the development of the lactating mammary gland during pregnancy, and for lymph node organogenesis in mouse embryos (Jones et al., 2006). RANK overexpression in human breast cancer cells increases their aggressiveness buy increasing the osteoclastongesis process that increase osteoclast degradation of bone and may result in poorer clinical outcome (Santini et al.,

2011). RANKL can be released as a soluble factor by the proteolytic effects of matrix metalloproteases (MMP-7, MMP-13) and membrane metalloproteases (MMP-1), which are produced during the formation of bone metastases. *In vitro*, soluble RANKL promotes the migration of tumour cells that express RANK. Thus, RANKL may share with chemokines the ability to help tumour cells to metastasise to the bone marrow (Clézardin, 2017). Data suggests that RANK expression status influences whether tumours predominantly migrate into bone. The association of high RANK expression with osteotropism in murine models was confirmed across diverse tumour cell types, including breast cancer and melanoma (Jones *et al.*, 2006). RANK overexpression in human breast cancer cells increases their aggressiveness and may result in a poorer clinical outcome. In fact, RANK expression progressively increases with pathologic grade and significantly correlates with a high proliferative index in clinical samples of breast cancer patients. Higher levels of RANK mRNA expression were found in (ER- PR-) tumours, which were consistent with the higher degree of RANK protein expression in these tumours. ER- PR- tumours are more aggressive, show a higher incidence of metastasis and worse prognosis than luminal tumours, and contain a higher frequency of CD44+/CD24- stem cell markers. Moreover, RANK/RANKL mRNA expression levels allow discrimination between metastatic and non-metastatic adenocarcinomas (Palafox et al., 2012).

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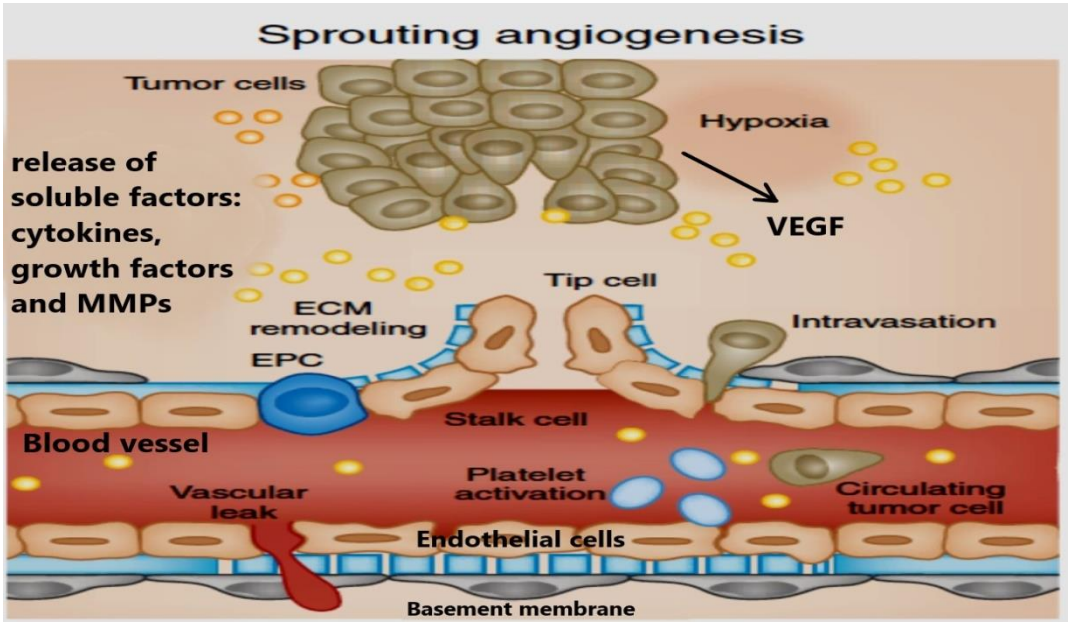


Figure 1.5: Multiple origins of tumour-induced neovascularisation. Showing the relation between tumour cells and the formation of new blood vessel (Weis & Cheresh, 2011)

1.8 Vicious cycle in bone microenvironment

The “Vicious Cycle” hypothesis provided the basis for the inhibition of bone resorption as a strategy to interfere with bone metastatic tumor growth. However, in the clinical setting inhibition of bone resorption while an effective strategy to reduce skeletal-related events may have little direct negative impact on cancer cell growth and tumour mass. This observation suggests that other mechanisms than bone resorption support cancer cell growth at the bone metastatic site (Hensel & Thalmann, 2016).

Bone is composed of hard-mineralized tissue; therefore it is highly resistant to invasion and destruction by cancer cells opposed to other metastatic sites. Osteoclasts have been described as the most efficient cells to induce bone resorption ‘‘bone-resorbing machines’’. Consequently, to grow within the bone matrix, the cancer cells must control the capacity to induce osteoclastic or

osteoblastic activation, which is the main cellular mechanism for cancer-induced bone destruction. Elevated osteoclastic bone resorption would then provide a microenvironment in which cancer cells can grow and induce further molecular interactions with the different cytokines within the bone microenvironment (Azim, Kamal & Azim, 2012). TGF-β which is deposited in the bone matrix by osteoblasts and released and activated by osteoclast during osteoclastic bone degradation. TGF-β is not the most abundant growth factor in bone, but it plays the most important role in the progression of osteolytic metastases (Buijs, Stayrook & Guise, 2011; Fox & Lovibond, 2005) .

It has been suggested that cancer cells produce soluble factors that activate directly (RANKL) or indirectly via osteoblasts parathyroid hormone-related peptide (PTHrP), IL-8, osteoclast differentiation and maturation. During bone degradation, osteoclasts release tumour supportive growth factors stored in the mineralized bone matrix (insulin-like growth factor-1, fibroblast growth factor, TGF- β, etc.). This vicious cycle has been proposed to accelerate tumour development in bone. RANKL/RANK blocking by soluble RANK (sRANK) or OPG released by osteoblasts (OBs) successfully prevented the development of bone metastases (Ando et al., 2008).

Tumour cells release several factors that stimulate osteoclast activity and bone resorption. Among them, parathyroid hormone-related peptide (PTHrP) was the first to be identified as involved in malignant osteolysis. PTHrP shares structural homology with parathyroid hormone and can, therefore, bind to its receptor PTH-1R, thus, stimulating the expression of RANKL, which in turn indirectly leads to the formation of new osteoclasts and increased bone resorption. PTHrP can also act in an autocrine form by promoting the production of connective tissue growth factor (CTGF). Connective tissue growth factor (CTGF) belongs

to the CCN family of cysteine-rich proteins and stimulates osteoclast formation in vitro. *In vivo*, the treatment of animals with an antibody to CTGF prevents the formation of osteolytic metastases induced by MDA-MB-231 cells (Clézardin, 2017).

1.9 Bone morphogenetic proteins (BMPs)

Members of the transforming growth factor (TGF) superfamily, which include bone morphogenetic proteins (BMP), are involved in the control of various biological processes, including differentiation, proliferation, apoptosis, and regulation of invasiveness (Buijs et al., 2007a). BMP-7 protein is expressed at higher levels in PCa bone and soft tissue metastasis when compared with primary PCa, supporting that BMP-7 signaling is related to clinical disease progression (Morrissey et al., 2010). Bone morphogenetic protein-6 (BMP-6) has been strongly involved in prostate cancer development and bone metastasis (Darby et al., 2008). Other BMPs may play a protective role against cancer progression such as BMP-9, that can inhibit cell growth, adhesion, invasion, and migration of prostate cancer cells in vitro (Ye, Kynaston & Jiang, 2008). In breast tumours, BMP-2, BMP-4, BMP-5 and BMP-7 expression has been described as elevated and the latter two associated with poor prognosis. The importance may be that BMPs have two way actions in breast cancer, such as BMP-4, which not only suppresses breast cancer cell growth, but also encourages invasion and migration. Studies related to BMP-4 expression with low proliferation tumours, but also increased recurrence (Zabkiewicz et al., 2017).

Runt-related transcription factor 2 (Runx2) and Osterix (Osx) are downstream targets of BMP during osteogenesis. Runx2 is expressed in chondrocyte and osteoblast and it plays many roles in the process of chondrogenesis and osteogenesis (Baek, Choi & Kim, 2014). It functions as a regulator of osteoblast differentiation at an early stage and plays a role in skeletal morphogenesis and tooth development. Further, it is known to regulate G1 transition in osteoblast cells (Vimalraj et al., 2015).

In normal prostate tissues, Runx2 is expressed but its physiological role is unknown, both Runx1 and Runx2 have been shown to regulate prostate specific antigen (PSA) through the presence of Runx2 binding sites in the regulatory region of the gene. Many studies indicate a higher level of Runx2 expression in prostate cancer and some shown that Runx2 is associated with disease progression (Chua et al., 2009; Lim et al., 2010). Other studies support a role for Runx2 in driving a more aggressive phenotype in prostate cancer. Hypoxic selection of LNCaP cells led to the outgrowth of an androgen independent subline that showed increased survival, invasiveness and tendency to metastasise *in vivo*. These changes were associated with up-regulation of Runx2 that was at least in part responsible for the more aggressive phenotype (Blyth et al., 2010).

1.10 Phytoestrogens as a potential treatment

The search for plant-derived anti-cancer agents started in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. The United States National Cancer Institute (NCI) launched a comprehensive plant collection program in 1960, concentrated mainly in temperate regions. This led to the discovery of various novel chemotypes displaying a range of cytotoxic activities, including

the taxanes and camptothecins, but their development into clinically active factors spanned a period of some 30 years, from the early 1960s to the 1990s. This plant collection program was ended in 1982, but the development of new screening technologies led to the revival of collections of plants and other organisms in 1986, with a focus on the tropical and sub-tropical regions of the world (Cragg & Newman, 2005).

In postmenopausal women, consumption of isoflavones was found to be associated with reduction of breast cancer incidence, mammary gland density and proliferation ability of mammary gland cells. These effects have been associated with the ability of isoflavones to increase serum Sex Hormone-Binding Globulin (SHBG) concentration, thereby reducing the bioavailability of sexual hormones in hormone-dependent tissues. Moreover, in peripheral tissues, isoflavones inhibit enzymes involved in the processes of cell proliferation (e.g. tyrosine kinases such as AKT and mitogen-activated protein) and reduce estradiol availability through the inhibitory effect on aromatase P450. (Lee, Lee & Sohn, 2005; Pilsakova, Riecanský & Jagla, 2010)

Hormone replacement therapy (HRT) after breast cancer raises many problems due to the oestrogen dependence of breast cancer, while the possible increased risk of breast cancer in healthy women taking HRT has to be considered for the treatment of oestrogen deprivation. Most physicians continue to avoid HRT in breast cancer survivors because of concerns regarding the enhancement of growth and dissemination of malignant cells while on HRT. Besides these (HRT) drugs, which are prescribed under medical control, soy phytoestrogens derived from plants are being promoted as the “natural alternative” to HRT, and have been available without restriction as nutritional supplements for many years.

After breast cancer, many women decide, sometimes 'by themselves', to have these phytoestrogens to mitigate menopausal symptoms (This et al., 2001).

Phytochemicals are chemical compounds found naturally in plants and consist of about 4000 different chemicals. A broad range of plant-derived compounds have been reported to have chemopreventive effects. Phytoestrogens are classified into four main groups: isoflavones (genistein, daidzein, glycetin and kaempferol), lignans (secoisolariciresinol, matairesinol, pinoresinol, lariciresinol), coumestan (coumestrol) and stilbenes (resveratrol). Western societies have been known to intake more foods containing lignans, while Asian populations eat more soy foods containing isoflavones (Lee, Hwang & Choi, 2016). Concentrations of phytoestrogens and their metabolites in plasma and urine have been recorded in several studies of humans and animals. In healthy people consuming diets without soy, plasma concentrations of isoflavones are usually in the nanomolar range (e.g., < 40 nmol/L). In contrast, plasma isoflavones concentrations increase considerably in the micromolar range after ingestion of isoflavones from soybean milk, soy meal, or baked soybean powder (Bhathena & Velasquez, 2002).

Paraskevi Moutsatsou, (2007) define phytoestrogens as a large family of plant-derived compounds having significant oestrogen agonist/antagonist activity. These, naturally occurring molecules, include the isoflavonoids, lignans, coumestans, stilbenes and the flavonoids quercetin and kaempferol . Their effects, work via the oestrogen receptor subtypes ERα and ERβ, one cell type/tissue specific and dose-dependent. Phytoestrogens may act as "natural" selective oestrogen modulators (SERMs) and may possibly be considered for the prevention of postmenopausal osteoporosis and cardiovascular disease without an adverse effect on breast and uterus. On the other hand, oestrogens

are steroid hormones with a complex mode of action, characterised by high tissue specificity and dose-dependent activity. They have pleiotropic effects on a diverse range of tissues, such as ovary, testis, prostate, breast, uterus, bone, liver, cardiovascular, central nervous system and immune system. Effects include decreasing nitric oxide production by genistein in cultured mice macrophage suggesting a modulation of immune responses and inhibition of cultured T cells in response to CD28 monoclonal antibodies. Also, inhibition of leukotriene B4, interleukins and its receptors. Genistein, at high concentrations, also inhibits cytotoxic T-cell mediated tumoricidal activity and activation of NK cells. Oestrogens promote breast and endometrial cancer in women and exacerbate autoimmune diseases, whereas the loss of oestrogens during menopause has been correlated with osteoporosis, coronary heart disease, depression and neurodegeneration. Compounds which inhibit the oestrogenic effects (antagonists) in some tissues, such as breast and uterus, while enhancing the oestrogens effects (agonists) in other tissues, such as bone, brain and cardiovascular cells, are known as selective oestrogen receptor modulators (SERMs) (Cooke, Selvaraj & Yellayi, 2006; Moutsatsou, 2007).

Many epidemiological studies indicate that diets rich in phytoestrogens (PE), particularly soy and unrefined grain products, may be associated with low risk of some cancers, especially steroid hormone-dependent, e.g. breast and prostate cancers (Wietrzyk, Grynkiewicz & Opolski, 2005). Phytoestrogens have an oestrogenic activity or are converted to oestrogenic compounds by bacteria in the gut. The first class of phytoestrogens is the isoflavonoids, which are present in soybean products, some fruits and vegetables, and red clover. Genistein, daidzein and glycitein are the main dietary-derived isoflavones. Most of these compounds have a strong affinity for one ER subtype (Pearce & Jordan, 2004).

Since the first endocrine agent tamoxifen was described in 1986, tamoxifen as the standard endocrine therapy has been shown to reduce ER-positive breast cancer. However, more new investigations are suggesting the positive roles of naturally occurring selective oestrogen-receptors modulators (SERM) such as phytoestrogens as an alternative therapeutic option to avoid increased risks of tamoxifen for its detrimental tissue specific results such as thromboembolic events, strokes. Also, tamoxifen has shown estrogenic activity in the uterus and therefore may increase the risk of endometrial cancer. For example, Targeting ERβ and PR by Larrea nitida extracts (LNE) may work as a potential suppressor of women's cancer that enhances differentiation and inhibits ERα-mediated proliferation. Data from the studies of naturally-occurring (SERM), both in cell culture and in animals have provided more details into a new dimension of the complex nature of ER action in health and disease. Furthermore, Larrea nitida extracts (LNE) had a selective binding affinity to hERβ rather than hERα and treatment of LNE reduced the cell proliferation in the presence of endogenous oestrogen (Ahn et al., 2014; Ososki & Kennelly, 2003a). Further to ER-mediated signalling mechanisms, there is rising experimental evidence that soy isoflavones play important ER-independent effects. Genistein has been shown to inhibit the growth of ER-negative breast cancer cells, indicating that other cellular mechanisms may play an important role in chemoprevention as well. Numerous *in vitro* studies have revealed that isoflavones inhibit cell proliferation and trigger apoptosis by inhibiting the activity of several enzymes, such as tyrosine protein kinase, mitogen-activated protein kinase or DNA topoisomerase II. additionally, isoflavones, especially genistein, promote antioxidant defence and DNA repair, inhibit the development of tumour angiogenesis and metastasis and also interfere in other ER-independent signal transduction pathways (Uifălean et al., 2015).

Over recent years, as indicated by This *et al*. 2001 many *in-vitro* studies have identified beneficial effects of the main phytoestrogens, especially genistein and daidzein. Study of the effect of genistein in cultures of MCF7 cells that express the oestrogen receptor alpha (ERα) shows dual effects on mammary cells. According to the concentrations of genistein, with high soy dose it increases cellular proliferation and cells division, which is dependent on oestrogen receptor. While genistein's effect with oestradiol, the normal oestrogen, acts as a competitive inhibitor for the binding site of oestradiol to oestrogen receptor and inhibits cell proliferation since it has a lower activity than oestradiol. While high doses of genistein (>10 μM/L) inhibit cells proliferation, and this effect is not ER-dependent and may related to the inhibition of the tyrosine kinase activity of growth factor receptors such as the blocking of AKT signaling cause growth arrest and apoptosis (Chandarlapaty et al., 2011; This *et al.*, 2001)

Genistein is the most abundant isoflavone in soybean products and is well known to have various biological activities. Among these, its anti-cancer effects against some cancers including breast and prostate carcinomas have been considered to be most remarkable. Genistein effectively suppressed BG-1 ovarian cancer cell proliferation promoted by bisphenol A (BPA) or 17β-estradiol (E2). This anti-proliferative effect of genistein was accomplished by reversing the effects of E2 or BPA on the expression of cell cycle-related genes. Unlike the actions of E2 or BPA, genistein suppressed the expression of cyclin D1 which with Cdk4 (cyclin dependent kinase 4) plays an essential role in G1 phase. It can phosphorylate Rb family proteins to disable their role as transcriptional suppressors and allow activation of E2Fgenes - dependent transcription to promote S phase entry and initiation of DNA synthesis and promoted the expression of p21, which also known as cyclin-dependent kinase inhibitor

1 or CDK-interacting protein 1, is a cyclin-dependent kinase inhibitor (CKI), that is capable of inhibiting all cyclin/CDK complexes when delivered with E2 or BPA, thus, leading to cell cycle arrest in G1 phase (Du, Tong & Ye, 2013; Hwang et al., 2013; Xiong et al., 1993).

Zhao and Mu (2010) indicated that intracellular mechanisms of phytoestrogen protection against cellular proliferation of breast cancers might be through: (i) binding to nuclear ER and inhibiting genomic ER-mediated gene expression, (ii) interaction with membrane-ER, blocking protein kinases and suppressing transcription factors, (iii) inhibiting growth factor receptor(GFR) activation and its downstream signaling networks, (iv) activating caspases to initiate cellular apoptosis process and (v) reducing the G-protein mediated signalling pathway in the ER-negative mammary cancer cell (Figures 1.6 and 1.7). The nuclear ER interaction is the most widely studied mechanism of phytoestrogen effect. Ginsenoside, a phytoestrogenic component extracted from ginseng root, preferentially activated ERα via the phosphorylation of a transcription factor (AF-1) without the ligand-receptor interaction. However, isoflavones were demonstrated to selectively activate ERβ rather than ERα in the breast cancer cell line MCF7, indicating a potential ERβ-related mechanism underlying protective effect of dietary phytoestrogen against a mammary tumour. Moreover, genistein (an isoflavone) might stimulate a self-limiting mechanism of E2-stimulated ERβ gene expression in breast cancer cells (Zhao & Mu, 2010). In addition, oleocanthal (a minor secoiridoid and type of natural phenolic compound found in extra-virgin olive oil) has been observed to inhibit invasion and migration of human breast (MCF7, MDA-MB-231) and prostate cancer (PC3) cell lines. Applying these results to humans suggests that consumption of olive

oil phenolics may play a role in reducing metastatic spread in humans (Hashim et al., 2014).

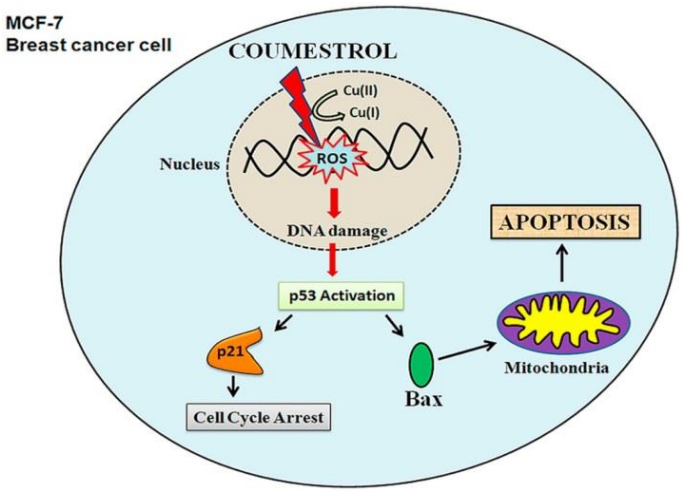


Figure 1.6 Phytoestrogen (coumestrol) effect on breast cancer proliferation (Zafar, Singh & Naseem, 2017).

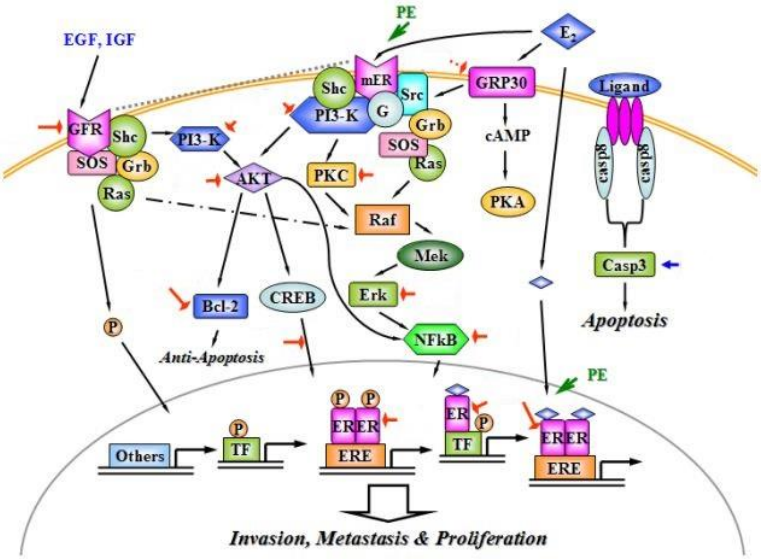


Figure 1.7 Phytoestrogen actions on modulating signalling pathways in the breast cancer cell. The arrows and hammers respectively present stimulation and inhibition TF: transcription factor, ERE: oestrogen responsive element. (Zhao & Mu, 2010).

1.11 Aim of this study

Phytoestrogens have beneficial effects on osteoblast and osteoclast activity and some such as genistein have been trialled in the clinic for the treatment of prostate cancer. However, while it is clear that PEs have effects on a range of cell types, there is little data in the literature on the expression of factors associated with preferential metastasis, disrupted remodelling and osteomimicry. Some data show a beneficial effect of genistein on markers but other PE have been poorly described and combinations typically seen in the diet have not been examined. Similarly, an interaction between PE has not been studied. Therefore, the proposed study aims to establish the effect of a range of phytoestrogens (genistein, daidzein and coumestrol) on cell proliferation, migration and the expression of genes with a role in EMT, preferential metastasis and modification of bone remodelling (CXCR4, snail, integrin α5, PTHrP, RANKL) and osteomimicry (Runx2, osterix and collagen type I) in breast and prostate cancer cell lines. The impact of cytokines associated with bone tumours (BMP-7, TGF-β and IL-33) on the response to selected PE will also be examined to ascertain if a more realistic bone environment alters their activity.

Chapter two

General Materials and Methods

2. Materials and Methods

2.1 Media, reagents and cell culture

Human MCF7 breast and PC-3 prostate cancer cells obtained from (ATCC, UK) were incubated in phenol red-free minimum essential medium or Ham's F-12 medium (Gibco ThermoFisher Scientific, UK) supplemented with 10% charcoal stripped foetal calf serum That decreased the levels of various hormones (Autogen Bioclear, U.K.), 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma-Aldrich, Poole, Dorset, UK). Incubations were performed at 37°C in 5% CO_2, and cultures fed every 2–3 days by replacing half of the culture volume with fresh medium. The non-selective oestrogen antagonist ICI 182,780 was obtained from Tocris Biosciences (Bristol, UK). All other reagents were obtained from (Sigma-Aldrich, Poole, Dorset, UK) unless stated.

2.2 Cell cryopreservation and reanimation

Cells of passage 3-7 were cryopreserved and then used for experiments as required. In brief, adherent cells were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA) solution for no longer than 5 minutes at 37°C. After detachment, cells were washed with the culture medium to deactivate trypsin and centrifuged at 1500rpm for 5 minutes at room temperature. After centrifugation, cells were resuspended in sterile 90% FCS and 10% dimethyl sulphoxide (DMSO) solution, placed in cryopreservation tube and frozen at -80°C for 24 hour before transfer to liquid nitrogen for long-term storage. To reconstitute frozen cells, vials were removed from liquid nitrogen and rapidly thawed and then immediately transferred into 5 ml of cell culture media, centrifuged to remove DMSO and then cultured in 25 cm2 flasks until 80% confluence was reached.

2.3 Measurement of cell viability

The effect of phytoestrogens on cell viability was assessed using a commercial proliferation assay. Cells were transferred to 96-well plates at a density of 10^5 cells per well and cultured with combinations of genistein, daidzein and coumestrol (10^{-5} to 10^{-9} M) for 72 hours. Viability was then assessed using an AQeous one cell viability assay which is a colorimetric technique for indicating the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The CellTiter 96® AQueous One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PEs has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution.

Assays are processed by adding a small volume of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 1-4 hours and then recording absorbance at 490nm with a 96-well plate reader. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture(Promega, UK). Cell viability in control cultures was signified as 1 and cell viability was calculated relative to this. Initial studies determined the profile of concentrations able to significantly alter viability. Experiments examining the role of oestrogen receptor signalling were performed by incubating cells with combinations of phytoestrogens shown to significantly modify viability in the presence of the oestrogen antagonist ICI 182,780 (10^{-5} M). Subsequent experiments examined the effect of combining these effective concentrations. Viability data was

normalized to un-treated control values and all assays were performed in triplicate.

2.4 Motility assay

To examine the effect of phytoestrogens on cell motility, cells were transferred to 24-well plates at a density of 10^5 cells per well and treated with genistein, daidzein and coumestrol (10^{-9} to 10^{-5} M) for 72 hours. A scratch was then made in the middle of each well using a sterile pipette tip. Wells were then washed three times in fresh medium to remove non-adherent cells and the cultures incubated for a further 48 hours in the presence of phytoestrogens. The separation of the edges of the scratch was used to quantify cell motility. To enable this, digital images were taken 0, 6, 12, 24 and 42 hours after scratching on an inverted microscope (Leica, Germany) fitted with a digital camera (Scopetek, DCM-510, China) at x40 magnification. Images were analysed by measuring the distance between the edges in mm of the scratch using ImageJ analysis software (fig 2.1) (National Institute of Health, USA).

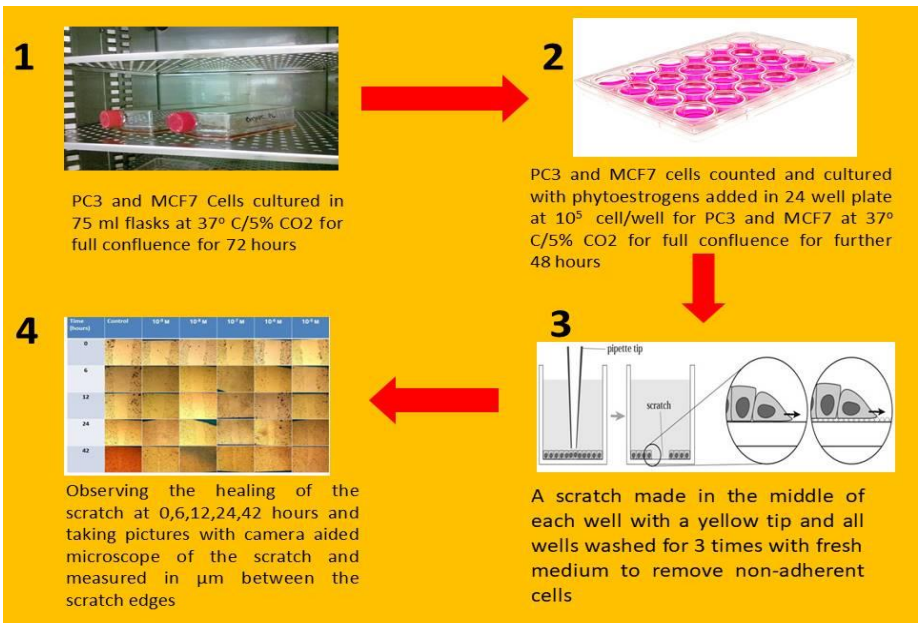


Figure 2.1: the wound healing assay technique steps used in the lab to study the motility of PC3 and MCF7 cells.

2.5 Molecular biology

2.5.1 RNA extraction and reverse transcription

Total RNA was isolated using a commercially available GenEluteTM mammalian total RNA miniprep kit (Sigma-Aldrich, Poole, Dorset, UK), which utilises a column based technique to isolate and purify RNA. After incubation, cells were washed with DPBS and total RNA extracted according to the manufacturer's protocol. All consumables and reagents used were free of contaminating DNAase and RNAase. Genomic DNA was removed using an on-column DNase-I treatment step. RNA and DNA quantity and purity were measured using a nanodrop spectrophotometer (ND-1000) (Labtech, Heathfield ,UK). Absorbance was measured with A260 for nucleic acid and the ratio A260:A280 was used to assess sample purity. RNA concentrations were then adjusted to 250µg/µl in molecular biology grade H_2O, RNA (1µg) was reversed transcribed to cDNA using M-MLV reverse transcriptase reaction in thin-walled PCR tubes (Sigma-Aldrich, Poole, Dorset, UK) using a GeneAmp PCR System 9700 machine. RNA was denatured at 70°C for 10 min in the presence of dNTPs (0.5 mM) (dATP, dCTP, dGTP, TTP) and random nanomers (1 µM). Reactions were cooled on ice for 5 minutes and then 1 unit of MMLV-reverse transcriptase was added to each reaction. Reactions were then incubated at room temperature for 10 min, 37°C for 50 min and 94°C for 5 min. RTs were stored at 4°C until used. Negative controls consisting of reactions lacking RT were run in all experiments.

2.5.2 Verification of PCR primers and RT

Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). 2 µl of cDNA was used for each PCR reaction. Each reaction contained 10 µM of forward and reverse primers, dNTPs (0.5 mM), Taq DNA polymerase (5

unit/µl) and PCR buffer (10x) in a final volume of 25 µl. Reaction conditions were 94oC for 2 minutes, followed by 40 cycles at 95oC for 30 seconds, 60oC for 30 seconds and 72oC for 30 seconds. Product size and primer specificity where then confirmed using agarose gel electrophoresis. PCR samples had 2.5 µl of loading buffer (orange G dye) added and was then loaded onto a 2% (w/v) TAE agarose gel. Gels were made by dissolving agarose (electrophoresis grade, Fisher Scientific, Loughborough,UK) in an appropriate volume of TAE buffer (40 mM Tris-base, l0 mM EDTA and 0.1% acetic acid) which was heated in a microwave for 2 minutes and then cooled to 50°C. 1 µl of ethidium bromide (10 mg/ml) was then added to the gel to enable visualization of DNA under UV light. Gels were run at 60-100V according to the size of the product for an appropriate time. Bands were checked for presence and size using UV gel documentation system (UVi Tech, Cambridge, United Kingdom) linked to a PC (Toshiba, Tokyo,Japan).

2.5.3 10x Tris-acetate-EDTA (TAE) buffer

0.4 M Tris-base, 0.5 M EDTA and 1M acetic acid were dissolved in 1 L of d.H_2O. The pH was adjusted to 8.5 and then diluted to make 1x TAE buffer.

2.5.4 Real-time quantitative PCR analysis of metastatic marker expression

Quantitative PCR is an advanced sensitive technique that enables the absolute quantification of gene expression in biological samples. This technique used in this study to detect the effect of PEs on the gene expression of key mediators of preferential metastasis using the $\Delta\Delta C_T$ methodology which is a simple formula used in order to calculate the relative fold gene expression of samples when performing real-time polymerase chain reaction (Livak & Schmittgen, 2001).

MCF7 or PC-3 cells (5 x 10^5 cells) were incubated in 25 cm^2 flasks for 24 or 72 hours with genistein, daidzein or coumestrol. Total RNA was extracted from these cultures using a Sigma GenElute RNA isolation kit and reversed transcribed with M-MLV reverse transcriptase using random nanomer primers. $\Delta\Delta C_T$ qPCR was performed on a StepOne PCR system (Applied Biosystems, Paisley, UK) using the DNA-binding dye SYBR green for detection of PCR products. A total of 1 µl of cDNA was added to a final reaction volume of 12.5 µl containing 0.05 U/µl Taq, SYBR green and specific primers (0.2 µM). Primers used prescribed in table 2.1.

	5´-3´ Forward primer	3´-5´ Reverse primer	Amplicon
Human β-actin	GCGCGGCTACAGCTTCACCA	TGGCCGTCAGGCAGCTCGTA	777-929
Human Runx2	AGACCCCAGGCAGGCACAGT	GCGCCTAGGCACATCGGTGA	816-972
Human CXCR4	GCGCAAGGCCCTCAAGA	GTGCGTGCTGGGCAGAGGTT	1010-1257
Human Snail	CGAGTGGTTCTTCTGCGCTA	CTGCTGGAAGGTAAACTCTGGA	27-183
Human Integrin α5β3	AATTTTACTGGCGAGCAG	TTGGTGGCATGCTTCGAG	1054- 1465
Human PTHrP	GTCTCAGCCGCCGCCTCAA	GGAAGAATCGTCGCCGTAAA	693-785
Human collagen type I	CCTGGCAGCCCTGGTCCTGA	CTTGCCGGGCTCTCCAGCAG	1766-1918
Human (osx)	GGCTCTAGCCCTCTGCGGGA	CGTGGGGGTTTGGCTCCACC	459-1123

Table 2.1 primers and their amplicons used in the experiments

The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR Green during the extension time. Reaction conditions were 94°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. At the end of each PCR run, a melt curve analysis was performed to show the absence of non-specific bands. The relative quantification (RQ) value for each group was calculated by the instrument's software using CT values for non-treated controls normalised to the

expression β-actin mRNA as the most widely used gene for normalization in the experiments of gene expression and to give the correct measurements when using the charcoal stripped (Rebouças et al., 2013). Samples were analysed in triplicate and experiments repeated separately three times.

2.6 Statistical analysis

Differences between groups were assessed using a Fisher's post-hoc analysis of variance test for pairwise comparisons between means (Statview; Abacus concepts, California, USA). The data corresponded to three independent observations from three separate repeats each consisting of three replicates. A p-value <0.05 was considered statistically significant.

Chapter Three

Individual phytoestrogens are more effective than combinations in reducing the viability of prostate and breast cancer cell lines, with no individual effect on their motility

3.1 Introduction

It has been suggested in some studies (Bilal et al., 2014; Hwang & Choi, 2015; Kyro et al., 2015) that Phytoestrogens, (PEs), a diverse group of plant-derived compounds with a structure and a function similar to that of oestradiol, have the capacity to reduce the incidence of tumour formation and the rate of cancer progression. In addition to their oestrogenic action, PEs have also been shown to inhibit tyrosine kinase and telomerase activity and to alter cellular redox status (Hwang & Choi, 2015). Spinozzi and his team indicated that exposure of in vitro cultured Jurkat cells, a T-cell leukemia line, to genistein resulted in a dose-dependent, growth inhibition. Cell-cycle analysis of genistein-treated cells revealed a G2/M arrest at low genistein concentrations. The imbalances in cell-cycle control were followed by apoptotic death of genistein-treated cells. Immunocytochemical analysis of cells showed that genistein effectively inhibit tyrosine kinase activity in cultured Jurkat cells. This indicate that the natural isoflavone genistein antagonises tumour cell growth by both cell-cycle arrest and induction of apoptosis and suggest that it could be an encouraging new agent in cancer therapy (Spinozzi et al., 1994). Epidemiological studies have been contradictory, suggesting variously that diets with a high phytoestrogen content may promote the risk of prostate and breast cancer, reduce the risk, or have no association at all with these forms of cancer (Trock, Hilakivi-Clarke & Clarke, 2006; Wu et al., 2008; Yan & Spitznagel, 2009).

Positive associations have been noted between soy protein intake and reduced incidence of breast cancer in both Asian populations, whose diets have a high soy content (Wu et al., 2008) and in Westerners given supplements (Trock, Hilakivi-Clarke & Clarke, 2006). Other studies, however, contest this assertion, and report that high soy consumption in Asian women living in Asia had no

effect on breast cancer incidence (Trock, Hilakivi-Clarke & Clarke, 2006). Also, the results of meta-analysis of epidemiological studies have indicated that soy consumption in Asian populations has no disadvantageous effects on the risk of breast cancer recurrence, and in some cases it significantly reduces the risk (Magee & Rowland, 2012). Similarly, a six-month isoflavones intervention involving Western women increased Ki-67 levels, a marker of proliferation, in breast epithelial cells of premenopausal women (Khan et al., 2012).

On the other hand, dietary soy isoflavones have been shown to stimulate metastatic tumour formation in lungs and increased Ki-67 protein expression in these metastasised tumours (Yang et al., 2015). In prostate tumours, additional factors, including PSA and IGFs, may also increase tumour growth (Abrahamsson, 2004). Genetic predisposition, inflammation and increased cell proliferation are some of the pre-element factors for prostate cancer commencement. Prostate cancer development depends on the decrease of androgen levels until a completely androgen-independent cancer is formed. Molecular and pathological analysis of human prostate cancer samples and animal model-based researches have both shown that infectious agents, oestrogenic hormones, age, race, genetics and other occupational factors can damage the prostate epithelium and provoke inflammatory responses leading to chronic or recurrent condition of prostate cancer (Joshi et al., 2015).

Alterations in molecules that regulate the cell cycle and apoptosis offer promising avenues for further investigation. P53, p27, p21, and Rb have been studied, resulting in variable levels of evidence that they participate in the pathogenesis of prostate cancer. In one study, loss of expression of the tumour suppressor protein p27 was strongly associated with the development of benign

prostatic hyperplasia, (BPH), whereas variable levels of p27 expression were noted in prostate cancers. In addition, there is a high frequency of somatic mutations in PTEN, a tumour suppressor gene, which suggests that this may be a frequent target for inactivation in sporadic prostate cancers growth factors. Their receptors also play an important role in the growth regulation of normal and cancerous cells. Several specific growth factors have been associated with the growth and survival of prostate cancer (Oh et al., 2003). It seems that several signalling cascades are involved in prostate cancer progression, and understanding the key signalling events and their complex inter-relationships has become essential to better therapeutic methods (Joshi et al., 2015).

The interaction of receptors with their specific growth hormone results in cell growth and proliferation. The overexpression of either or both growth factors and receptors contribute to the consecutive signalling in cancer (Perona, 2006). Growth factor TGF-β causes growth arrest of most of the cell types. The mechanisms, which differ somewhat between cell types, involve the inhibition of the expression of the transcription factors Myc and members of the Id family, and induction of the cell cycle inhibitor tumour cells becomes increasingly resistant to the cytostatic effects of TGF-β, which is a vital factor in the shift from being a tumour suppressor to a tumour promoter. During tumourigenesis, TGF-β promotes cell survival and metastasis of breast cancer cells by increased expression of the anti-apoptotic factor (DEC1) (Heldin, Landström & Moustakas, 2009).

The conflicted nature of the literature may reflect differences between menopausal status and short term and long term effects of PE, with earlier exposure being more beneficial. It may also reflect interactions between the diverse ranges of PE that can be obtained from a diet, which could also impact

on the outcome, according to supplementation studies. While it is clear that most individuals will be exposed to a range of dietary phytoestrogens, which may have contrasting effects, in vitro studies examining the cellular impact of PEs have typically assessed the impact of PEs in isolation. Thus, the current study initially investigates the effect of individual and combinations of PEs on breast (MCF7) and prostate (PC3) cancer cell lines' viability to ascertain which is more effective after incubation for 72 hours. This is followed by a wound healing assay to study the effect of phytoestrogens on motility, and to observe whether there is a connection with invasion and metastases of these cell lines.

3.2 Materials and Methods

Methods used here in chapter 3 are similar to what been mentioned in chapter 2 (materials and methods) for the points:

2.1 media reagents and cell culture

2.2 cell cryopreservation and reanimation

2.3 measurements of cell viability

2.4 motility assay

2.6 statistical analyses

3.3 Results

3.3.1 Combinations of phytoestrogens have less effect on viability

Experiments were undertaken to establish how prostate (PC3) and breast (MCF7) cancer cells respond to PE individually, in combination and to non-selective oestrogen antagonist ICI 182,780.

PC3 and MCF7 cells were incubated with phytoestrogens for 72 hours to have long exposure time of cells to PE. Genistein significantly decreased PC3 and MCF7 cell viability, PC3 cells response at 10^{-7} M was (15%, P = 0.01) and for 10^{-6} M was (11%, P = 0.04) (Figure 3.1 A); for MCF7 cells response at 10^{-6} M was (15%, P = 0.04) and for 10^{-5} M was (25%, P = 0.04) (Figure 3.1 B). Daidzein significantly reduced viability of PC3 cells, 10^{-8} M (18%, P = 0.04) and for 10^{-7} M (22%, P = 0.01) (Figure 3.1 A), whereas it had no effect on MCF7 cell viability at any concentration (Figure 3.1 B). In contrast coumestrol reduced MCF7 viability at 10^{-7} M (29%, P = 0.03,) 10^{-6} M (34%, P = 0.01) and 10^{-5} M (37%, P = 0.007) (Figure 3.1 B), but had no effect on PC3 cells and increase cell proliferation compared to control (Figure 3.1 A). The inhibitory effect of all phytoestrogens was reversed by incubating with the oestrogen receptor antagonist ICI 182, 780 and this may be related to the effect of endogenous oestrogen and to the ER-independent pathway (Figure 3.2 A and B).

To determine the nature of any interaction between phytoestrogens the effect of incubating cells with combinations of phytoestrogens shown to significantly reduce viability was examined. Surprisingly combinations of phytoestrogens were antagonistic, as while individual phytoestrogens reduced viability no significant effect was noted when cells were incubated with combinations (Figure 3.3 A and B). In light of this, all subsequent studies examined effective concentrations of individual PE rather than combinations.

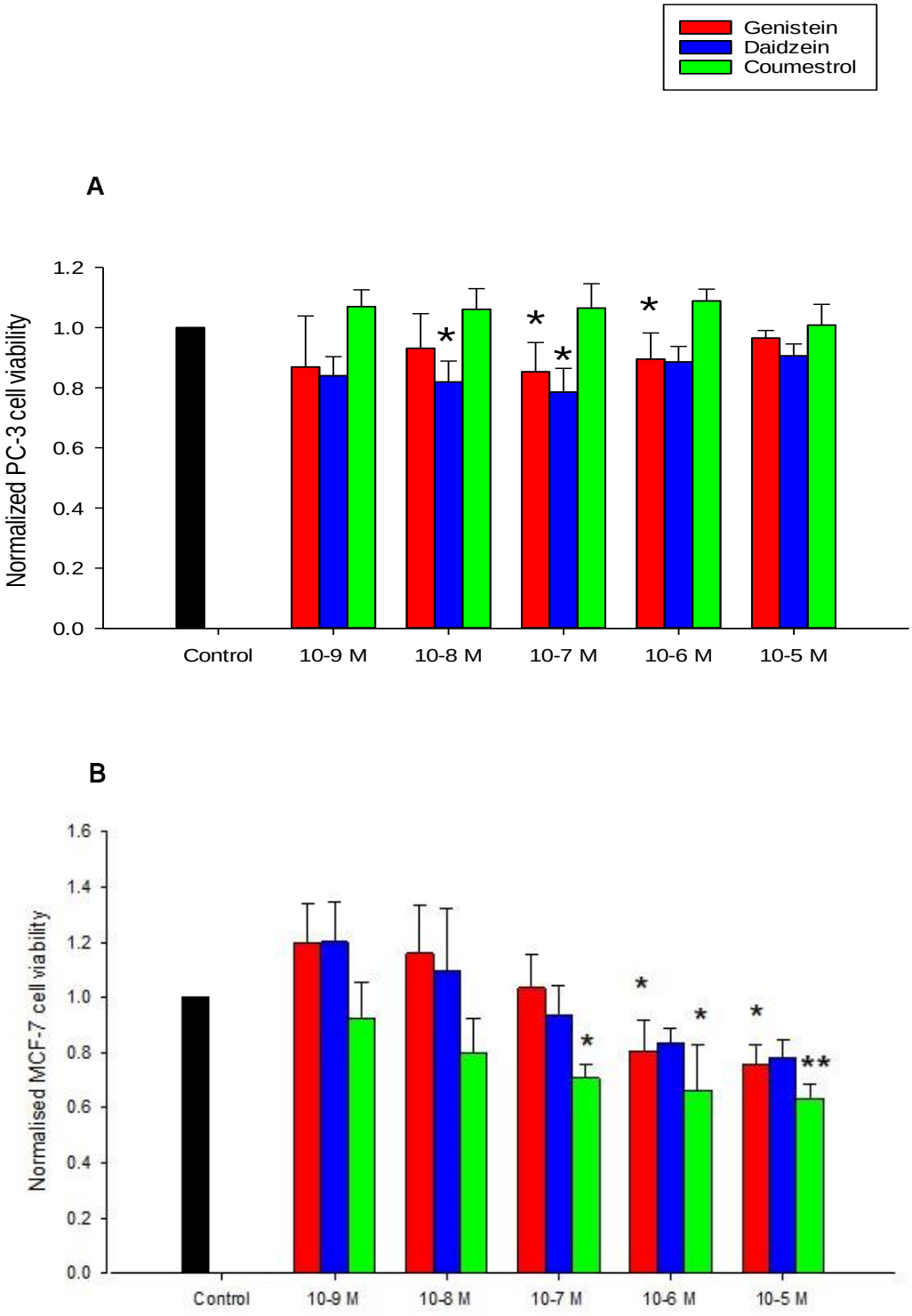


Figure 3.1 Effect of phytoestrogens on cell viability. Concentrations of genistein, daidzein and coumestrol that significantly reduced A. PC3 and B. MCF7 cell viability. Cells were transferred to 96-well plates at a density of 10^5 cells per well and cultured with combinations of genistein, daidzein and coumestrol (10^{-5} to 10^{-9} M) for 72 hours. Viability was then assessed using an AQueous one cell viability assay (Promega UK) according to manufacturer's instructions. Results are the mean ± SEM for three repeat experiments. * $P<0.05$, ** $P<0.01$ versus control.

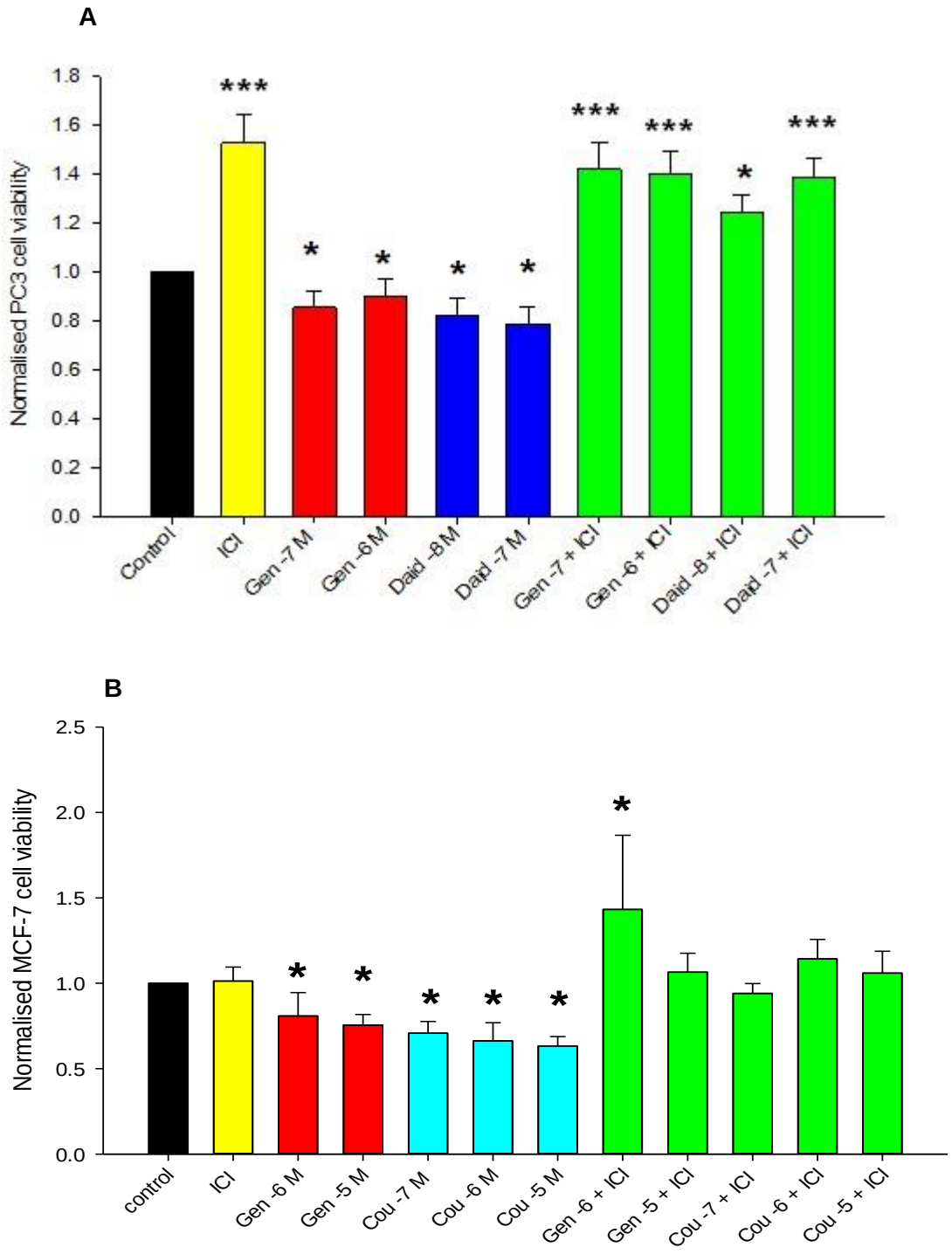


Figure 3.2 The non-selective oestrogen antagonist ICI 182,780. (10^{-5} M) prevents the effect of phytoestrogens on cell viability in A. PC3 and B. MCF7 cells. Cells were transferred to 96-well plates at a density of 10^5 cells per well and cultured with combinations of genistein, daidzein and coumestrol (10^{-5} to 10^{-9} M) and ICI 182,780 for 72 hours. Viability was then assessed using an AQueous one cell viability assay (Promega, UK) according to manufacturer's instructions. Results are the mean ± SEM for three repeat experiments. * P<0.05 and *** P<0.0001versus control

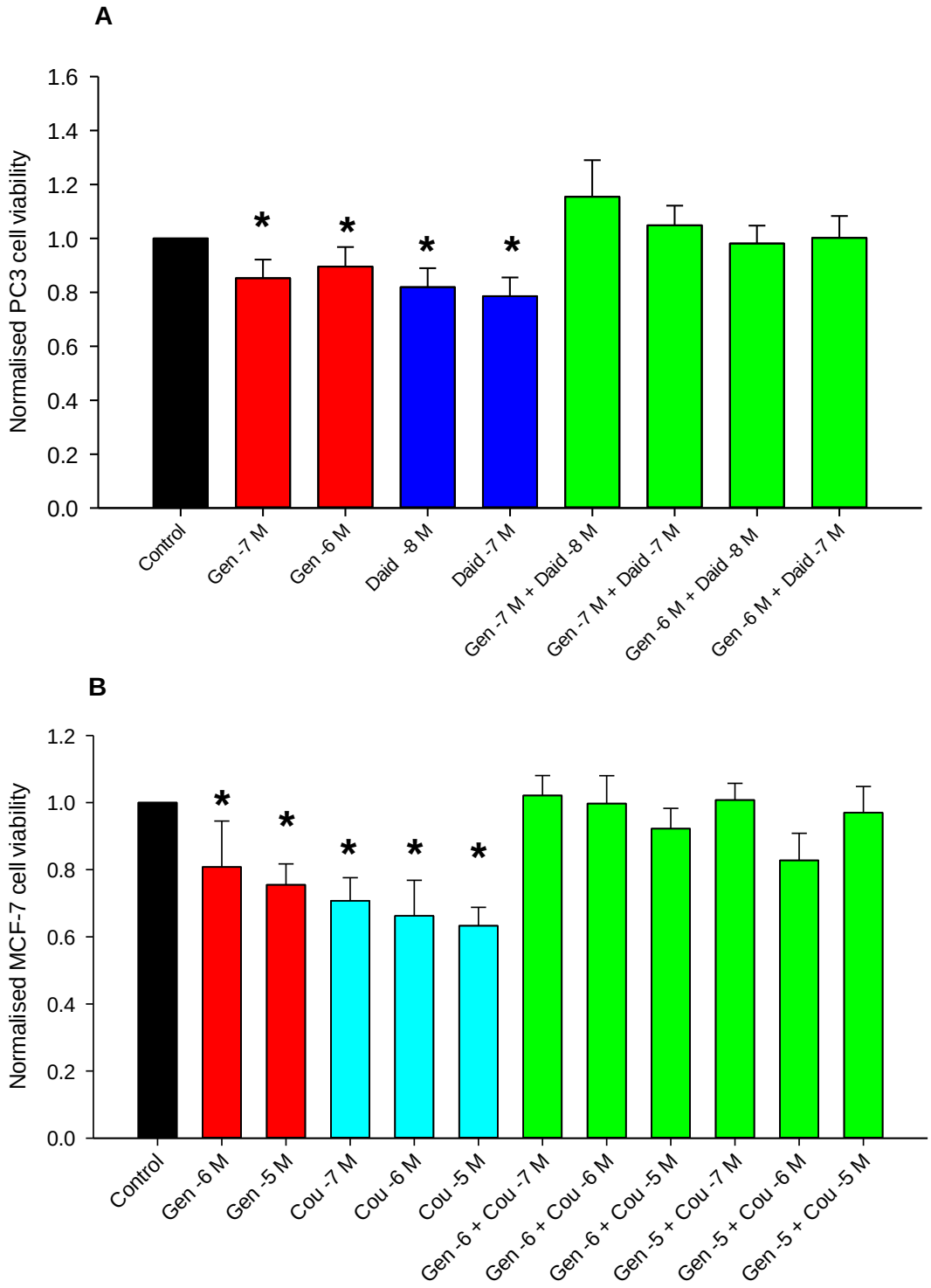
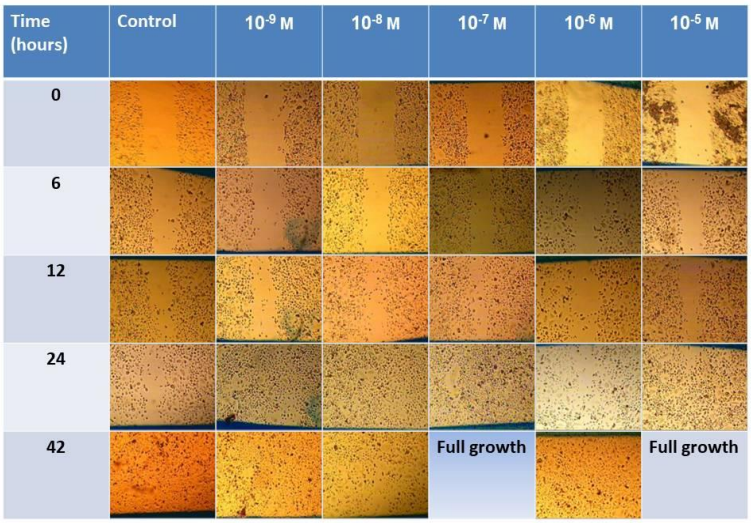


Figure 3.3 The inhibitory effect of phytoestrogens is lost when effective concentrations are combined in A. PC3 and B. MCF7 cells. Cells were transferred to 96-well plates at a density of 10^5 cells per well and cultured with effective combinations of genistein, daidzein and coumestrol for 72 hours. Viability was then assessed using an AQueous one cell viability assay (Promega, UK) according to manufacturer's instructions. Results are the mean ± SEM for three repeat experiments. * $P<0.05$ versus control.

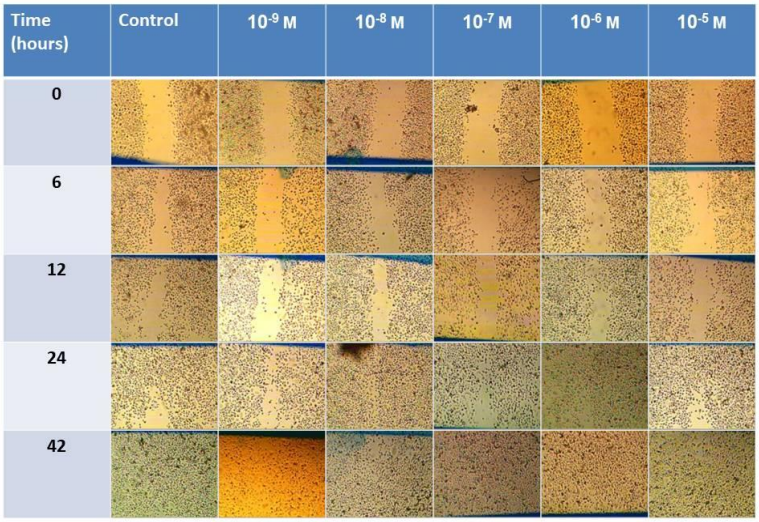
3.3.2 Motility

As expected, breast and prostate cancer cells closed the scratch within 42 hours with little to no gap present between the edges of the scratch at this time point. In cultures of PC3 cells (Fig 3.4) there was little effect of phytoestrogens on the rate of closure with only one statistically significant effect noted with daidzein (10^{-7} M) and was (33%, P = 0.01), which promoted the rate of closure at 12 hours table (3.2). Genistein and daidzein had no significant effect on MCF7 motility table (3.1), whereas coumestrol (Fig 3.5) significantly decreased scratch width at 6 hours for 10^{-8} M (70%, P = 0.02), 10^{-7} M (68%, P = 0.003) and 10^{-6} M (71%, P = 0.04); at 12 hours for 10^{-7} M (51%, P = 0.01) and 10^{-6} M (53%, P = 0.04); and for 24 hours the effect was from concentrations (10^{-9} to 10^{-5} M) and was for 10^{-9} M (33%, P = 0.01), 10^{-8} M (30%, P = 0.002), 10^{-7} M (24%, P = <0.0001)10^{-6} M (28%, P = 0.0005) and for 10^{-5} M was (35%, P = 0.03) (Table 3.1 B).

PC3 coumestrol



PC3 daidzein



PC3 genistein

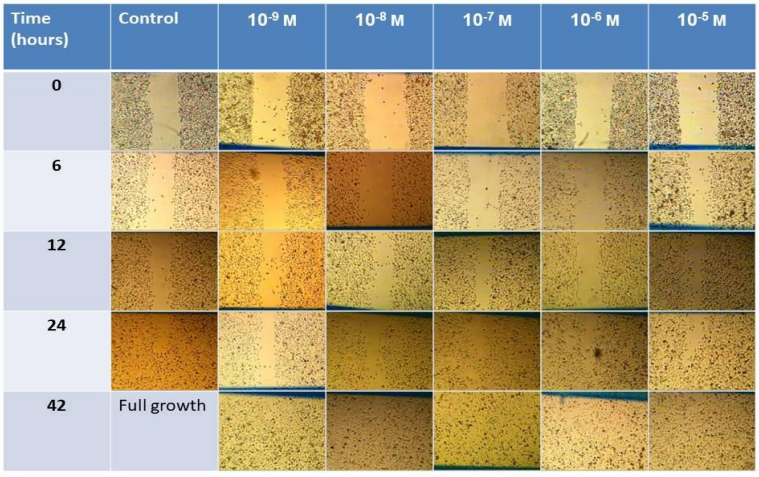
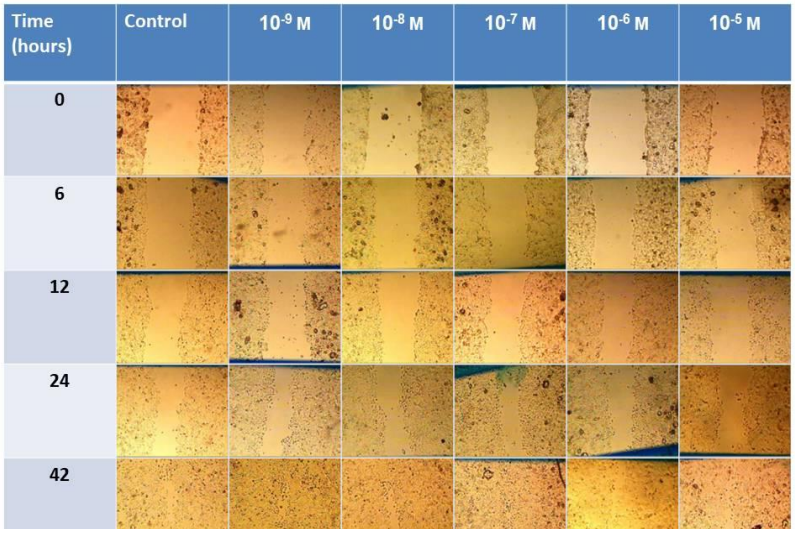
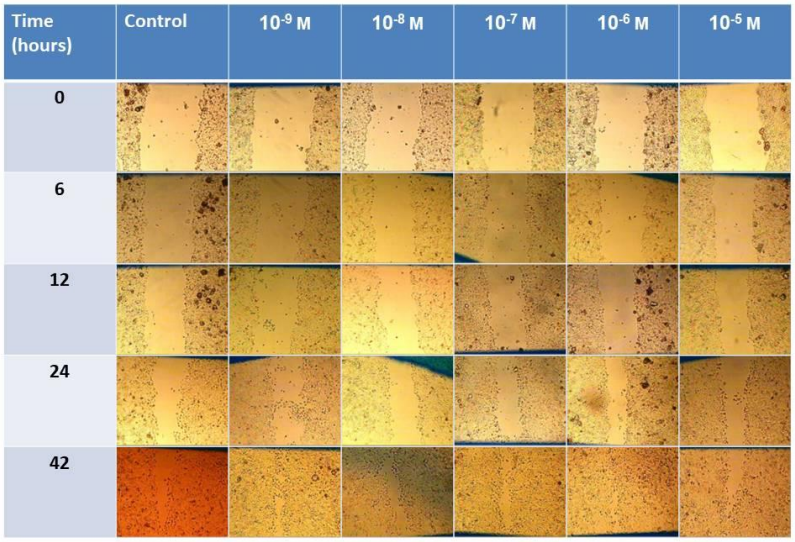


Figure 3.4: showing the growth of the PC3 cells and closure of the scratch at 0 time and after 6, 12, 24 and 42 hours.

MCF7 coumestrol



MCF-7 daidzein



MCF-7 genistein

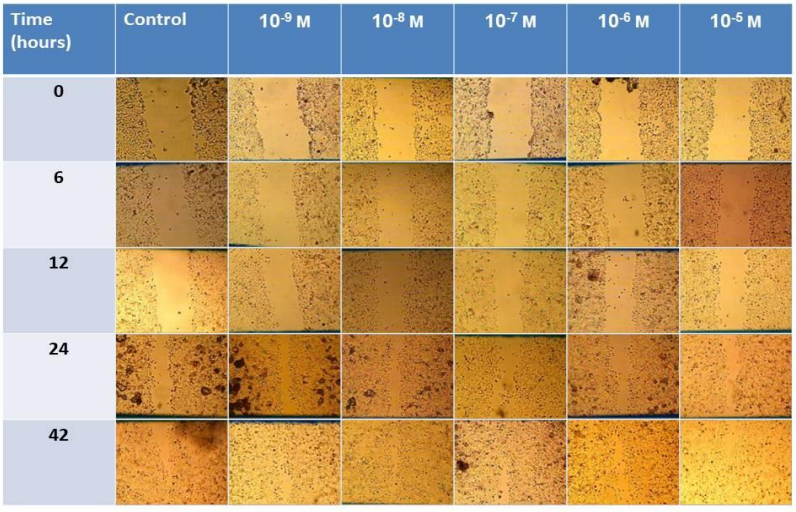


Figure 3.5: showing the growth of the MCF-7 cells and closure of the scratch at 0 time and after 6, 12, 24 and 42 hours.

Table 3.1 Coumestrol significantly increases the rate of wound closure of MCF7 cells in a scratch assay.

A

Concentrations (M)	Scratch width 6 hr (mm)	SEM	Scratch width 12 hr (mm)	SEM	Scratch width 24 hr (mm)	SEM	Scratch width 42 hr (mm)	SEM
Control	0.77	0.02	0.62	0.03	0.41	0.04	0.20	0.03
-9 M	0.80	0.05	0.66	0.06	0.47	0.06	0.28	0.07
-8 M	0.74	0.03	0.60	0.03	0.36	0.03	0.19	0.02
-7 M	0.72	0.02	0.53	0.03	0.34	0.04	0.15	0.03
-6 M	0.72	0.02	0.52	0.02	0.28	0.03	0.11	0.03
-5 M	0.78	0.03	0.62	0.04	0.37	0.04	0.20	0.04

B

Concentrations (M)	Scratch width 6 hr (mm)	SEM	Scratch width 12 hr (mm)	SEM	Scratch width 24 hr (mm)	SEM	Scratch width 42 hr (mm)	SEM
Control	0.76	0.02	0.61	0.03	0.45	0.05	0.20	0.04
-9 M	0.71	0.03	0.60	0.05	0.33	0.05 *	0.17	0.05
-8 M	0.70	0.02 *	0.57	0.02	0.30	0.03 *	0.18	0.02
-7 M	0.68	0.02 *	0.51	0.03*	0.24	0.03 *	0.15	0.02
-6 M	0.71	0.01*	0.53	0.02*	0.28	0.02*	0.16	0.02
-5 M	0.75	0.02	0.59	0.03	0.35	0.03*	0.15	0.03

C

Concentrations (M)	Scratch width 6 hr (mm)	SEM	Scratch width 12 hr (mm)	SEM	Scratch width 24 hr (mm)	SEM	Scratch width 42 hr (mm)	SEM
Control	0.76	0.02	0.64	0.03	0.39	0.04	0.18	0.04
-9 M	0.75	0.02	0.64	0.03	0.42	0.05	0.22	0.04
-8 M	0.78	0.02	0.63	0.03	0.43	0.04	0.18	0.03
-7 M	0.76	0.02	0.60	0.02	0.41	0.04	0.17	0.03
-6 M	0.81	0.02	0.66	0.03	0.45	0.04	0.17	0.03
-5 M	0.75	0.02	0.58	0.03	0.37	0.04	0.14	0.03

MCF7 cells were treated with genistein, daidzein and coumestrol for 72 hours. A scratch was then made in the middle of wells using a sterile pipette tip. Digital images were taken after scratching on an inverted microscope (Leica, Germany) fitted with a digital camera (Scopetek, DCM-510, China) at x40 magnification. The separation of the edges of the scratch was used to quantify motility by measuring the distance between the edges in mm using ImageJ analysis software (National Institute of Health, USA). * P<0.05 versus control. (A) daidzein, (B) coumestrol and (C) genistein.

Table 3.2 shows there was no effect of coumestrol and genistein on wound clouser just for daidzein after 12 hours in PC3 cell culture in the scratch assay.

A

Concentrations (M)	Scratch width 6 hr (mm)	SEM	Scratch width 12 hr (mm)	SEM	Scratch width 24 hr (mm)	SEM	Scratch width 42 hr (mm)	SEM
Control	0.63	0.06	0.52	0.07	0.32	0.08	0.18	0.06
-9 M	0.69	0.05	0.52	0.06	0.33	0.07	0.22	0.07
-8 M	0.60	0.03	0.45	0.05	0.29	0.07	0.10	0.04
-7 M	0.58	0.04	0.33	0.06*	0.15	0.05	0.11	0.07
-6 M	0.69	0.05	0.50	0.06	0.23	0.06	0.13	0.05
-5 M	0.61	0.04	0.40	0.06	0.25	0.06	0.10	0.04

B

Concentrations (M)	Scratch width 6 hr (mm)	SEM	Scratch width 12 hr (mm)	SEM	Scratch width 24 hr (mm)	SEM	Scratch width 42 hr (mm)	SEM
Control	0.54	0.03	0.38	0.04	0.17	0.03	0.06	0.03
-9 M	0.58	0.03	0.40	0.03	0.22	0.04	0.04	0.03
-8 M	0.57	0.03	0.41	0.03	0.17	0.03	0.09	0.05
-7 M	0.53	0.03	0.36	0.03	0.14	0.03	0.05	0.03
-6 M	0.57	0.03	0.33	0.02	0.20	0.04	0.11	0.05
-5 M	0.56	0.03	0.36	0.03	0.19	0.03	0.03	0.02

C

Concentrations (M)	Scratch width 6 hr (mm)	SEM	Scratch width 12 hr (mm)	SEM	Scratch width 24 hr (mm)	SEM	Scratch width 42 hr (mm)	SEM
Control	0.60	0.03	0.47	0.03	0.26	0.04	0.08	0.03
-9 M	0.58	0.03	0.43	0.03	0.23	0.04	0.11	0.03
-8 M	0.62	0.03	0.51	0.04	0.30	0.03	0.10	0.03
-7 M	0.66	0.02	0.51	0.03	0.22	0.04	0.06	0.02
-6 M	0.58	0.04	0.44	0.04	0.26	0.06	0.07	0.02
-5 M	0.57	0.03	0.42	0.04	0.25	0.05	0.11	0.04

PC3 cells were treated with genistein, daidzein and coumestrol for 72 hours. A scratch was then made in the middle of wells using a sterile pipette tip. Digital images were taken after scratching on an inverted microscope (Leica, Germany) fitted with a digital camera (Scopetek, DCM-510, China) at x40 magnification. The separation of the edges of the scratch was used to quantify motility by measuring the distance between the edges in mm using ImageJ analysis software (National Institute of Health, USA). * $P<0.05$ versus control. (A) daidzein, (B) coumestrol and (C) genistein.

3.4 Discussion

There is conflicting data in the literature regarding the effect of phytoestrogens on breast and prostate cancer. Studies indicate variously that diets rich in phytoestrogens or phytoestrogen supplementation can have beneficial or detrimental effects on risk, cell viability and gene expression (Bilal et al., 2014). While many previous studies have examined the effect of individual phytoestrogens, there is little data on the impact of combinations of phytoestrogens, studies in which PE with different variables have been combined (Charles et al., 2007) or studies focussing on genomic effects (Dip et al., 2009). These areas are worthy of further investigation, as it is highly likely that individuals are exposed to many PE at different concentrations, due to the varied nature of PE obtained from dietary sources. Even with supplementation with distinct PE, individuals will still be receiving a range of PE, which could modify the action of the supplement. This study indicates that while genistein, daidzein and coumestrol reduce breast and prostate cell viability individually in an oestrogen receptor dependent manner, this beneficial action is lost when previously effective concentrations are combined. Antagonism between chemotherapeutic agents is not a novel phenomenon: genistein has been shown to modulate the anti-proliferative effect of cisplatin on breast and colonic tumour cells. Genistein at 10^{-4} M inhibits cell growth and induces apoptosis in breast cancer MCF7 cells and colon cancer cells HT29. In contrast, co-treatment of genistein with the same concentration of 10^{-4} M with cisplatin results in an additive effect and abolishes the anti-proliferative effect of cisplatin on breast and colon cancer cells. (Hu et al., 2014). It has also been reported to induce resistance to doxorubicin and mitoxantrone by increasing ABC transporter expression, which increases the expulsion of the tumour suppression drugs from the cells (Rigalli et al., 2016). The basis of the

antagonistic action observed in the current study is not known, but in the light of the concentration-dependent effect on viability may relate to combinations exceeding effective concentration windows. For example, no PE reduced viability at 10^{-5} M, but a significant effect was noted with 10^{-6} M genistein. Exposing cells to additional PE in the presence of 10^{-6} M genistein would, therefore, be expected to reduce viability as overall concentrations rise. The work of Choi supports this, and the biphasic concentration-dependent effects of genistein and daidzein noted in these studies were attributed to differential actions, mediated through ER (Choi & Kim, 2013). In the current study, the inhibitory effect of PEs was also ER-dependent, as it was prevented by a non-selective oestrogen antagonist. It is not clear, however, whether this action was mediated through ERα or through β, which have opposing actions on breast and prostate cell viability (Omoto & Iwase, 2015). While ERα is typically associated with greater proliferation and increased tumour burden in breast and prostate cancer, overexpression of ER β *in vitro* reduces oestrogen-driven proliferation and tumour formation (Paruthiyil et al., 2004). Similarly, in clinical studies, ERβ expression has been shown to correlate with increased proliferation and higher grade invasive tumours in breast cancer (Huang et al., 2014). While evidence supports a beneficial effect of ERβ and a detrimental effect of ERα, there is added complication due to splice variants with opposing actions on tumour cell activity (Dey et al., 2012). ER β1 is often lost in advanced cases and has a tumour suppressive action, whereas ER β2 is elevated in more advanced cases and promotes proliferation (Dey et al., 2012). Thus, there is a crosstalk between ER influences transcription of ER responsive genes through the receptors own intrinsic activity and competition for binding to oestrogen-responsive DNA elements. Thus, oestrogen receptor dependent ligands, such as phytoestrogens,

induce a unique cellular response that is determined by their specific affinity for ER variants, and modified by the presence of other ER binding ligands. Phytoestrogens have differential binding affinities for ER α and β. Studies have indicated that genistein and daidzein have a significantly higher affinity for ER beta than coumestrol, which has similar affinities for ER α and β (Harris et al., 2005; Kostelac, Rechkemmer & Briviba, 2003). However, data on phytoestrogen affinities for splice variants is lacking in breast and prostate cancer is lacking, and it is possible that they have significantly different EC50. Thus, the antagonistic response seen in the current study could arise due to crosstalk between ER signalling and differences between proliferative and apoptotic actions.

Phytoestrogens have additional ER-independent effects that modify mitosis and cell death. These include changes in redox status and inhibition of tyrosine kinase activity (Lee et al., 2012; Ullah et al., 2016; Zafar, Singh & Naseem, 2016). Therefore, an alternative explanation for the antagonistic effect is that it is due to crosstalk between ER dependent and independent actions. Whatever the explanation, it is clear from the present data that individual PEs are more effective than combinations in reducing cancer cell viability. This antagonism may, in part, explain the lack of consensus within the literature, as absolute control of dietary PE sources is unfeasible. This has important implications for their clinical utilisation. The overall dietary profile and an individual's ER status should be considered as part of any supplementation strategy to maximise a response.

One of the limitations of the wound healing assay, however, was the non-use of more sophisticated techniques, such as like biomimetic hydrogels, microchannel devices, grooved substrates, microcontact-printed and micropatterned lines, vertical confinement devices and micropost arrays. .

This study shows that PEs have a varied effect on breast and prostate cancer cells *in vitro,* with genistein showing the greatest potential. In contrast to daidzein and coumestrol, it reduced viability in both breast and prostate cancer cells, and had a broader effect on gene expression. However, not all the effects of coumestrol were beneficial, it increased breast cancer mobility, which could enhance the metastatic potential of primary breast cancer cells.

The results of this study suggest that some of the conflicting data in the literature may have arisen due to differences in the concentration and types of dietary PE present and stages of tumour development. If PEs are to be considered as having potential for reducing the incidence or severity of breast and prostate cancer, then further analysis of each individual's phytoestrogen intake and ER status should be considered.

3.5 summary of chapter 3 results

Table 3.5.1 Summary of PEs effect on PC3 in individual treatment

Concentration (M)	Genistein		Coumestrol		Daidzein	
	% of control	P Value	% of control	P Value	% of control	P Value
10^{-9}	87 ↓	0.17	107 ↑	0.43	84 ↓	0.06
10^{-8}	93 ↓	0.47	106 ↑	0.48	82 * ↓	0.04
10^{-7}	85 * ↓	0.01	107 ↑	0.46	78 * ↓	0.01
10^{-6}	89 * ↓	0.04	109 ↑	0.31	89 ↓	0.19
10^{-5}	97	0.70	101 ↔	0.92	91 ↓	0.27

Note: " ↑ " , " ↓ " and " ↔ " means upregulation, down-regulation and little or no modulation of the indicated target respectively. * P<0.05 versus control.

Table 3.5.2 Summary of PEs effect on MCF7 in individual treatment

Concentration (M)	Genistein		Coumestrol		Daidzein	
	% of control	P Value	% of control	P Value	% of control	P Value
10^{-9}	120 ↑	0.42	92 ↔	0.56	120 ↑	0.22
10^{-8}	116 ↑	0.51	89 ↓	0.13	110 ↑	0.54
10^{-7}	103 ↑	0.88	29 * ↓	0.03	94 ↓	0.70
10^{-6}	85 * ↓	0.04	66 * ↓	0.01	84 ↓	0.32
10^{-5}	75 * ↓	0.04	63 ** ↓	0.007	78 ↓	0.19

Note: " ↑ ", " ↓ "and " ↔ " means upregulation, down-regulation and little or no modulation of the indicated target respectively. * P<0.05, ** P<0.01 versus control

Table 3.5.3 Summary of PEs effect on PC3 with non-selective oestrogen modulator antagonist ICI 182,780

Concentration (M)	% of control	P Value
Gen 10^{-6} + ICI 10^{-5}	140 *** ↑	0.001
Gen 10^{-7} + ICI 10^{-5}	142 *** ↑	0.0009
Daid 10^{-7}+ ICI 10^{-5}	139 *** ↑	0.001
Daid 10^{-8}+ ICI 10^{-5}	124 ↑	0.05
ICI 10^{-5}	153 *** ↑	0.001

Note: "↑" means upregulation of the indicated target. *** P<0.001 versus control.

Table 3.5.4 Summary of PEs effect on MCF7 with non-selective oestrogen modulator antagonist ICI 182,780

Concentration (M)	% of control	P Value
Gen 10^{-5} + ICI 10^{-5}	106 ↑	0.23
Gen 10^{-6} + ICI 10^{-5}	143 ↑	0.64
Cou 10^{-5} + ICI 10^{-5}	106 ↑	0.74
Cou 10^{-6} + ICI 10^{-5}	114 ↑	0.79
Cou 10^{-7} + ICI 10^{-5}	94 ↓	0.66
ICI 10^{-5}	101 ↔	0.98

Note: "↑", "↓"and "↔" means upregulation, down-regulation and little or no modulation of the indicated target respectively.

Table 3.5.5 Summary of PEs effect on PC3 in combination of the effective individual treatment

Concentration (M)	% of control	P Value
Gen 10^{-6} + Daid 10^{-7}	100 ↔	0.99
Gen 10^{-6} + Daid 10^{-8}	98 ↓	0.88
Gen 10^{-7} + Daid 10^{-7}	105 ↑	0.69
Gen 10^{-7} + Daid 10^{-8}	115 ↑	0.20

Note: "↑", " ↓ "and " ↔ " means upregulation, down-regulation and little or no modulation of the indicated target respectively.

Table 3.5.6 Summary of PEs effect on MCF7 in combination of the effective individual treatment

Concentration (M)	% of control	P Value
Gen 10^{-5} + Cou 10^{-5}	97 ↓	0.79
Gen 10^{-5} + Cou 10^{-6}	83 ↓	0.12
Gen 10^{-5} + Cou 10^{-7}	101 ↔	0.93
Gen 10^{-6} + Cou 10^{-5}	92 ↓	0.49
Gen 10^{-6} + Cou 10^{-6}	100 ↔	0.97
Gen 10^{-6} + Cou 10^{-7}	102 ↑	0.85

Note: "↑", " ↓ "and " ↔ " means upregulation, down-regulation and little or no modulation of the indicated target respectively.

Chapter Four

Transforming growth factor-β (TGF-β) and bone morphogenic protein 7 (BMP7) interfere with the effect of phytoestrogens in breast and prostate cancer cell lines

4.1 Introduction

After studying the effect of individual phytoestrogens on the viability of prostate and breast cancer cells, this chapter will study their effect on gene expression and in the presence or absence of cytokines typical of that seen in the bone microenvironment. The transforming growth factor-β (TGF-β) family of cytokines has 33 members in humans, including TGF-β isoforms, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs). These control cell growth, survival, migration and differentiation, and have significant roles during embryonic development and adult tissue homeostasis (Heldin, Vanlandewijck & Moustakas, 2012). TGF-β family members are involved in fibrotic conditions, cancer and autoimmune disease As a regulatory 'switch', it can, in combination with other cytokines, can 'reprogramme' effector T cell differentiation along different pathways (Veldhoen et al., 2008); enhance the proliferation of mouse $CD8^{+}$; increase TNF-a; and accelerate T-cell death (Wan & Flavell, 2008). In carcinogenesis, TGF-β has a dual role; originally it represses tumorigenesis by inducing growth arrest and promoting apoptosis. In advanced cancers, however, where TGF-β is often overexpressed, it promotes tumorigenesis by induction of epithelial-mesenchymal transition (EMT), whereby tumour cells become more invasive and prone to form metastases. The tumour-promoting effects of TGF-β also introduce effects on non-malignant cells; promoting angiogenesis (Heldin, Vanlandewijck & Moustakas, 2012) and inhibiting the production of IL-2, a chemokine known to potently activate T cells, and this suppresses immune monitoring cells, such as NK, CD4+ and CD8+ (Kehrl et al., 1986; Ribatti, 2017).

Growth factors, including TGF-β, liberated during the bone destruction associated with breast and prostate cancer, acts on cancer cells to induce a positive feedback response, in which matrix factors induce the production of additional osteolytic factors such as PTHrP, IL-6 and IL-11, which in turn stimulate more osteoclastic resorption and increase TGF-β release. Therefore, TGF-β plays a central role in this vicious cycle of bone metastasis and, consequently, the TGF-β signalling pathway in tumour cells and its cross talk within the bone microenvironment present an attractive therapeutic target (Dorai et al., 2014).

Since metastasis is the primary cause of cancer lethality, a wider understanding of the mechanisms that promote metastasis is required. TGF-β signaling has been linked to the process of metastasis in many cancer types, including those of the breast, prostate and lung. Moreover, TGF-β signaling is triggered in PCa bone metastatic patients because TGF-β is one of the most abundant growth factors in bone and is released during osteoclastic bone resorption. In breast cancer bone metastases, TGF-β enhance the expression of genes that shown to be associated with bone metastases, including PTHrP, IL-11, CTGF, CXCR4, and MMP1(Nguyen, Bos & Massagué, 2009). Evidence supports the concept that EMT encourages invasiveness and intravasation into lymph or blood vessels of cancer cells as one of the early manifestations of metastasis (Thiery et al., 2009). In addition to the direct effects of EMT on cancer cells, invasive tumour cells that started EMT secrete many growth factors and chemokines that promote and recruit stromal cells, facilitating migration and intravasation of the tumour cell. Regarding TGF-β, it is important to know whether TGF-β continues to play an active role during additional steps in the pathway that guides tumour cells to form metastases (Heldin, Vanlandewijck & Moustakas, 2012). Further

investigations are required in order to augment our knowledge of the dynamics and interactions that exist between EMT and MET programmes in regulating cell reprogramming, and to determine the therapeutic potential of targeting these differences (Morrison, Parvani & Schiemann, 2013).

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily, and are known to be strong inducers of bone formation. More than 30 BMP-related proteins have been recognized. They are synthesized by skeletal cells, and have an important role in early embryogenesis, skeletogenesis and in the maintenance of bone mass in the mature skeleton. They play a role in the differentiation of marrow stromal cells toward osteoblasts, chondrocytes and adipocytes, and also in a variety of extraskeletal tissues, particularly in vascular and neural development and diseases (Biver, Hardouin & Caverzasio, 2013).

BMP receptors activate intracellular signalling pathways. Smad is the major route, in which phosphorylated Smad 1/5/8 form heterocomplexes with Smad 4, which then translocate to the nucleus. Nuclear Smad complexes control transcription of BMP target genes by binding to sequence motifs in the promoter regions of BMP-responsive genes, and through interaction with transcription factors or transcriptional co-activators and/or repressors (Attisano & Wrana, 2002; Miyazono, 1999). In breast cancer cells, BMP7 induces different phenotypic changes. The capacity of BMP7 to dramatically stimulate breast cancer cells is displayed in MDA-MB-231 cells, in which a remarkable increase in cell growth, migration, and invasion was detected. Thus, it is concluded that BMP7 has a substantial impact on breast cancer cells and is an important factor determining breast cancer responses in bone (Alarmo et al., 2009).

BMP7 indirectly control the expression of important genes involved in promoting the EMT process by ZEB1, ZEB2, Snail1, Snail2, N-cad and Vimentin, by

abolishing TGFβ-induced activation of theses genes. Also, BMP7 significantly abolished TGFβ-induced suppression of E-cadherin, suggesting a possible antagonising effect of BMP7 against TGFβ-mediated EMT in breast cancer cells (Ying, Sun & He, 2015). Buijs and co-workers showed that, in prostate cancer cells, bone morphogenic protein-7, (BMP7), is an antagonist of the TGF-β and can inhibit osteolytic metastases that are characteristic of prostate cancer *in vivo*. Buijs showed that while TGF-β alone decreases E-cadherin expression and generates epithelial mesenchymal transition, (EMT), in prostate cancer cells which facilitate the invasive and metastatic phenotype, a combination of TGF-β and BMP7 promote E-cadherin expression and enhance the epithelial phenotype and repress the development of prostate cancer bone metastases (Buijs *et al.*, 2007a; Buijs et al., 2007b; Buijs et al., 2007c). In prostate cancer cell line LNCaP, BMP7 promotes an epithelial phenotype by increasing E-cadherin expression, decreasing proliferation, and vimentin expression which is a marker of mesenchymal cells (Morrissey *et al.*, 2010).

BMP pathways have been correlated with mechanisms determining the osteoclastic and osteoblastic response to cancer metastases. BMPs and their receptors are expressed by human cancer cell lines and human biopsy specimens from bone metastatic sites (especially BMP-2, 4, 6 and 7), with clear effects on proliferation, migration and invasion of tumour cells, and expression pattern, according to the primary cancer (Biver, Hardouin & Caverzasio, 2013). BMP -2, -4, and -7 are generally expressed in breast and prostate cancers, and mostly BMP7 in breast cancer. Other cell line specific differential expression of BMPs is observed in gastric and colon adenocarcinoma. Expression of Bone morphogenic protein receptors (BMPRs) (type I and type II) has been noticed in various prostate cancer cell lines and tissues. Other BMPRs such as BMP type

1A, 1B and II are also expressed in lung cancer and osteosarcoma (Singh & Morris, 2010)

Lack of BMP7 expression by the cancer cells, or resistance of these cells to the BMP7 produced by the surrounding normal epithelial or stromal cells drives the malignant cells to operate with a TGF-β bias, promoting loss of E-cadherin expression and metastasis (Dorai *et al.*, 2014). BMP7 inhibits bone metastasis formation *in vivo* and decreased BMP7 expression has been shown in primary breast cancer. Daily BMP7 treatment to nude mice inhibited the growth of prostate cancer cells in bone, suggesting that BMP7 and TGF-beta have important impacts on the outcome of prostatic metastases in bone (Biver, Hardouin & Caverzasio, 2013).

Most of the literature and a majority of researchers have not focussed particularly on the relation of phytoestrogens and BMP-7 in prostate and breast cancer in their studies, although there may be ongoing investigations in this area that have yet to be announced. For this reason, it was a challenge to decide working on this relation here in this chapter that focused on BMP-7 in prostate and breast cancer cell lines in the presence of phytoestrogens. Unfortunately, there are no direct studies that combine the PEs/TGF/BMP-7 model and compare the responses of the prostate cell lines and the breast cancer cell lines.

As shown with individual PEs it appears to be more effective than combinations on the viability of prostate and breast cancer cells. In this chapter, there will be a detailed observation of their impact on genes involved in the disease process, and attempts will be made to replicate more closely the cytokines (TGF-β and BMP7) that these cells would experience in-vivo, in order to see whether this modulates the response to individual PEs.

Therefore, the effect of PEs on breast and prostate cancer expression of genes involved in preferential metastasis to the skeleton will be investigated. Also in this chapter, there will be an attempt to model the status that the cells would experience in the bone microenvironment and to discover whether the PEs would still have an effect similar to that of their individual effect in the presence of TGF- β and BMP7 cytokines. Further, the chapter will study the possible effect on genes that are important in the EMT process (Integrin α5β3) and osteomimicry (Runx2, Osx and collagen type I) in PC3 cells as runx2 has been proposed to mediate gene expression and associated with increased motility and invasiveness of PCa cells, and the aggressiveness of osteolytic bone disease that occur with PCa metastasis to bone (Akech et al., 2010). It will also study these cytokines as a model of what prostate and breast cancer cells may encounter in the presence and absence of PEs and the expression of genes involved in EMT (Snail and Integrin α5β3) and bone vicious cycle (PTHrP and collagen type I) in MCF7 cells as PTHrP expression was investigated as previous studies have proposed that production of PTHrP is more common in metastatic breast cancer cells in bone than in the primary tumour and may be responsible for the local bone destruction taking place in patients with breast cancer (Guise et al., 1996). Also collagen type I is regarded as simply a physical barrier against cancer invasion and tumour cells migration and the essential for tumour invasion is collagen degradation, for which matrix metalloproteinases (MMPs) play an important role (Fang et al., 2013).

4.2 Materials and methods

Methods used here in chapter 4 are similar to what has been mentioned in chapter 2 (materials and methods) for the points:

2.5.1 RNA extraction and reverse transcription

2.5.2 Verification of PCR primers and RT

2.5.3 10x Tris-acetate-EDTA (TAE) buffer

2.5.4 Real-time quantitative PCR analysis of metastatic marker expression

2.6 Statistical analysis

4.2.1 cytokines

Recombinant Human TGF-β1 (Human Cells) and Purified recombinant protein of Human bone morphogenetic protein 7 (BMP7) was purchased from Insight Biotechnology Limited (Wembley Middlesex, United Kingdom). Concentrations used for the cytokines TGFβ and BMP7 were 0.1 ng/mL(Vo et al., 2013) and 30 ng/mL (Chen et al., 2014) respectively.

	5′-3′ Forward primer	3′-5′ Reverse primer	
Human β-actin	GCGCGGCTACAGCTTCACCA	TGGCCGTCAGGCAGCTCGTA	777-929
Human Runx2	AGACCCCAGGCAGGCACAGT	GCGCCTAGGCACATCGGTGA	816-972
Human CXCR4	GCGCAAGGCCCTCAAGA	GTGCGTGCTGGGCAGAGGTT	1010-1257
Human Snail	CGAGTGGTTCTTCTGCGCTA	CTGCTGGAAGGTAAACTCTGGA	27-183
Human Integrin α5β3	AATTTTACTGGCGAGCAG	TTGGTGGCATGCTTCGAG	1054- 1465
Human PTHrP	GTCTCAGCCGCCGCCTCAA	GGAAGAATCGTCGCCGTAAA	693-785
Human collagen type I	CCTGGCAGCCCTGGTCCTGA	CTTGCCGGGCTCTCCAGCAG	1766-1918
Human (osx)	GGCTCTAGCCCTCTGCGGGA	CGTGGGGGTTTGGCTCCACC	459-1123

Table 4.1 PCR primers and their amplicons used with individual phytoestrogens, TGF-β and BMP7 experiments

4.3 Results

4.3.1 Expression of genes involved in metastasis

The effect of phytoestrogens on expression of genes associated with epithelial to mesenchymal transition (snail), preferential metastasis (CXCR4 and integrin α5β3) and disruption of bone remodelling (Runx2 as mechanistically linked to androgen responsive pathways that support prostate cancer cell growth and PTHrP as breast cancer cells in bone express parathyroid hormone-related protein (PTHrP) more frequently) was determined by quantitative PCR. Genistein (10^{-7} - 10^{-6} M) significantly reduced PC3 snail expression at 24 hours was (30%, P = 0.02) and (36%, P = 0.02) at 72 hours for genistein 10^{-6} M and for genistein 10^{-7} M the reduction in expression for snail was (25%, P = 0.02) at 24 hours and (2%, P = 0.002) at 72 hours (Figure 4.1 A), CXCR4 and integrin α5β3 expression were also significantly reduced at 24 (1%, P = 0.005) and (11%, P = 0.0002) at 24 hours and (3%, P = <0.0001) and (13%, P = 0.002) at 72 hours for genistein 10^{-6} M, respectively, and for 10^{-7} M the reduction in expression was significant only at 24 hours (2%, P = <0.005) for CXCR4 and (20%, P = 0.0005) for Integrin α5 (Figure 4.1 B and D). Genistein had a bimodal effect on PC3 Runx2 expression reducing expression at the earliest time point (11%, P = <0.0001) for 10^{-6} M and (20%, P = 0.0001) for 10^{-7} M and significantly increasing expression at 72 hours (10^{-7} M) (160%, P = <0.47) for 10^{-6} M and (282%, P = 0.4) for 10^{-7} M probably because of the oestrogen receptor availability during the 24 hour and become less available after 72 hour (Figure 4.1 C). Daidzein also reduced gene expression in a dose and time dependent manner in PC3 cells, significantly decreased snail, CXCR4, integrin α5β3 and Runx2 expression. The effect of daidzein (10^{-7} - 10^{-8} M) was more pronounced at later time points and was (27%, P = <0.01) for snail and (4%, P = 0.0001) for CXCR4 and (7%, P

= <0.04) for Integrin α5 for 10^{-7} M (Figure 4.1 A, B and D) and for 10^{-8} M the reduction was (18%, P = <0.006) for snail and (3%, P = 0.0001) for CXCR4 and (9%, P = <0.01) for Integrin α5β3. Apart from Runx2 expression where a significant decrease was only noted at 24 hours for daidzein (10^{-7} - 10^{-8} M) and was (14%, P = <0.0001) and (18%, P = <0.0001) respectively (Figure 4.1 C).

In MCF7 cells, genistein significantly suppressed CXCR4 and integrin α5β3 expression at all concentrations and time points and was (42%, P = 0.0004) and (50%, P = 0.01) at 24 hours for (10^{-6} - 10^{-5} M) respectively for CXCR4 except for genistein 10^{-5}M after 72 hour and was (61%, P = 0.08) (Figure 4.3 B), for Integrin α5β3 the reduction in expression was for both genistein (10^{-6} - 10^{-5} M) at 24 and 72 hours and was (12%, P = 0.0001) and (0%, P = <0.0001), respectively, and at 72 hours was (12%, P = <0.0001) and (9%, P = <0.0001) for (10^{-6} and 10^{-5} M), respectively. Coumestrol also significantly reduced integrin α5β3 at 24 and 72 hour for Coumestrol (10^{-7} , 10^{-6} and 10^{-5}M) and was (0%, P = <0.0001) , (6%, P = <0.0001) and (19%, P = <0.0001) for 24 hours ,respectively, and the reduction at 72 hours was (14%, P = <0.0001) , (16%, P = <0.0001) and (7%, P = <0.0001) respectively(Figure 4.2C). For the reduction in CXCR4 expression was at 24 hours only in Coumestrol (10^{-7} , 10^{-6} and 10^{-5}M) and was (53%, P = <0.001) , (23%, P = <0.0001) and (9%, P = <0.0001) respectively (Figure 4.2 B). Genistein had a differential effect on MCF7 snail and PTHrP expression that significantly increased at 10^{-6} and 10^{-5} M after 24 hours (329%, P = <0.0001) and (495%, P = <0.0001), respectively, for snail and for PTHrP the increase was not significant and was (126%, P = 0.84) and (179%, P = 0.3) for 10^{-6} and 10^{-5} M respectively, then a suppressive expression at 72 hours was significant in snail for 10^{-6} and 10^{-5} M and was (20%, P = <0.0001) and (34%, P = 0.0003), respectively, but was not significant decrease in PTHrP and was

(67%, P = 0.74) and (11%, P = 0.29) for 10^{-6} and 10^{-5} M respectively (Figure 4.2 A and D). Coumestrol decreased PTHrP and snail genes expression after 24 and 72 hours for 10^{-6} M and 10^{-5} M but not to a significant levels and was higher at 10^{-7} M for both snail and PTHrP at 24 and 72 hours (305%, P = 0.0001), (126%, P = 0.08) and (180%, P = 0.27) (127%, P = 0.04) respectively(Figure 4.2 A and D).

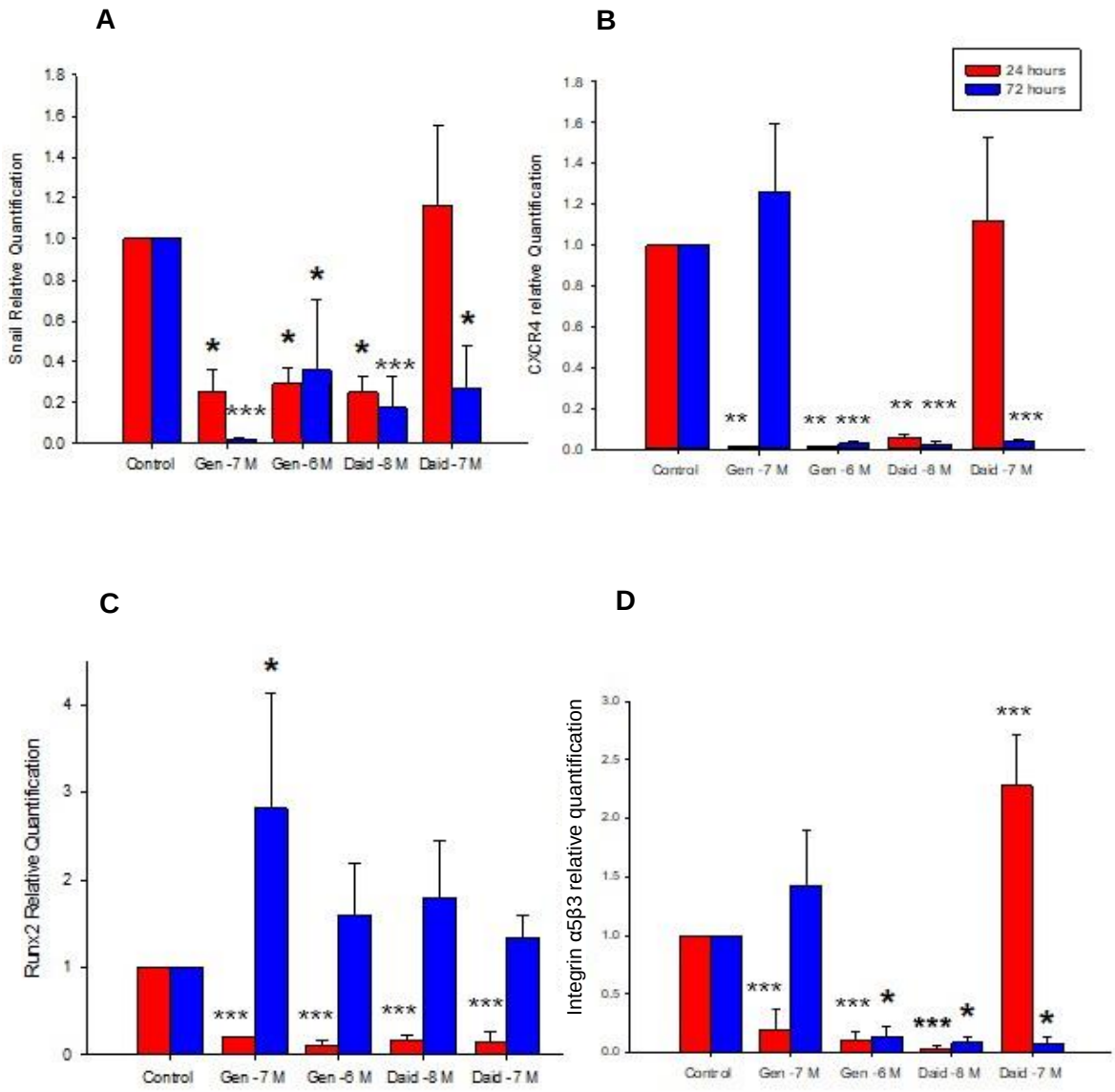


Figure 4.1 PC3 cells genes expression with individual phytoestrogens. Concentrations of genistein and daidzein shown to reduce cell viability also modify the expression of genes implicated in EMT (snail), preferential metastasis (CXCR4 and integrin α5β3) and osteomimicry (Runx2) in PC3 cells. Cells were cultured with genistein or daidzein for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔcT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * $P<0.05$ and *** $P<0.0001$

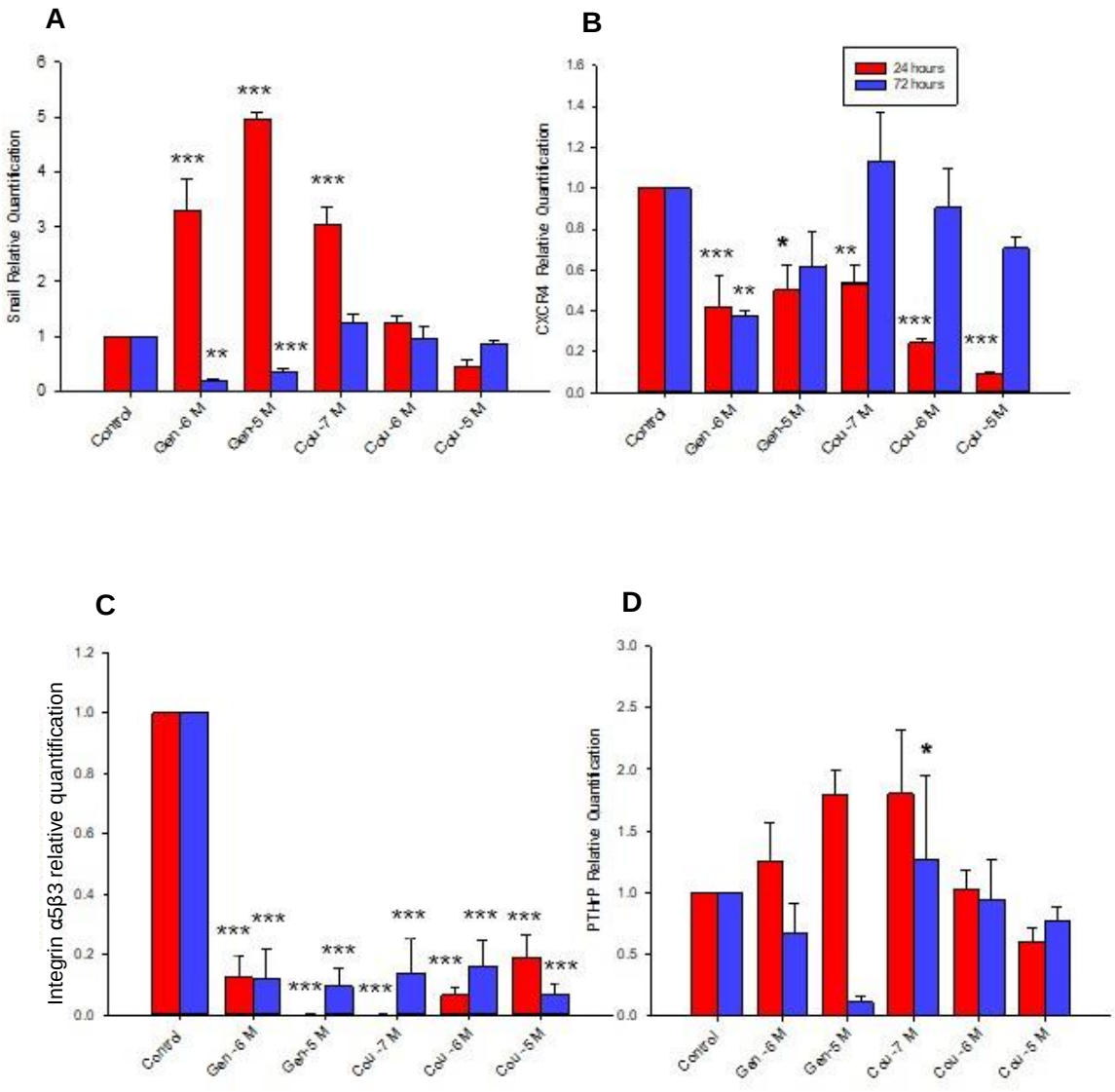


Figure 4.2 MCF7 genes expression with individual phytoestrogens. Concentrations of genistein and coumestrol shown to reduce cell viability also modify the expression of genes implicated in EMT (snail), preferential metastasis (CXCR4 and integrin α5β3) and osteolysis (PTHrP) in MCF7 cells. Cells were cultured with genistein or daidzein for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔcT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * $P<0.05$, ** $P<0.01$ and *** $P<0.0001$versus control

4.3.2 Modulation of phytoestrogens effect by TGF-β on genes involved in the metastatic process in prostate and breast cancer cells.

This experiment will look at the role of TGF on phytoestrogens that induced changes to genes expression in a microenvironment that mimic what phytoestrogen will encounter in this microenvironment in PC3 and MCF7 cells. Genes that been studied in PC3 are (Runx2, osterix and collagen type I) as the most abundant protein within the bone and that prostate and breast cancer cells bound with high affinity to this protein) and looking at (PTHrP, snail and collagen type I) expression in breast cancer cells (MCF7).

TGF-β at 0.1 ng/mL for 72 hours significantly reduced PC3 Runx2 (74%, P = 0.008) and osx (66%, P = 0.03) expression and increased collagen type I expression significantly (159% at P 0.02). Daidzein 10^{-7} M/TGF-β decrease Runx2 (60%, P = 0.008) and osx decreased (47%, P = <0.0001), while in genistein 10^{-6} M/TGF-β Runx2 decreased (58%, P = 0.003) and osx (77% at P= 0.01) after 72 hours This results of significant Runx2 reduction was opposite to the individual effect of these phytoestrogens in the absence of TGF-β on Runx2 gene expression after 72 hours. TGF-β induced collagen type I expression reduced by PE, returning near the control levels. Also, collagen type I expression was less in combinations comparing to its high expression with the individual treatment of TGF- β (Figure 4.3).

In MCF7 cells, treatment with TGF- β significantly increased Snail expression (148%, P = 0.01). Collagen type I expression also increased but not to significant levels (139%, P = 0.16) while PTHrP stay lower but not to significant level (81%, P = 0.13). When TGF-β at 0.1 ng/mL combined with and coumestrol (10^{-5} M) for 72 hours significantly reduced the expression of the PTHrP (25%, P = 0.03), collagen type I (12%, P = <0.0001) and snail (33%, P = 0.008) which is

similar to the significant effect of individual genistein on both PTHrP and snail gene expression in the absence of TGF-β. Genistein (10^{-5} M) become more effective in the presence of TGF-β than when used individually and reduced PTHrP (22%, P = 0.001), collagen type I (22%, P = <0.0001) and snail (44%,P = 0.003) significantly as shown in (Figure 4.4).

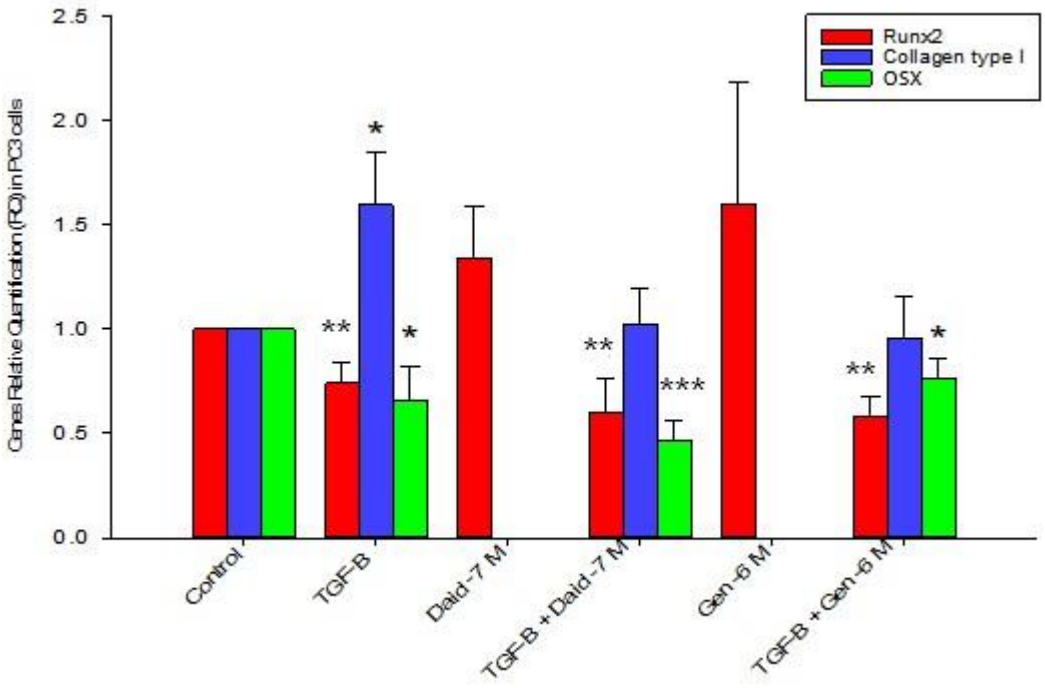


Figure 4.3 Transforming growth factor β (TGF-β) in PC3 cells. shown to interfere the effect of phytoestrogens and modify their individual action on the expression of genes involved in osteomimicry (Runx2) in PC3 cells. Cells were cultured with genistein or daidzein for 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05, ** P<0.01 and *** P<0.0001versus control

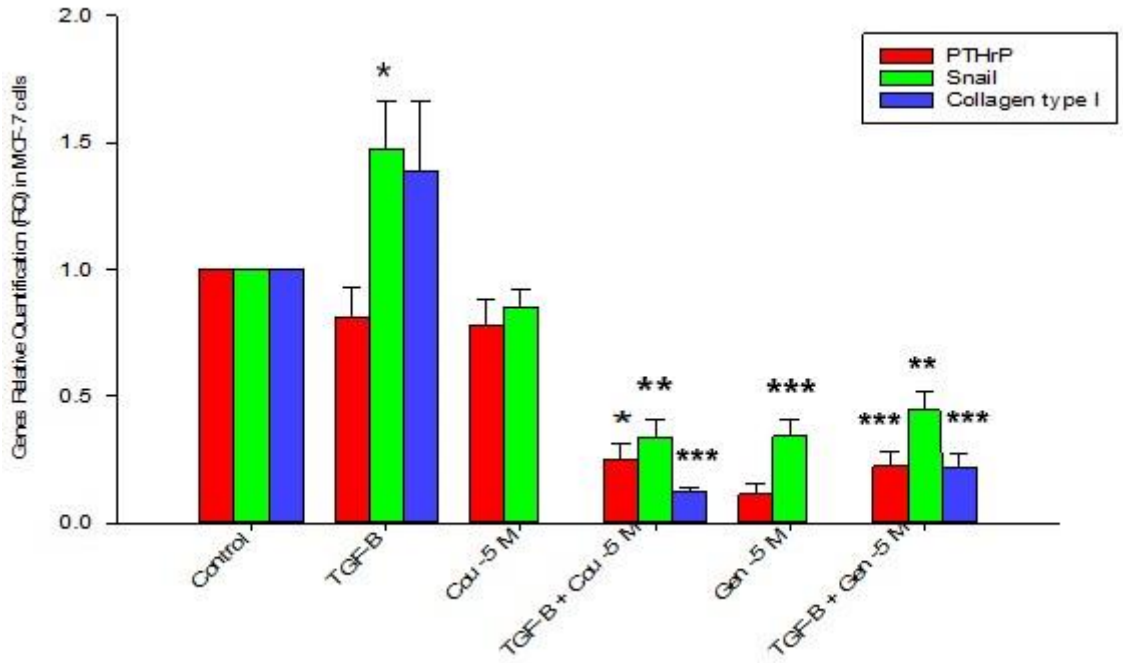


Figure 4.4 Transforming growth factor β (TGF-β) shown to interfere the effect of phytoestrogens and modify their individual action on the expression of genes involved in bone vicious cycle (PTHrP) and metastasis process of EMT (Snail) in MCF7 cells. Cells were cultured with genistein or coumestrol for 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05, ** P<0.01 and *** P<0.0001versus control

4.3.3 Bone morphogenic protein 7 (BMP 7) modify the individual effect of phytoestrogens on gene expression in prostate and breast cancer cell lines.

Bone morphogenic protein 7 (BMP 7), a bone formation and remodelling factor, and combinations of phytoestrogens with BMP7 or TGF-β were studied. Runx2 and integrin α5β3 expression was investigated in PC3 cells while expression of snail and integrin α5β3 were investigated in MCF7 cells after 72 hours to give phytoestrogens more time of engaging with cytokine environment containing BMP7 and to determine the importance of osteomimicry and the ability to initiate the EMT process in these cell lines.

BMP7 reduced PC3 cell integrin α5β3 expression to significant levels (18%, P = 0.09), while Runx2 expression was not reduced significantly by BMP7 exposure (79%, P = 0.70). Previously, individual Daidzein and genistein reduced Integrin α5β3 significantly in the absence of BMP7.In the presence of BMP7 the expression of Integrin α5β3 highly increased (99%, P = 0.98) but not to a significant level. Runx2 gene expression did not show any decrease but stayed on its high non-significant levels in the presence or absence of BMP7 (167%, P = 0.22) (Figure 4.5).

Combining BMP7 and TGF-β at 0.1 ng/mL also had no significant effect on Runx2 (68%, P = 0.60) and integrin α5β3 (79%, P = 0.65) expression. Genistein or daidzein and BMP7 combination non-significantly increased the expression of Runx2 (169%, P = 0.21) and (167%, P = 0.22) and integrin α5β3, (155%, P = 0.26) and (99%, P = 0.98) respectively, which is similar to the previous result with individual phytoestrogens in the absence of BMP7 (Figure 4.5).

In MCF7 cells, BMP7 significantly reduced integrin α5β3 expression (43%, P = 0.009). Combining BMP7 and TGF-β also significantly reduced integrin α5β3 expression (28%, P = 0.001). Previously, individual genistein significantly decreased snail expression in the absence of BMP7. In the presence of BMP7, genistein alters its effect and increased snail expression significantly (377%, P = 0.009). Individual coumestrol had a similar non-significant effect in the presence and absence of BMP7 (131%, P = 0.76) and (85%, P = 0.31) respectively, neither decreased nor increased snail expression significantly. Previous individual genistein and coumestrol significantly reduced the expression of Integrin α5β3 in the absence of BMP7 (9%, P = <0.0001) and (7%, P = <0.0001) respectively. In the presence of BMP7, phytoestrogens genistein 10^{-5} M and coumestrol 10^{-5} M lost their significant effect (81%, P = 0.39) and (68%, P = 0.1) respectively (Figure 4.6).

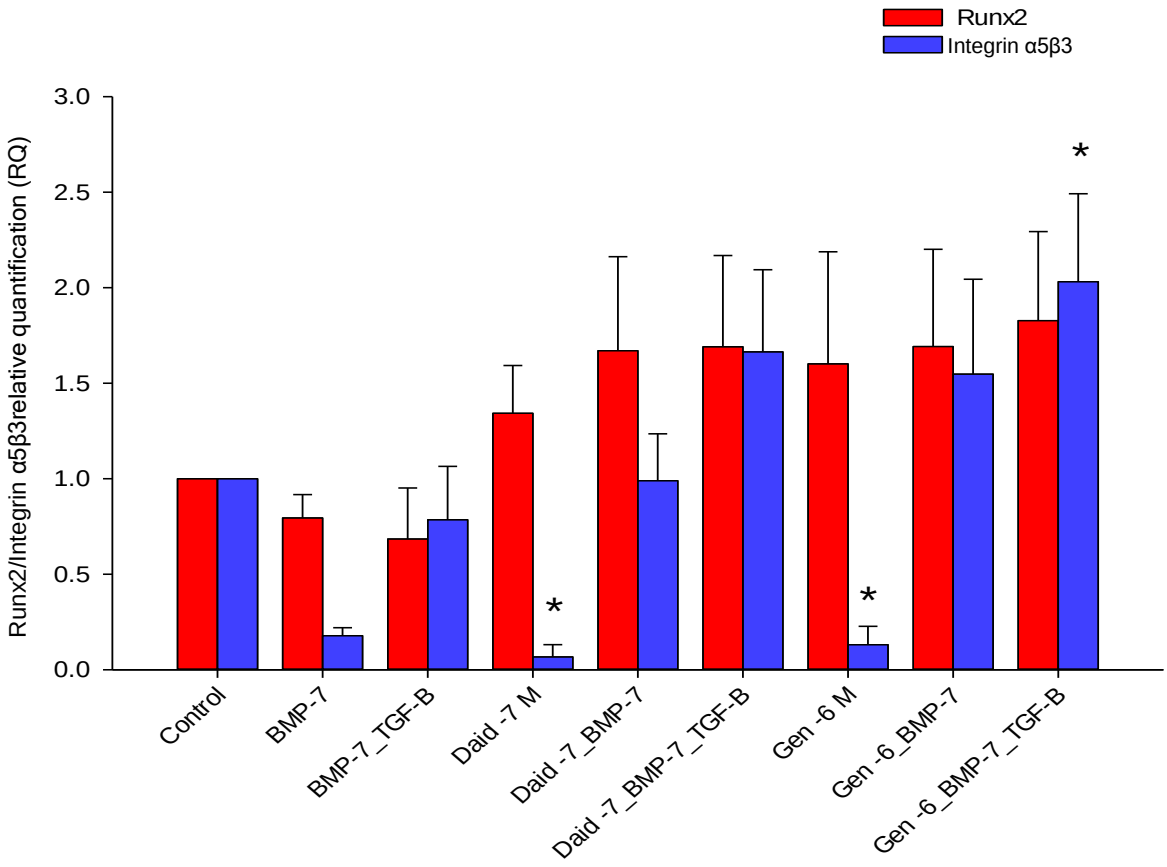


Figure 4.5 Modification of Runx2 and Integrin α5β3 gene expression in PC3 cell line by BMP7. BMP7 interfere the effect of phytoestrogens and modify their individual action on the expression of genes involved in osteomimicry (Runx2) and metastasis process of EMT (Integrin α5β3) in PC3 cells. Cells were cultured with genistein or daidzein for 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * $P<0.05$ versus control.

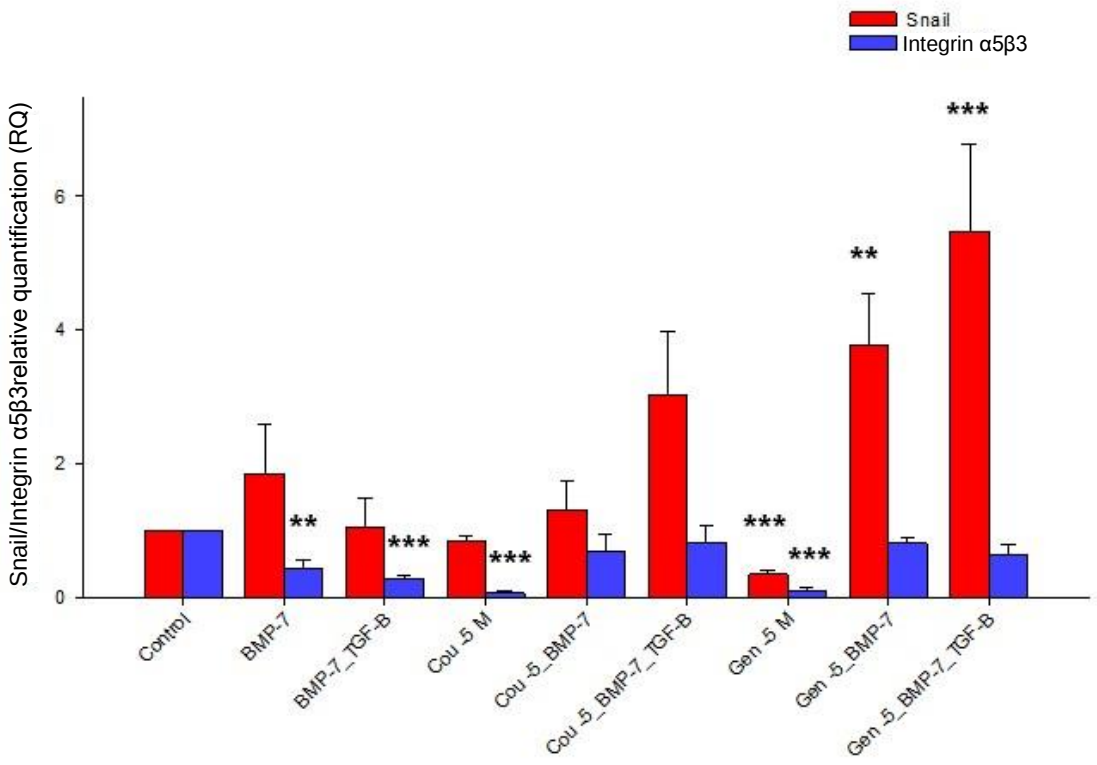


Figure 4.6 Modification of snail and gene expression in MCF7 cell line by BMP7:BMP7interfere the effect of phytoestrogens and modify their individual action on the expression of genes involved in metastasis process of EMT (Snail and Integrin α5β3) in MCF7 cells. Cells were cultured with genistein or coumestrol for 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05, ** P<0.01 and *** P<0.0001versus control.

4.4 Discussion

The potential benefit of individual phytoestrogens is underlined by their ability to modify the expression of genes implicated in tumour progression when cytokines typical of the bone environment are absent. Snail, a key transcription factor that induces integrin and cadherin expression during epithelial to mesenchymal transition, was suppressed by genistein and daidzein in the absence of TGF-β and BMP7 (Figure 4.2 A). In keeping with this, genistein and daidzein also reduced integrin α5β3 and CXCR4 expression (Figure 4.2 B and D). CXCR4 plays a major role in preferential metastasis of breast and prostate cancer to the skeleton, as bone marrow is the major source of CXCR4's ligand. A decrease in CXCR4 expression would, therefore, be expected to reduce the incidence of skeletal secondaries. These pose a major therapeutic challenge due to the tumour promoting stimulus provided by the bone environment. Central to this is the binding of aberrantly expressed cell adhesion molecules, such as integrin α5β2, to Arginylglycylaspartic acid (RGD) containing proteins in the bone matrix. This sequesters tumour cells to bone, provides additional proliferative stimuli and promotes pro-angiogenic growth factor connective tissue. Growth factor (CTGF) induced angiogenesis, contributing to the disproportionate impact of skeletal metastases in late-stage disease (Heldin, Vanlandewijck & Moustakas, 2012). Decreased integrin α5β3 and CXCR4 expression would, therefore, reduce disease progression and morbidity. However, it could be argued that cells would disseminate to other organs, delaying, rather than limiting, progression.

While, overall, the tumour burden is augmented in the skeleton, bone tissue is not a supportive environment for its development, as its mineralised nature limits growth and lacks a dense capillary network. This paradox is explained by

the ability of tumour cells to disrupt bone remodelling and, as a consequence, circumvent these limitations and generate a more conducive environment for growth. Breast cancer cells generate a developmental niche through the production of PTHrP, which indirectly stimulates osteoclast formation and bone resorption, by increasing the RANKL expression on osteoblasts and bone marrow stromal cells (Patel et al., 2011). Prostate cancer, in contrast, is associated with an osteosclerotic response, caused by a process that is termed osteomimicry. Osteomimicry forms part of the EMT, during which the tumour cells express transcription factors such as Runx2 that are typically restricted to the osteoblastic lineage (Cox et al., 2012; Jadaan, Jadaan & McCabe, 2015). The current data indicate that individual phytoestrogens modify the expression of these genes. Genistein and daidzein transiently reduced Runx2 expression in prostate cells and genistein significantly reduced PTHrP expression in breast cells. These changes would be expected to further limit the ability of skeletally disseminated cancer cells to survive and prosper in bone tissue, further reducing skeletal morbidity, such as hypercalcemia of malignancy.

The addition of TGF-β at (0.1 ng/mL) and BMP7 at (30 ng/mL) to the culture environment was to construct a cytokine model which the cells would typically encounter in bone, and to assess whether this modified the response of the PEs. Interestingly, the addition of rhTGF-β was found to block the signalling of rhBMP7, thus blocking the expression of genes important in bone formation (Ehnert et al., 2012). In contrast, according to a study by Zeisberg et al., BMP7 was found to counteract the TGF-β signalling mechanism (Zeisberg et al., 2003).

Transforming growth factor β, (rhTGF-β), shows an antagonist effect on phytoestrogen-induced changes, in combinations with daidzein and genistein.

The same results were observed when studying the effect on Runx2 and Osx, but not for collagen type I. This result suggests an important role for TGF-β on PE (daidzein and genistein) in early stages of metastasis, by inhibiting the expression of the osteomimicry genes (Runx2 and Osx) which are responsible for the direction of the cancer cell to the bone microenvironment in many cancers, including prostate cancer (Han et al., 2012). TGF-β blocks any effect of PE and increases collagen type I gene expression, but did not display this effect on Runx2 and osx, which both decreased significantly. This is contrary to the findings of Enhert *et al.*, that TGF- β effectively blocks BMP7 in osteoblast, thus suggesting that blocking of the process of osteomimicry in cancer cells (Ehnert *et al.*, 2012). Therefore, in the presence of TGF-β, daidzein and genistein, which increase the expression of Runx2, lost their effect on the expression of genes responsible for osteomimicry (Runx2 and Osx).

The inhibitory effect of genistein on PTHrP and snail is also lost in the presence of TGF-β. The latter reduced the non-inhibitory effect of individual coumestrol on PTHrP and snail gene expression to a significant extent (Figure 4.5).This suggest a strong effect of TGF-β on coumestrol and genistein action presented in a bone microenvironment vicious cycle.

Furthermore, low Snail expression suggest a decrease in the EMT process by elevating levels of epithelial phenotype, markers such as E-cadherin and integrin α5β3, at the primary tumour site. In contrast, TGF-β alone increased the expression of PTHrP, collagen type I and Snail in breast cancer cells, indicating a negative effect of TGF-β on the invasive, migration and proliferation of cancer cells which were inhibited when combined with phytoestrogens (Figure 4.5). It is suggested that TGF-β might be of importance on genistein action which has

been clear in this study results , and indicates that a relation between PEs and growth factors really exists (Adlercreutz, 2002).

BMP7 has shown an inhibitory effect on the phytoestrogens daidzein and genistein, and increased the expression of Runx2 in prostate cancer cell lines (Figure 4.6). Also, BMP7 inhibited the effect of genistein and coumestrol in breast cancer cell lines and increase the expression of snail to a high level. This is opposite to what had been observed of the individual effect of genistein (Figure 4.7). Individual coumestrol and genistein effects were noticeably greater than when combined with BMP7,and lost the significant decrease of integrin α5β3 (Figure 4.7).

Here, the current results demonstrated that BMP7 interferes and abolishes the inhibitory effect of individual phytoestrogens (daidzein, genistein and coumestrol), increases the expression of genes important in osteomimicry, (Runx2), EMT process (snail and Integrin α5β3) and enhances the migration of prostate and breast cancer cells. (Alarmo et al., 2009).

The data summarised here sets the scene for further research and investigations into this new class of plant-derived compounds that are capable of interfering with very complicated diseases, such as prostate and breast cancer, and its interaction with important growth factors and cytokines, such as TGF-β and BMP7, that may cause further complications to the patient.

4.5 summary of chapter 4 results

Table 4.5.1 phytoestrogens effect on expression of genes involved in metastasis in PC3 for 24h and 72h

Concentration (M)	Snail				CXCR 4				Runx2				Integrin α5			
	24h		72h		24h		72h		24h		72h		24h		72h	
	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value
Gen 10^{-7}	25* ↓	0.02	2 *** ↓	$2x10^{-3}$	2** ↓	$5x10^{-3}$	126 ↑	0.1	20*** ↓	$<10^{-4}$	282 ↑	0.4	20*** ↓	$<5x10^{-4}$	142 ↑	0.2
Gen 10^{-6}	30* ↓	0.02	36 * ↓	0.02	1** ↓	$5x10^{-3}$	3*** ↓	10^{-4}	11*** ↓	$<10^{-4}$	160 ↑	0.47	11*** ↓	$<2x10^{-4}$	13* ↓	0.02
Daid 10^{-8}	25* ↓	0.01	18*** ↓	$<6x10^{-3}$	6** ↓	0.01	3*** ↓	10^{-4}	18*** ↓	$<10^{-4}$	179 ↑	0.29	3*** ↓	$<10^{-4}$	9* ↓	0.01
Daid 10^{-7}	117 ↑	0.55	27* ↓	0.01	112 ↑	0.67	4*** ↓	10^{-4}	14*** ↓	$<10^{-4}$	134 ↑	0.48	228*** ↑	$<10^{-4}$	7* ↓	0.04

Note: " ↑ " and " ↓ " means upregulation, down-regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Table 4.5.2 phytoestrogens effect on expression of genes involved in metastasis in MCF7 for 24h and 72h

Concentration (M)	Snail				CXCR 4				PTHrP				Integrin α5			
	24h		72h		24h		72h		24h		72h		24h		72h	
	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value
Gen 10^{-6}	*** 329 ↑	$<10^{-4}$	20** ↓	$<10^{-4}$	42*** ↓	$<4x10^{-4}$	37** ↓	0.01	126 ↑	0.84	67 ↓	0.74	12*** ↓	$<10^{-4}$	12*** ↓	$<10^{-4}$
Gen 10^{-5}	*** 495 ↑	$<10^{-4}$	34*** ↓	$<3x10^{-4}$	50** ↓	0.01	61 ↓	0.08	179 ↑	0.3	11 ↓	0.29	0*** ↓	$<10^{-4}$	12*** ↓	$<10^{-4}$
Cou 10^{-7}	*** 305 ↑	$<10^{-4}$	126 ↑	0.08	53** ↓	$<10^{-3}$	113 ↑	0.53	180 ↑	0.27	* 127 ↑	0.04	0*** ↓	$<10^{-4}$	14*** ↓	$<10^{-4}$
Cou 10^{-6}	125 ↑	0.45	98 ↓	0.87	23*** ↓	$<10^{-4}$	90 ↓	0.64	103 ↑	0.94	94	0.43	6*** ↓	$<10^{-4}$	16*** ↓	$<10^{-4}$
Cou 10^{-5}	46 ↓	0.11	85 ↓	0.31	9*** ↓	$<10^{-4}$	71 ↓	0.17	60 ↓	0. 39	78 ↓	0.86	19*** ↓	$<10^{-4}$	7*** ↓	$<10^{-4}$

Note: "↑" and " ↓ " means upregulation, down-regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Table 4.5.3 TGFβ interfere the individual effect PEs action on expression of genes involved in osteomimicry in PC3 cells

Concentration (M)	Runx2		Collagen type I		Osx	
	% of control	P Value	% of control	P Value	% of control	P Value
TGFβ	74** ↓	0.008	159* ↑	0.02	66* ↓	0.03
Daid 10^{-7}	134 ↑	0.48				
TGFβ + Daid 10^{-7}	60** ↓	0.008	103 ↑	0.88	47*** ↓	<0.0001
Gen 10^{-6}	160 ↑	0.47				
TGFβ + Gen 10^{-6}	58** ↓	0.003	96 ↓	0.82	77* ↓	0.01

Note: " ↑ " and " ↓ " means upregulation, down-regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Table 4.5.4 TGFβ interfere the individual effect PEs action on expression of genes involved in bone vicious cycle in MCF7 cells

Concentration (M)	PTHrP		Collagen type I		Snail	
	% of control	P Value	% of control	P Value	% of control	P Value
TGFβ	81 ↓	0.13	139 ↑	0.16	148* ↑	0.01
Cou 10^{-5}	78 ↓	0.86			85 ↓	0.31
TGFβ + Cou 10^{-5}	25* ↓	0.03	12*** ↓	<0.0001	33** ↓	0.008
Gen 10^{-5}	11 ↓	0.29			34*** ↓	0.0003
TGFβ + Gen 10^{-5}	22*** ↓	0.001	22*** ↓	<0.0001	44** ↓	0.003

Note: " ↑ " and " ↓ " means upregulation, down-regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Table 4.5.5 BMP7 interference the effect of individual PEs action on expression of genes involved in osteomimicry and metastasis process EMT in PC3 cells

Concentration (M)	Runx2		Integrin α5β3	
	% of control	P Value	% of control	P Value
BMP7	79 ↓	0.7	18 ↓	0.09
BMP7+TGFβ	68 ↓	0.6	79 ↓	0.65
10^{-7}	134 ↑	0.48		
Daid 10^{-7} +BMP7	167 ↑	0.22	99 ↓	0.98
Daid 10^{-7} + BMP7 + TGFβ	169 ↑	0.21	166 ↑	0.17
Gen 10^{-6}	160 ↑	0.47		
Gen 10^{-6} + BMP7	169 ↑	0.21	155 ↑	0.26
Gen 10^{-6} + BMP7 + TGFβ	183 ↑	0.14	203 ↑	0.03

Note: " ↑ " and " ↓ " means upregulation, down-regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Table 4.5.6 BMP7 interference the effect of individual PEs action on expression of genes involved in metastasis process EMT in MCF7 cells

Concentration (M)	Snail			Integrin α5β3		
	% of control		P Value	% of control		P Value
BMP7	186	↑	0.37	43**	↓	0.009
BMP7+TGFβ	186	↑	0.95	28***	↓	0.001
Cou 10^{-5}	85	↓	0.31	7***	↓	<0.0001
Cou 10^{-}+ BMP7	131	↑	0.76	68	↓	0.1
Cou 10^{-5} + BMP7 + TGFβ	303	↑	0.06	82	↓	0.49
Gen 10^{-5}	34***	↓	0.0003	9***	↓	<0.0001
Gen 10^{-5} + BMP7	377**	↑	0.009	81	↓	0.39
Gen 10^{-5} + BMP7 + TGFβ	547***	↑	0.0001	64	↓	0.11

Note: " ↑ " and " ↓ " means upregulation, down-regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Chapter Five

Modulatory effect of IL-33 on phytoestrogen-induced changes in the gene expression of breast and prostate cancer cell lines

5.1 Introduction

Interleukin IL-33, a member of IL-1 cytokine family, is a dual-function protein that acts both as an alarming extracellular cytokine and as an intracellular nuclear factor. Abundant nuclear expression of IL-33 in endothelial cells from both large and small blood vessels in most normal human tissues, as well as in human tumours IL-33 is constitutively expressed in epithelial barrier tissues and lymphoid organs, maintaining the barrier function in normal conditions, and is released as a danger signal upon cellular damage or stress. By binding to its membrane ST2 receptor, IL-33 exerts an important role in inflammation, and allergy, and promotes the production of large amounts of IL-5 and IL-13 as part of type-2 innate immunity, IL-33 also induces the phosphorylation and activation of ERK1/2, JNK, p38 and PI3K/AKT signaling modules causing a production and release of pro-inflammatory cytokines(Hu et al., 2017; Koyasu & Moro, 2011).

The IL-33/ST2 pathway augments breast cancer progression and metastasis by promoting intra-tumoural accumulation of immunosuppressive cells, such as macrophages, myeloid-derived suppressor cells (MDSCs), mesenchymal stromal cells (MSCs) and by decreasing innate anti-tumour immunity (Figure 5.1). Furthermore, IL-33 was over-expressed in various cancers. A high expression of IL-33 was reported in colorectal cancer(CRC) tissues (Liu et al., 2014b), and the serum of breast cancer (Lu et al., 2014), and non-small cell lung cancer (NSCLC) patients (Hu et al., 2013). Further studies shown that IL-33 is constitutively expressed to high levels in the nuclei of endothelial and epithelial cells *in vivo* and that it can be released in the extracellular space after cellular damage. Thus, IL-33 was suggested to function as an endogenous danger signal or alarmin to alert cells of the innate immune system of tissue

damage during trauma or infection and a full-length IL-33 does not require processing for biological activity (Lefrançais et al., 2012).

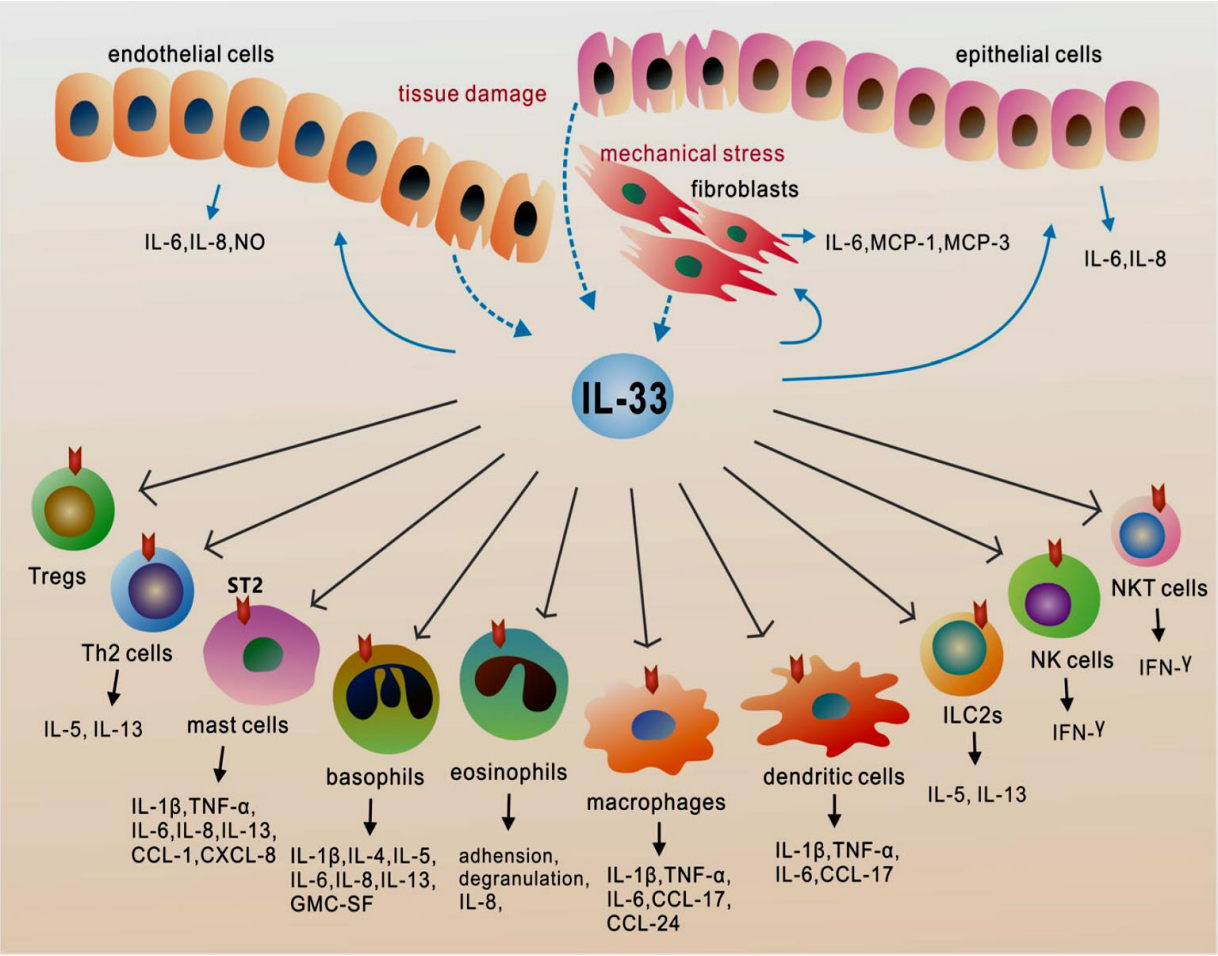


Figure 5.1: Cellular sources and targets of IL-33. IL-33 is released from endothelial cells, epithelial cells and fibroblasts in response to tissue damage or mechanical stress (dotted arrow). After release, IL-33 functions as an alarmin and activates different types of cells , including Th2 cells, Tregs, basophils, mast cells, eosinophils, macrophages, dendritic cells, innate lymphoid cells (ILC2s), NK cells and NKT cells. These activated cells respond to IL-33/ST2 signaling by producing both pro-inflammatory and anti-inflammatory mediators (Xu et al., 2017).

Toll-like receptors (TLRs) and other pattern recognition receptors, (PRRs), enhance the immune response after the recognition of conserved motifs expressed by pathogens. TLRs are also triggered by endogenous danger signals, termed danger-associated molecular patterns (DAMPs), which are released from the damaged host tissue after trauma or stress and may be considered as a signalling pathway for IL-33 (Xu et al., 2017).

It has been reported that transgenic expression of IL-33 may activate CD8 (+) T cells and NK cells, and inhibit tumour growth and metastasis in B16 melanoma and Lewis lung carcinoma metastatic models. It has also been shown to have direct effects on cancer cells, activating multiple intracellular signalling cascades to promote stemness and inhibit chemotherapy-induced cell death. Thus, IL-33, through direct and in-direct actions, has an important role in promoting tumour progression. Therefore, like other cytokines, IL-33 may modulate the response to potential therapeutic agents, leading to poorer outcomes. Currently, there is an absence of literature studying the effect of IL-33 in the presence of phytoestrogens or any other phytochemicals in breast and prostate cancer.

This chapter will try to model a breast and prostate cancer environment for IL-33 in the presence of phytoestrogens, and will attempt to study the effect of IL-33 on phytoestrogen-induced gene expression in PC3 cells (CXCR4, Runx2, snail and Integrin α5β) and (CXCR4, snail and Integrin α5β) in MCF7 cells. This chapter will also investigate whether phytoestrogens, will keep their individual beneficial effects on expression of these genes (as in Chapter Three) and will attempt to find a relationship between this expression and the presence of IL-33.

5.2 Materials and methods

Methods used here in chapter 5 are similar to what has been mentioned in chapter 2 (materials and methods) for the points:

2.5.1 RNA extraction and reverse transcription

2.5.2 Verification of PCR primers and RT

2.5.3 10x Tris-acetate-EDTA (TAE) buffer

2.5.4 Real-time quantitative PCR analysis of metastatic marker expression

2.6 Statistical analysis

5.2.1 IL-33 Cytokine

Recombinant Human IL-33 was purchased from Insight Biotechnology Limited (Wembley Middlesex, United Kingdom) and concentration used was 10 ng/mL (Choi et al., 2009). IL-33 added to two sets one for individual IL-33 for 24 and 72 hours and one for IL-33 and phytoestrogens also for 24 and 72 hours.

Primers used were as following:

	5´-3´ Forward primer	3´-5´ Reverse primer	
Human β-actin	GCGCGGCTACAGCTTCACCA	TGGCCGTCAGGCAGCTCGTA	777-929
Human Runx2	AGACCCCAGGCAGGCACAGT	GCGCCTAGGCACATCGGTGA	816-972
Human CXCR4	GCGCAAGGCCCTCAAGA	GTGCGTGCTGGGCAGAGGTT	1010-1257
Human Snail	CGAGTGGTTCTTCTGCGCTA	CTGCTGGAAGGTAAACTCTGGA	27-183
Human Integrin α5β3	AATTTTACTGGCGAGCAG	TTGGTGGCATGCTTCGAG	1054- 1465

Table 5.1 PCR primers and their amplicons used with IL-33 experiments

5.3 Results

5.3.1 IL-33 effect on phytoestrogen-induced changes in CXCR4 gene expression in PC3 cells

Here in this experiment the effect of phytoestrogens in the presence of IL-33 on CXCR4 was studied. IL-33 alone had no significant effect on CXCR4 expression in either 24 or 72 hours and was (117%, P = 0.58) and (120%, P = 0.57). However, the suppressive effect of daidzein and genistein seen in previous chapters was abolished in the presence of IL-33. After 24 hours, CXCR4 expression increased significantly in the presence of IL-33 plus daidzein (189%, P = 0.02) or genistein (187%, P = 0.03). After 72 hours, there was an increase in CXCR4 expression in both combinations (239%, P = 0.01) for IL-33 with daidzein and (168%, P = 0.19) for IL-33 with genistein, but this increase was significant only with daidzein (Figure 5.2).

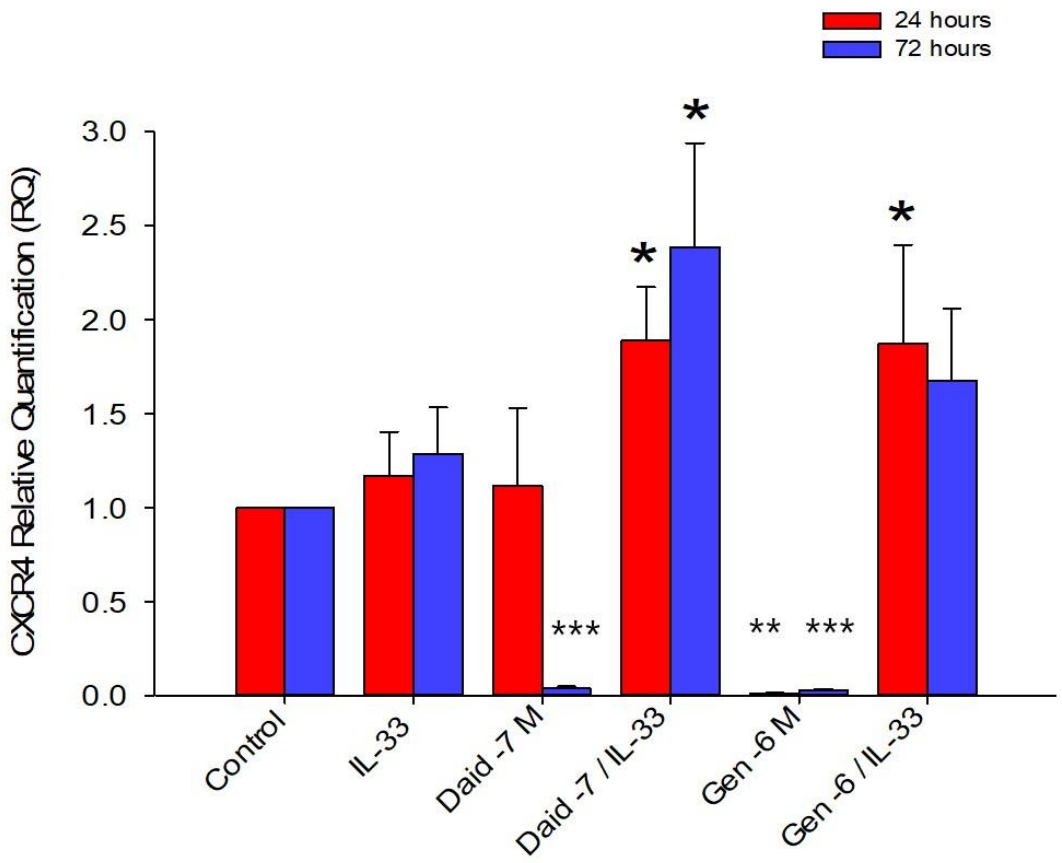


Figure 5.2 Interleukin 33 (IL-33) prevents the inhibitory effect of phytoestrogens on CXCR4 in PC3 cells. Cells were cultured with genistein or daidzein for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05, ** P<0.01 and *** P<0.0001versus control

5.3.2 IL-33 has negative effect on Snail gene expression in the presence of phytoestrogens in PC3 cells.

IL-33 alone had no significant effect on Snail expression at 24 and 72 hours (130%, P = 0.47) (170%, P = 0.15) respectively. However, in the presence of IL-33 the inhibitory effect of daidzein and genistein on snail expression was lost and a significant increase was noted at 72 hours for daidzein (199%, P = 0.04) (Figure 5.3).

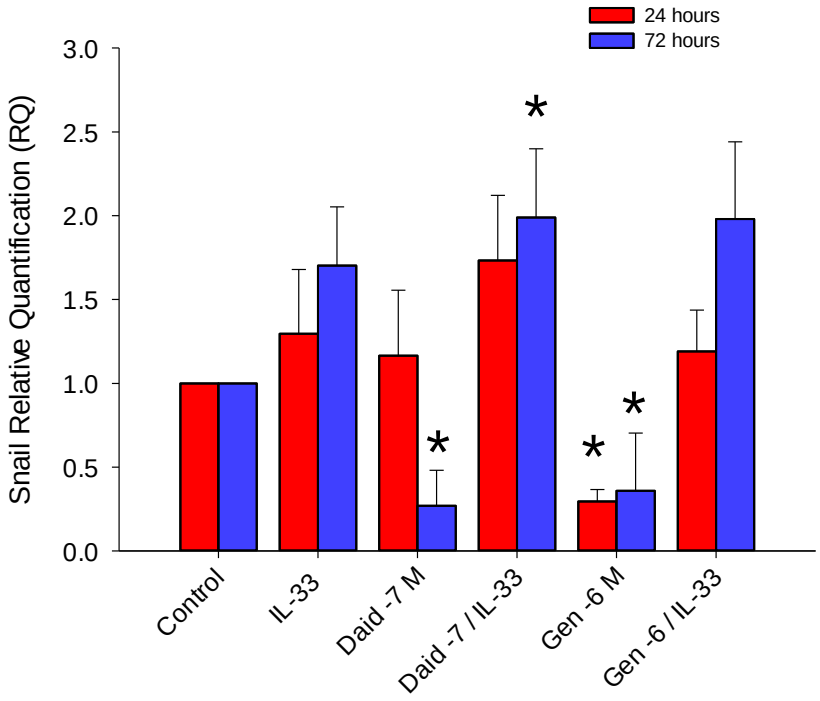


Figure 5.3 Interleukin 33 (IL-33) modifies the response to PE on snail expression in PC3 cells. Cells were cultured with genistein or daidzein for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05 versus control.

5.3.3 IL-33 increased Runx2 gene expression in the presence or absence of phytoestrogens in PC3 cells

There was no significant inhibitory effect of IL-33 alone on Runx2 gene expression at 24 or 72 hours and was (156%, P = 0.13) (134%, P = 0.34) respectively. However, the inhibitory effect of genistein and daidzein on runx2 expression was lost in the presence of IL-33 for genistein at 24 hours was significant (332%, P = <0.0001) and at 72 hour was (143%, P = 0.18) and for daidzein at 24 hours was (125%, P = 0.46) and at 72 hours was (82%, P = 0.58) (Figure 5.4).

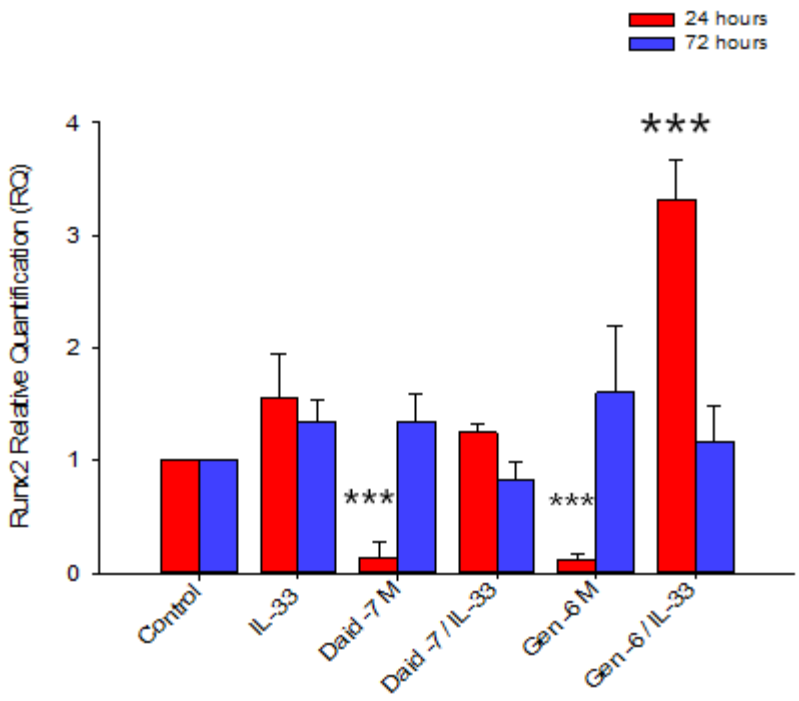


Figure 5.4 Interleukin 33 (IL-33) modifies the response to PE on runx2 expression in PC3 cells. Cells were cultured with genistein or daidzein for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. *** P<0.0001versus control.

5.3.4 Effect of IL-33 on phytoestrogen-induced effects on integrin α5β3 gene expression in prostate cancer PC3 cells.

IL-33 alone decreased the expression of integrin α5β3 significantly after 72 hours (34%, P = 0.009) which indicate for a protection against the loss of cell-cell contact which is a known behaviour in cancer initiation for migration. IL-33 prevented the inhibitory effect of genistein on integrin α5β3 expression and led to a significant increase in expression after 24 hours for genistein (452%, P = <0.0003) and decreased sharply after 72 hours with genistein, this might be related to unknown behaviour of IL-33 at these time points. On the other hand, the individual daidzein significant effect on integrin α5β3 gene expression decreased but not to a significant level (49%, P = 0.44) after 24 hours with IL-33 (Figure 5.5).

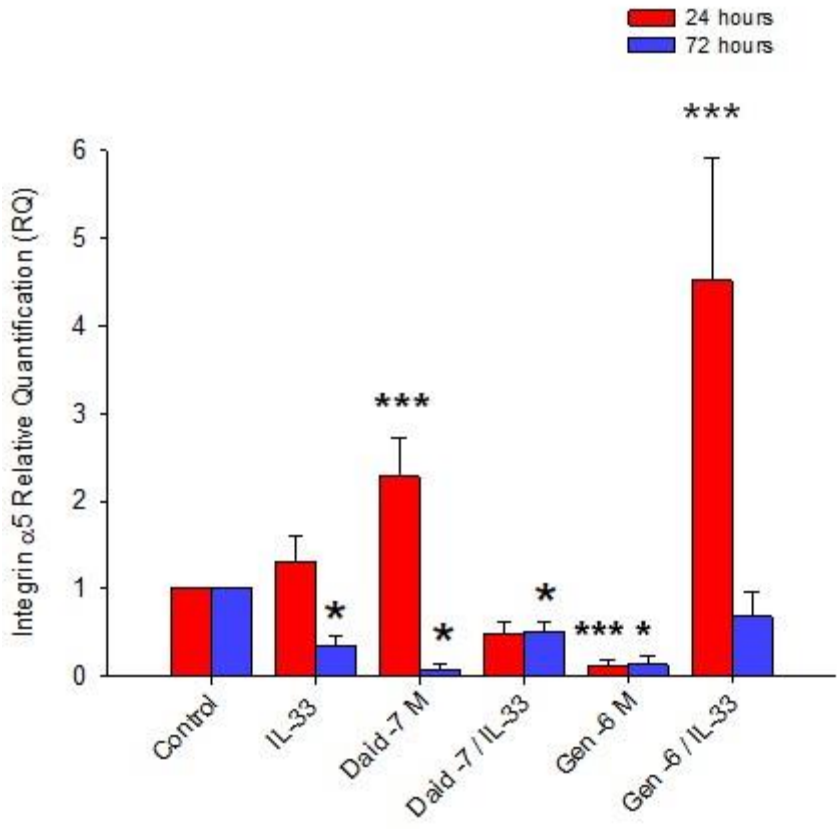


Figure 5.5 Interleukin 33 (IL-33) modifies the response to PE on integrin α5β3 expression in PC3 cells. Cells were cultured with genistein or daidzein for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * $P<0.05$ and *** $P<0.0001$ versus control.

5.3.5 The effect of IL-33 on phytoestrogen-induced changes in CXCR4 gene expression in MCF7 cells.

IL-33 alone had no significant effect on CXCR4 expression at 24 and 72 hours and was (63%, P = 0.053) and (120%, P = 0.65) respectively. IL-33 had no effect on the inhibitory action of genistein or coumestrol at 24 hours but prevented the inhibitory effect genistein on CXCR4 expression at 72 hours and was (123%, P = 0.59) and (124%, P = 0.58) respectively (Figure 5.6).

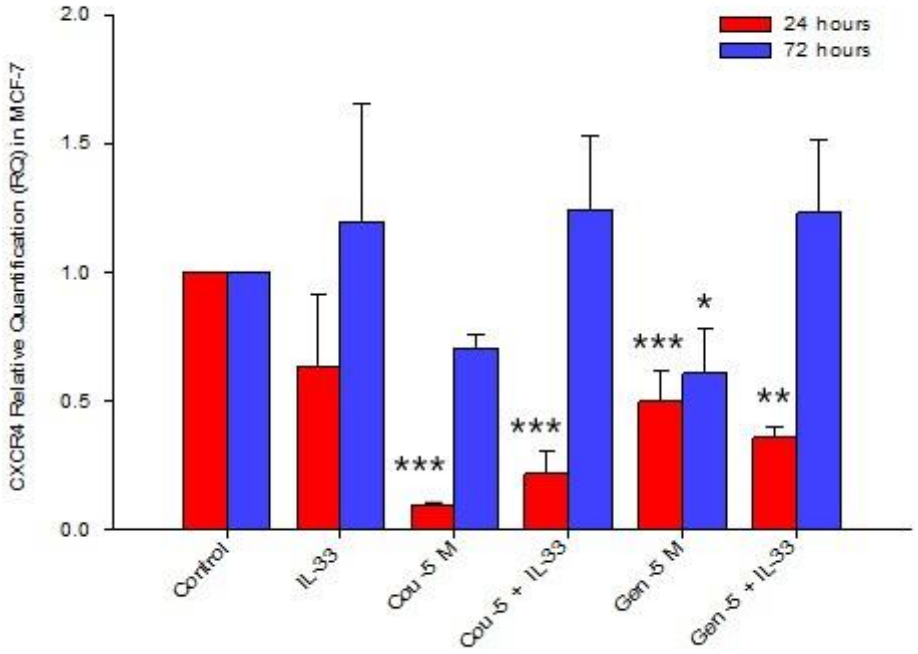


Figure 5.6 Interleukin 33 didn't interfere the inhibitory action of coumestrol and genistein on CXCR4 gene expression in MCF7 cells at 24 hours but modifies later responses to genistein on CXCR4 expression in MCF7 cells. Cells were cultured with genistein or coumestrol for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05, ** P<0.01 and *** P<0.0001versus control

5.2.6 IL-33 prevented the inhibitory effect of genistein on snail expression at later stage in MCF7 cells

IL-33 has been tested here to observe whether there is any effect on EMT related gene (snail) expression with and without phytoestrogens. It has been noticed that IL-33, alone, significantly decreased snail expression at 72 hours (38%, P = 0.02). Also, IL-33 prevented the inhibitory effect of genistein at 72 hours (81%, P = 0.45) and decreased it at 24 hours but not to a significant level compared to individual genistein, whereas the effect with coumestrol was not changed (Figure 5.7).

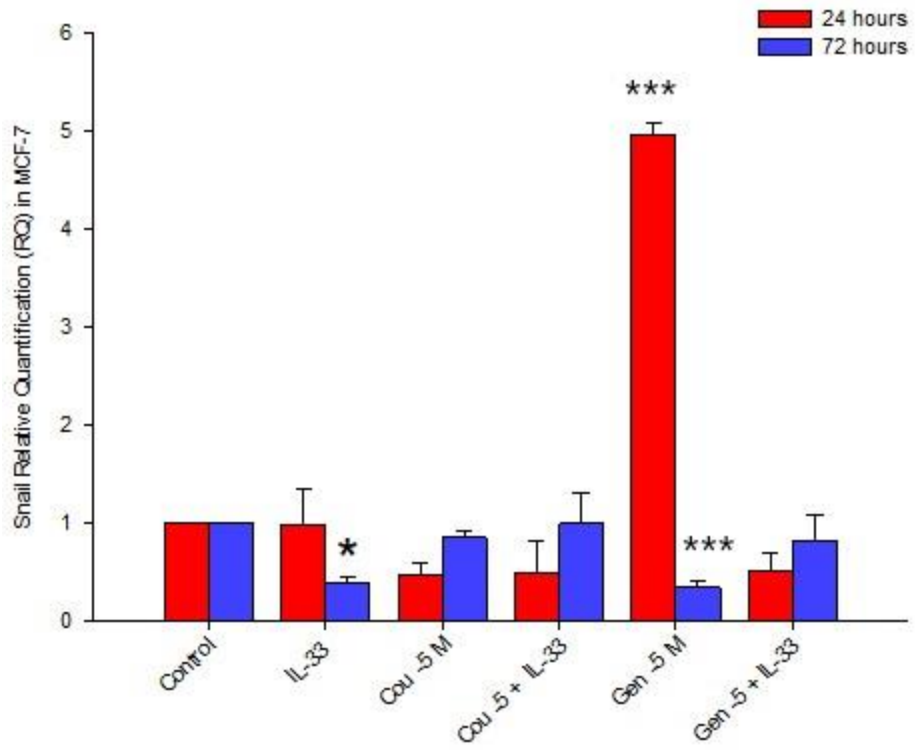


Figure 5.7 Interleukin 33 (IL-33) modifies the later response to genistein on snail expression in MCF7 cells. Cells were cultured with genistein or coumestrol for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05 versus control. * P<0.05 and *** P<0.0001

5.2.7 IL-33 modifies the response to phytoestrogen-induced changes in integrin α5β3 expression in MCF7 cells.

IL-33 significantly decreased integrin α5β3 expression at 72 hours (53%, P = 0.01) and 24 hours but was not significant decrease (24%, P = 0.07). IL-33 modified the response to genistein and coumestrol and prevented the decrease in expression at 24 hours (158%, P = 0.13) and (65%, P = 0.30), however the same significant decrease at 72 hours remained for genistein and coumestrol (Figure 5.8).

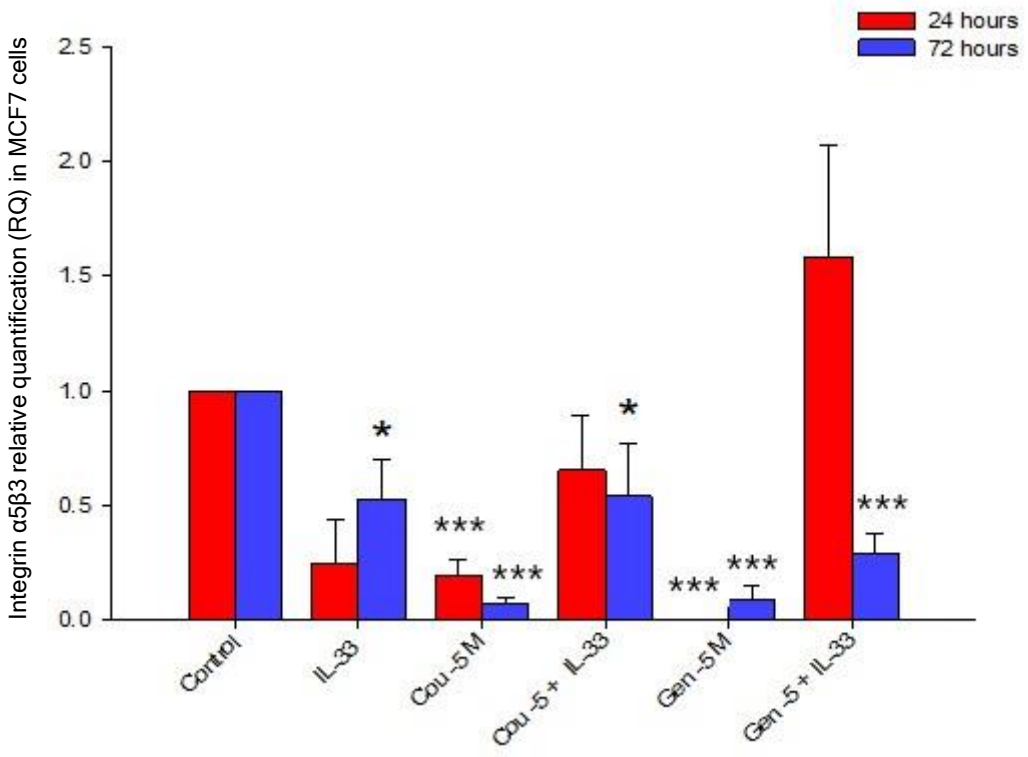


Figure 5.8 Interleukin 33 in MCF7 cells and Integrin α5β3 gene expression. Interleukin 33 (IL-33) modifies the early response to genistein and coumestrol on integrin expression in MCF7 cells. Cells were cultured with genistein or coumestrol for 24 or 72 hours prior to mRNA isolation. Gene expression was then assessed using ΔΔCT qPCR and the DNA-binding dye SYBR green for detection of PCR products. The relative quantification (RQ) value for each group was calculated using CT values for non-treated controls normalized to the expression β-actin mRNA. Samples were analysed in triplicate and experiments repeated separately three times. * P<0.05 and *** P<0.0001

5.3 Discussion

IL-33 has been studied mainly for its role in Th2 immunity and Th2-related diseases, such as asthma, atopic dermatitis, and anaphylaxis. IL-33 is released from a variety of tissues as a pro-inflammatory cytokine (Villarreal & Weiner, 2014). Serum levels of IL-33 and sST2 in breast cancer patients were significantly higher than in healthy women, and it is thought to be a prognostic marker in several cancers (Lu et al., 2014). ST2 was initially shown to be selectively expressed on Th2, but not Th1 or regulatory T cells (Treg). More studies have shown that IL-33 can activate murine dendritic cells directly driving polarisation of naïve T cells towards a Th2 phenotype, and it can act directly on Th2 cells to increase secretion of Th2 cytokines such as IL-5 and IL-13. Moreover, IL-33 can also act as a chemo-attractant for Th2 cells. Limited studies have addressed the role of IL-33/ST2 signaling on anti-tumour immune responses, tumour growth and or metastasis. However, recent studies revealed that ST2 negative mice with mammary tumour have attenuated tumour growth and metastasis, with increased circulating levels of pro-inflammatory cytokines and activated NK and CD8+ T cells. Therefore, IL-33 may be an important mediator in tumour escape from immune control and in tumour angiogenesis and thus warrants further investigation (Miller, 2011).

Moreover, IL-33 and its receptor sST2 were found to have a significant correlation with serum VEGF, which indicates an association of IL-33 and sST2 with angiogenesis (Lu *et al.*, 2014). Zhang *et al.* also indicated that tumour derived IL-33 enhanced the recruitment of myeloid cells. such as macrophages, that secrete VEGF to promote tumour angiogenesis and metastasis by remodelling the tumour microenvironment, rather than by modifying the intrinsic propensity of tumour cells to invade, migrate, or undergo EMT (Zhang et al.,

2017). IL-33 has been shown to enhance liver inflammation and fibrosis, thus, providing a substrate for hepatobiliary tumour development. By affecting multiple pro-tumour processes, directly and indirectly, IL-33, through AKT and Yes-associated proteins, promotes oncogene-induced cholangiocarcinoma (CCA) in mice (Yamada et al., 2015). Jovanovic *et al*. found that exogenously administrated IL-33 enhanced primary 4T1 (mouse breast cancer) growth and inhibited innate anti-tumour immunity (Jovanovic et al., 2012). In gastric cancer, IL-33 enhanced invasion and migration in a dose-dependent manner (Yu et al., 2015).

It has been observed that serum levels of IL-33 are higher in patients with ER-positive tumours, suggesting that the IL-33/ST2 axis may be involved in hormone receptor signaling (Liu et al., 2014a). Hormone therapy is a first-line treatment for ER-positive breast cancer, with tamoxifen as the most widely used anti-oestrogen drug. Haiyan and his team indicated an important function of IL-33 in producing an endocrine resistance of breast cancer patients by promoting expressions of stem cell genes, including ALDH1A3, OCT4, NANOG and SOX2, promoting tumourigenesis, leading to the development of breast cancer stem cells. In clinical breast cancer patients, higher serum IL-33 levels predict tumour recurrence, while increased IL-33 expression in cancer cells predicts tamoxifen resistance, due to less ER expression (Hu *et al*., 2017).

IL-33 was studied herein to find whether it modified the effect of phytoestrogens on expression of genes important in osteomimicry (Runx2), cell migration (CXCR4), EMT (snail and integrin α5β3). In prostate cancer, interleukin-33 abolishes the significant reduction of CXCR4 gene expression caused by individual genistein and daidzein and increases it to higher levels. Also, IL-33

affects the expression of runx2 that increased with both individual treatment of IL-33 and when combined with phytoestrogens but not to a significant levels only increased significantly with genistein at 24 hours. Also IL-33 increased snail expression but was significant only with daidzein at 72 hours and increased integrin α5β3 expression and was significant only when combined with genistein after 24 hours. Thus, in the presence of IL-33 the potentially beneficial effect of PEs on osteomimicry, preferential metastasis and epithelial transition is lost.

The data on the role of IL-33 in cancer progression is limited, especially with regard to the function mediated in human breast cancer cells (Liu *et al.*, 2014a). IL-33 was used to inhibit the individual effect of genistein and coumestrol on important genes in metastasis and cell migration. IL-33 modified the individual effect of PEs, and altered their effect by upregulation of CXCR4, snail and integrin α5β3 gene expression. This data suggests that the presence or expression of IL-33 in the breast cancer environment have a role in promoting the epithelial-mesenchymal transition process and cell migration.

There was a difference between individual genistein phytoestrogen and when combined with IL-33 in both prostate and breast cancer cell lines at 24 and 72 hours. This might be the availability of oestrogen receptors ER and that IL-33 and its reaction with its receptor ST2 affect the ER signaling of genistein and abolish its effect. A further detailed investigation should elucidate this activity.

In conclusion, IL-33 might have an important role in both prostate and breast cancers. Due to the shortage of literatures regarding the cross-talk between IL-33 and phytoestrogens in cancers, particularly breast and prostate cancers, this study tried to add to the evidence that IL-33 plays a non-protective role, enhancing the progression of the disease and worsening the situation, both *in vitro* and *in vivo*. Further expansion of research on IL-33 interaction with the phytochemical are necessary if we are attain a sound knowledge of the function of phytoestrogens.

5.4 summary of chapter 5 results

Table 5.4.1 IL-33 modification to the response to PEs in PC3 cells at 24h and 72h

Concentration (M)	CXCR 4				Snail			
	24h		72h		24h		72h	
	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value
IL-33	117 ↑	0.58	120 ↑	0.57	130 ↑	0.47	170 ↑	0.15
Daid 10^{-7}	112 ↑	0.67	4*** ↓	0.0001	117 ↑	0.55	27* ↓	0.01
Daid 10^{-7} + IL-33	189 * ↑	0.02	239* ↑	0. 01	173 ↑	0.1	199* ↑	0.04
Gen 10^{-6}	1** ↓	0.005	3*** ↓	0.0001	30* ↓	0.02	36* ↓	0.02
Gen 10^{-6} + IL-33	187* ↑	0.03	168 ↑	0.19	119 ↑	0.7	198 ↑	0.0505

Concentration (M)	Runx2				Integrin α5			
	24h		72h		24h		72h	
	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value
IL-33	156 ↑	0.13	134 ↑	0.34	131 ↑	0.64	34** ↓	0.009
Daid 10^{-7}	14*** ↓	0.0001	134 ↑	0.48	*** 228 ↑	0.0001	7* ↓	0.04
Daid 10^{-7} + IL-33	125 ↑	0.46	82 ↓	0.58	49 ↓	0.44	51* ↓	0.04
Gen 10^{-6}	*** 11 ↓	0.0001	160 ↑	0.47	*** 11 ↓	0.0002	13* ↓	0.02
Gen 10^{-6} + IL-33	*** 332 ↑	<0.0001	143 ↑	0.18	*** 452 ↑	0.0003	68 ↓	0.14

Note: " ↑ " and " ↓ " means upregulation, down-regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Table 5.4.2 IL-33 modification to the response to PEs in MCF7 cells at 24h and 72h

Concentration (M)	CXCR 4				Snail				Integrin α5			
	24h		72h		24h		72h		24h		72h	
	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value	% of control	P Value
IL-33	63 ↓	0. 053	120 ↑	0.65	98 ↓	0.95	38* ↓	0.02	24 ↓	0.07	53* ↓	0.01
Cou 10^{-5}	9*** ↓	$<10^{-4}$	71 ↓	0.17	46 ↓	0.11	85 ↓	0.31	19*** ↓	$<10^{-4}$	7*** ↓	$<10^{-4}$
Cou 10^{-5} + IL-33	22*** ↓	$<10^{-4}$	124 ↑	0.58	48 ↓	0.12	99 ↔	0.98	65 ↓	0.30	54* ↓	0.02
Gen 10^{-5}	42*** ↓	$4x10^{-4}$	37* ↓	0.01	*** 329 ↑	$<10^{-4}$	20*** ↓	$<10^{-4}$	12*** ↓	$<10^{-4}$	12*** ↓	$<10^{-4}$
Gen 10^{-5} + IL-33	36** ↓	0.002	123 ↑	0.59	52 ↓	0.18	81 ↓	0.45	158 ↑	0.13	29*** ↓	$7x10^{-4}$

Note: " ↑ ", " ↓ " and " ↔ " means upregulation, down-regulation and little or no regulation of the indicated target respectively. * P<0.05, ** P<0.01 and *** P<0.0001

Chapter six

General Discussion

6. General discussion

Of particular interest in relation to human health are the class of compounds known as the phytoestrogens, which are comprised of various groups of non-steroidal oestrogenic compounds that are broadly distributed within the plant kingdom. There is an increasing body of evidence that consumption of some of these plants or their molecules could be an additive effective tool to prevent and treat several diseases associated with ageing, metabolism, malignant transformation and cardiovascular disease (Sirotkin & Harrath, 2014).

Soybeans, along with other phytoestrogen- rich food such as legumes, are rich with phytochemicals, are processed into various food products for digestibility, taste and bioavailability of nutrients and bioactive compounds (Zaheer & Humayoun Akhtar, 2017). The oestrogenic compounds (i.e. isoflavones) in phytoestrogens are found in the form of glucosides, malonylglucosides, acetylglucosides and aglycones. In the gut, the isoflavone glycosides present in soy products are deglycosylated by β- glucosidases in the small intestine. The metabolism of soy isoflavones by bacteria varies among individuals. Inter-individual differences in phytochemical metabolism may be affected by gut microbial identity and activity, and stability and variation in concentrations of endogenous compounds that may modulate biotransformation pathways. Furthermore, gut bacterial modification of soy isoflavones results in metabolites that differ in biological activity from the main compounds (Miura et al., 2016). Thus, human gut microflora have been shown to employ metabolic activities on isoflavones that influence bioavailability and bioactivity (Turner, Thomson & Shaw, 2003)

Oestrogens impact on the growth and functioning of female and male reproductive tissue, support the skeletal and central nervous system, contribute to cardio-protective effects, and protect against colon cancer and skin aging. In the light of the numerous effects on the human body of oestrogens, it is not unusual to consider the importance of phytoestrogens for human health. They are perceived as a more natural way to complement medical health care, and many women turn to phytoestrogens as an alternative to hormone replacement therapy (HRT) in order to circumvent undesirable side effects, such as increased risk of breast and endometrial cancer and irregular bleeding (Ososki & Kennelly, 2003b).

One may ask why the role of phytoestrogens in prostate carcinogenesis should be studied when prostate growth and development are regulated by testosterone. However, epidemiological studies indicate a lower rate of prostate and breast cancers in Asian communities than in Western communities, which is explainable in terms of diet, chiefly the regular intake of soy phytoestrogens (Adjakly et al., 2015). The phenolic ring structures of isoflavones enable these phytochemical compounds to bind oestrogen receptors (ER) and mimic oestrogen (E2). In addition to genistein and daidzein binding ER, it is with a lower affinity when compared with estradiol, and with greater affinity for ERβ than for ERα. Furthermore, phytoestrogens have been described as acting like natural selective ER modulators (SERMs) at various tissue sites throughout the body (Lund et al., 2004).

The results here indicate that phytoestrogens (genistein and daidzein) have a significant effect on reducing prostate cancer cell growth. Genistein and daidzein can bind to ERβ and mimic the action of oestrogens. This binding between these phytoestrogens and the receptor could partly explain the anti-

cancer effects of these molecules and their impact as a focus for prostate pathology research (Adjakly *et al.*, 2015; Mentor-Marcel et al., 2001). Furthermore, Piccolella et al. indicated that genistein in prostate cancer shows antiproliferative action through ERβ (Piccolella et al., 2014). *In vitro* studies demonstrate a role for phytoestrogens (tectorigenin and irigenin) in controlling prostate cancer cell numbers by inhibiting proliferation through cell cycle regulation. These compounds would reduce cell numbers and increase the ability of antiandrogens to induce cell death in prostate cancer cells and prove that many other phytoestrogens beside genistein and daidzein have a significant effect on prostate cancer cell inhibition (Morrissey et al., 2004). Another study on prostate cancer cells showed that genistein directly inhibits Akt and NF-κB pathways, which leads to activation of apoptosis (Russo et al., 2016). Many studies suggest that phytoestrogens like genistein may compete with, and prevent, endogenous oestrogens from binding to the oestrogen receptor (ER), thus inhibiting cellular proliferation and promoting differentiation. Also, several clinical studies suggest high soy consumption may facilitate prostate cancer prevention, but its role as therapy for an established tumour is conflicting (Masko, Allott & Freedland, 2013). Results of other studies indicate the functional beneficial effects of coumestrol in promoting apoptosis of human prostate cancer cells PC3, thus reducing cell numbers. The study suggests a mechanism for coumestrol action in prostate cancer that an intrinsic apoptotic pathways and inhibition of PI3K/AKT and activation of MAPKs (ERK1/2 and JNK) cell signaling may be involved. These mechanistic conclusions support the suggestion that coumestrol has anti-cancer effects on prostate cancer cells, affecting their viability and mitochondrial functions by a significant loss of mitochondrial membrane potential. Additionally, cleavage of caspase-3 and -9,

which are apoptotic proteins associated with mitochondria, also altered in response to coumestrol. Furthermore, coumestrol caused mitochondrial dysfunction resulting in an increase in ROS production in PC3 and LNCaP cells, although that finding contradicts the current results, which concluded that coumestrol did not suppress cell proliferation in prostate cancer cells, but rather enhanced it (Lim et al., 2017)

In breast cancer, phytoestrogens have been studied to see their effect on cell viability. Phytoestrogens (genistein and coumestrol) have the greatest significant effect on breast cancer cell growth reduction in my studies, whereas daidzein acted as a promoter ofr breast cancer cell growth. It has been found that dietary phytoestrogen intake during adolescence may be associated with a decreased risk of adult breast cancer. This finding has significant implications for breast cancer prevention, since diet is a potentially modifiable determinant (Thanos et al., 2006). Furthermore, it has been observed that phytoestrogens (genistein, glycitein, daidzein,O-Desmethylangolensin (O-Dma), coumestrol and equol) shown a potent inhibitory effect on MDA-MB-231 breast cancer cell invasion, while lignans exerted a minimal effect in matrigel experiments (Magee, McGlynn & Rowland, 2004).

This study produced outcomes similar to those of Wang et al., which suggested that genistein above 10^{-5} M inhibited growth of MCF7 cells, while below this concentration genistein stimulated growth (Wang, Sathyamoorthy & Phang, 1996). The proposed mechanism for phytoestrogen effects in breast tumour cells is that the set of genes upregulated by ERβ activation promote cell cycle progression and consequently suppress proliferation, while activation of ERα appears to do largely the opposite (Patisaul & Jefferson, 2010). There is some epidemiological evidence of increased phytoestrogen intake being associated

with a reduced risk of breast cancer, but it is inconsistent. Unlike soy flour which had no effect, soy extracts and purified isoflavones caused growth stimulation of MCF7 cells transplanted into ovariectomised athymic mice (Velentzis et al., 2008). It has been suggested that dietary consumption of phytoestrogens, including coumestrol, reduces the risk of breast cancer. However, phytoestrogens also share antagonistic characteristics that affec the risk of developing breast cancer (Zafar, Singh & Naseem, 2017). In a previous study on MCF7 cells, phytoestrogens seem to have had the effect of increasing cell numbers, which contradicted the findings of my own study, interestingly; however, this increase is not the result of a stimulation of proliferation, butis the consequence of the inhibition of the rate of apoptosis. These results support the hypothesis that phytoestrogens may stimulate the progression of an existing tumour (Schmidt, Michna & Diel, 2005). Another suggestion was that genistein phytoestrogen treatment resulted in a greater cell survival in MCF7 cells, probably due to its oestrogenic activity through its binding to ERα. However, the interaction of genistein with the ERβ perhaps suggests that the most important part of the study was that of phytoestrogens in combination. Furthermore, these results suggest that the anti-oestrogenic activity of phytoestrogens was established by a mechanism similar to tamoxifen or ICI 182,780, leading to a reduction in cell proliferation (Collins, McLachlan & Arnold, 1997; Pons et al., 2016) . Furthermore, Mário L de Lemos concluded that genistein and daidzein may stimulate existing breast tumour growth and antagonise the effects of tamoxifen. He warned women with breast cancer that they should be aware of the risks of possible tumour growth when taking soy products. Thus, adding phytoestrogens will return with negative effect on the HRT as the study here indicated an increase in most of the genes expression in prostate and breast

cancers, thus adding to the evidence of that adding phytoestrogen has a negative effect on HRT. which is an indication of the bad effect of phytoestrogens and IL33 in combinations(de Lemos, 2001).

Various studies have focused on the effect of single phytoestrogens in isolation and have mostly ignored the effect that these phytochemicals may have in combination with one another. It is important to examine these compounds in suitable combinations, correlated with their origin in the environment, metabolism, and presence *in vivo*. It has been suggested that when environmental oestrogens in general are found in combination with one another, they may either cancel each other out, resulting in no physiological effect a synergistic oestrogenic impact, or a synergistic anti-oestrogenic effect on oestrogen-sensitive pathways (Willard & Frawley, 1998). After studying the effect of individual phytoestrogens to establish effective concentrations in experiment model, a further investigations applied to study the impact of these PEs in combination. Phytoestrogens, when combined, lost their inhibitory effect on cell viability for both cell lines. Cell numbers increased and no synergistic effect was observed. This suggests the antagonist behaviour of each phytoestrogen, and could be a mechanism of ER-dependent and independent antagonistic interactions, including kinase activation, that work against each other and cause loss of growth inhibition. The current results both differ from, and agree , the small number of studies that have examined combinations (Dong et al., 2013; Kumar et al., 2011; Willard & Frawley, 1998). It has been suggested that a combination of phytoestrogens had no stimulatory effect at all, or may be antagonistic, depending on the precise combination of PEs (Mousavi & Adlercreutz, 1992; Willard & Frawley, 1998) and these findings concur with the current study.

When studying the motility and migration in both prostate and breast cancer cells, the effect of phytoestrogens on PC3 cells' rate of closure was found to be minimal and only daidzein showed an enhancement of the closure rate. On the other hand, in MCF7 cells, genistein and daidzein displayed no effect on motility rate, while coumestrol decreased the scratch width at in the early stages. Moreira et al. noticed a decrease in the proliferation rate of breast cancer cells and motility when phytoestrogens were used. This is in contrast to other current findings i.e., that for most phytoestrogens there was no effect (Moreira et al., 2012). Genistein and other phytochemicals inhibited the proliferation of prostate cancer cells and also tumour growth in mice, via modulation of Gli1 expression. It was also shown that genistein inhibited Hedgehog (Hh) signalling in prostate cancer cells, thus suppressing their stem cell properties and reducing cell motility by maintaining epithelial properties. It has been found also that daidzein metabolites, such as equol, were significantly more active than daidzein, implying that there was a similarly enhanced effect of daidzein and its metabolites on regulation of *in vivo* invasion and migration in breast tumours (Bao et al., 2014). This may be an area for further exploration. Also, both R- and S- equol enantiomers were found to inhibit the growth of breast cancer cell line MDA-MB-231 and the prostate cancer cell lines LNCap, and to inhibit the invasion of MDA-MB-231 and PC3 cancer cells through matrigel. Furthermore, S-equol was unable to prevent DNA damage in MCF7 cells but R- eqoul was protective in the same cell line, suggesting a variation in the chemoprotective properties of equol *in vivo* (Magee et al., 2006).

The data from the current study clearly shows differences between the effects of individual phytoestrogens on prostate and prostate cell viability and motility. While individual PEs significantly reduced cell numbers, this reduction was lost

when they were in combination. As a result, these observations add more indefinite characteristics to PE and their use in prostate and breast cancer patients. Hence, further and deeper investigations are required to determine the precise clinical use of PE.

After studying the effect of PEs on prostate and breast cancer viability and motility, there followed a further investigation of their effect on gene expression levels. The genes that were studied have important roles in promoting cell migration, EMT process, osteomimicry and bone vicious cycles. Two of the PEs that had been shown previously to affect a significant reduction in cell numbers were deployed to study the effect of these phytoestrogens on the genes responsible for cell migration, epithelial-mesenchymal transition EMT and metastasis such as in snail, CXCR4 and integrin α5β. Genistein and coumestrol displayed no significant effect, the only effect was that of Gen 10^{-5}M on the PTHrP gene expression in breast cancer cells after 72 hours, suggesting no roles for these phytoestrogens on bone vicious cycle and thus more TGF-β and osteoclast differentiation and more osteolysis.

Runx2 expression was studied as a marker of osteomimicry in prostate cancers. The results indicated a favourable impact of phytoestrogens on genes related to metastasis EMT and osteomimicry. The results also indicated that the phytoestrogens under study (genistein, daidzein and coumestrol) resist transformation into mesenchymal stem cells and detachment from the primary site instead of heading to its next target organ. It has also been demonstrated that genistein is capable of inhibiting invasion through a variety of mechanisms in multiple cell types (Pavese, Farmer & Bergan, 2010). The studies here agree with the latest studies that have explained that phytoestrogens, particularly genistein, may have the potential to inhibit cancer metastasis by particularly

adjusting the EMT process via diverse signaling pathways and their effect on gene expression under study in prostate and breast cancer has been demonstrated (Lee, Hwang & Choi, 2016). It can be concluded that there was a clear and important effect of the phytoestrogens under investigation on the gene expression in prostate and breast cancer cells. As discussed earlier, individual phytoestrogens affect cell viability that may involve both ERα and ERβ dependent and independent signaling pathways. These signaling routes may apply to the same effect of phytoestrogens on genes that consider very important and associated with vital cell pathology and morphology like cell migration, EMT process, osteoclast differentiation, vicious cycle and osteolysis through the same signaling pathways. Therefore, further and deeper studies are needed in order to investigate phytoestrogens, possible active receptors or channels, their connection to gene expression and their effect on cell pathological activity.

The study tried to model the environment that prostate and breast cancer cells may face in bone. To investigate this, a three cytokines: TGF-β, BMP7 and IL-33 has been used. In prostate cancer TGF-β appear to abolish the activity of phytoestrogens, decrease the gene expression of Runx2 and Osx, and increase collagen type I expression. This suggests that TGF-β promotes a late stage of osteomimicry in prostate cancer in the presence of phytoestrogens, compared to the same phytoestrogens when studied individually. In breast cancer, TGF-β significantly decreased the expression of PTHrP, collagen type I and snail, which suggests that TGF-β causes less EMT, reduced vicious cycles and osteomimicry, and plays a protective role, which is opposite to its role as a promoter of tumourigenesis in breast cancer. In conclusion, TGF-β can have strong tumour suppressive properties in the earlier stages of the disease, while

having a tumour-promoting effect at more advanced stages. Thus, treatments that target TGF-β too early in the disease process may be detrimental, and the timing of certain therapies needs careful consideration. Thus, phytoestrogens may play a dual role in prostate and breast cancer in the presence of TGF-β (Principe et al., 2014).

The results show that BMP7 in prostate cancer enhances the progression of the disease progression through interference with the action of phytoestrogens by increasing the expression of Runx2 and integrin α5β3. In breast cancer cells, BMP7 showed an increase in snail and integrin α5β3 expression that might suggest an acceleration of tumour burden and of a late stage EMT type response. However, other studies suggest that not all BMPs induce EMT, and some appear to promote MET, reducing the aggressive properties of tumour cells. In murine mammary epithelial cells , BMP-7 was not able to induce EMT, whereas TGF-β could suggest a protective role for BMP7, which is opposite to results suggested here (Zabkiewicz *et al.*, 2017).

In the IL-33 prostate cancer model experiment, the addition of phytoestrogens results in increased gene expression (CXCR4, snail, Runx2 and integrin α5β3), causing IL-33 to prevent or cancel the effect of phytoestrogens when added individually. These results might suggest that IL-33 had a detrimental effect at the early stage of tumours. Furthermore, the IL-33 effect on breast cancer in the presence of phytoestrogens also showed an ablation of their effect on CXCR4 and snail expression, but didn't affect expression of integrin α5β, compared to the individual phytoestrogens. These results in breast cancer may suggest that IL-33 has a detrimental effect by promoting cell migration and metastasis, which increases the tumour burden at the early stages. The data on the role of IL-33 in cancer progression is limited, especially the data concerning the function

mediated in human breast cancer cells (Liu *et al.*, 2014a). For this reason, intensive and detailed studies are recommended for IL-33 involvement in cancer diseases, and efforts need to be made to elucidate its important role in the presence of phytoestrogens. This study contributes to the literature in a small way, but further study will be required if we are to grasp the whole picture.

There were positive and negative aspects to this study. The *in vitro* results add some data to the contemporary studies of phytoestrogens; but, on the negative side, the phytoestrogens were not studied *in vivo* and conjoined with the *in vitro* results to achieve a better understanding of their effect. I hope in future to study further the effect of phytoestrogens on other cancer cell lines and to include *in vivo* studies on animals. There will be a greater focus on the *in vivo* outcomes, and, hopefully, more advanced molecular techniques applied.

In conclusion, this study showed that phytoestrogens are effective when administered individually but lose this effect when they are combined. Also, growth factors should be taken in considerations if recommend phytoestrogens as hormone replacing therapy. Hence, before considering phytoestrogens as a supplement, clinicians must take into consideration the overall profile of phytoestrogens before administration. Furthermore, what applied to the epidemiological effect of phytoestrogens in one area may not apply in others. Hence, administrating phytoestrogens at an early stage might be of more benefit than at a later stage. Finally, intensely focussed studies of phytoestrogens are most certainly required, and must include further agents and factors involved in the progression and pathology of tumours.

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Appendices

Genistein and Daidzein inhibit PC3 cell viability

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Introduction

Epidemiological evidence suggests that dietary phytoestrogen (PE) intake is associated with a reduced risk of prostate cancer and may account for the lower incidence of prostate and breast cancer in Asian populations. Phytoestrogens (PE) are a diverse group of oestrogen-like compounds found in a range of foods. In addition to having oestrogenic actions some also act as reactive oxygen species scavengers and tyrosine kinase inhibitors. They have been shown to have beneficial effects on osteoblast and osteoclast activity and some such as genistein have been trialled in the clinic for the treatment of prostate cancer. Prostate cancer is the second leading cancer-related cause of mortality in American men. Epidemiological studies have shown that the incidence of latent prostatic lesions in men appear to be uniform across Asian and Western countries, but prostate cancer outcome is considerably higher in Western countries (1)

Aims

The aim of this study is to determine the effect of Phytoestrogens (PE) on prostate cancer cell viability and motility.

Methodology

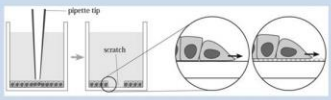
MTS assay

Human prostate cancer PC3 cells were cultured in 96 well plates (greiner bio-one, UK) (1x 10^5 cells per well) in the presence of the phytoestrogens coumestrol, genistein or daidzein (10^{-5} – 10^{-9} M). Viability was assessed using an AQeous non-reactive cell proliferation assay kit according to manufacturer's instructions (Promega, UK). All studies were performed in triplicate and analysed using Statview (Abacus Concepts, USA). Cells were grown in F-12 Nut mix (ham) (1X) (Gibco life technologies , UK). Cells were washed with DPBS (1x) then trypsinized using (1X) versene (Gibco life Technologies, UK). After trypsinization cells were centrifuged using a (Denlen BR401) centrifuge at (1500 rpm) for 5 minutes then counted and diluted. Cells were incubated in the presence of Phytoestrogens for 72 hours before detecting the viability of the cells, 20 µl of medium was removed from each well and replaced with 20 µl of the AQeous non-reactive cell proliferation assay kit (Promega, UK) and the plates incubated at 37^{0}C / 5% CO2 for 1 hour. After 1 hour the plates were read with a microplate reader (Softmax Pro5 software) wave length 490 nm. Data was then analysed using Excel (Microsoft office) and Statview (Abacus Concepts, USA)

Cell Motility Assay

Human prostate cancer PC3 cells after they were washed with DPBS (1X) (Gibco, Life Technologies) and trypsinized with Versene (1X) (Gibco, Life Technologies), were cultured in RPMI 1640 (Gibco, Life Technologies) in 24 well plates (greiner bio-one)(1x10^5 cell per well) for 3 days at 37ºC / 5% CO2. A scratch was then made in the middle of the well (Figure.1) using a yellow pipette tip and the phytoestrogens coumestrol, genistein or daidzein (10^{-5} – 10^{-9} M) added. The separation of the edges of the scratch were then quantified and used as a measure of cell migration. Photographs were take at 0, 6, 12, 24 and 42 hours using a 4X objective lens on an inverted microscope (Motic AE2000) fitted with a digital camera (DCM-510 USB 2.0). Images were analysed by measuring the distance between two edges using ImageJ software (National institutes of Health) and the data analysed using Statview (Abacus Concepts, USA).

Methodology



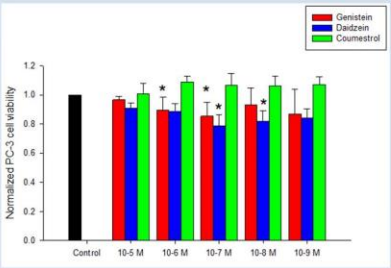
(Figure.1) shows the technique use to make the scratch in the well.

Statistical Analysis
After collecting the data from both MTS and motility assays it was analysed using Statview (Abacus Concepts, USA). A one way ANOVA was used to compare between groups and a P value of <0.05 was considered significant.

Results

Cell Viability

A statistically significant decrease in PC3 cell number was detected following incubation with genistein (10^{-6}, 10^{-7} and 10^{-9} M). Viable cell number was 87%, 82% and 82% of control (Figure.2). Daidzein (10^{-7}, 10^{-8} and 10^{-9} M) also significantly reduced PC3 viability, cell number being 78%, 80% and 83% of control. In contrast coumestrol had no significant effect on viability (Figure.2).



(Figure.2) shows the effect of genistein and daidzein on the viability of PC3 cells. Cell number decreased significantly comparing to the control. On the other hand coumestrol had no effect on PC3 cell viability. *P <0.05

Cell Motility Assay

Motility was investigated for PC3 cells treated with genistein, daidazein and coumestrol for 0, 6, 12, 24 and 42 hours. The observations revealed no significant effect for all the drugs used (Figure.3)

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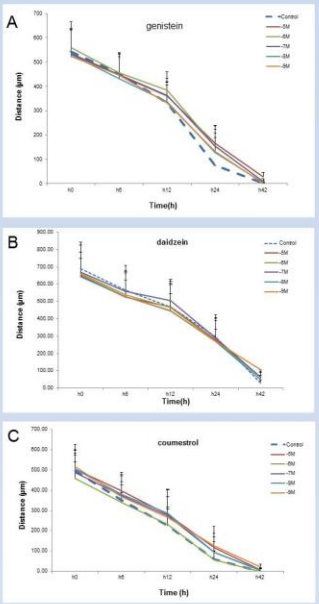
Results



(Figure.3) shows A: genistein, B: daidzein and C: coumestrol.

Discussion

The data indicates that genistein and daidzein reduce PC3 prostate cancer cell viability and provides some evidence to support epidemiologic studies showing a beneficial effect of phytoestrogens on prostate cancer development. The mechanism of action is unknown but could include several potential pathways. Genistein has been shown to antagonize oestrogen receptors and inhibit several tyrosine kinases thought to be involved in the control of cell proliferation by inhibiting PTK mediated signalling. Genistein can also inhibit topoisomerase I and II and protein histidine kinase which may contribute to the anti-proliferative or pro-apoptotic effects. Daidzein and genistein can both influence expression of genes implicated in cell cycle and angiogenesis, such as CDKN1A, a CDK inhibitor involved in the regulation of the cell cycle at both the G0/G1 and G2/M phases. While genistein, daidzein and coumestrol showed non significant effect on cell motility which may need further investigation or longer pre-exposure to reflect prolonged treatment (2).

Conclusion

In conclusion, genistein and daidzein reduced prostate cancer (PC3) cell viability, while coumestrol didn't show any effect. This agrees well with other epidemiologic studies where the mediating effect of phytoestrogens on cancer development and progression has been highlighted. Conversely, cell motility seems not to be affected under the current experiment conditions. This might suggest that longer pre-treatment and/or exposure needs to be applied in further investigations.

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Downregulation of CXCR4 gene expression in breast and prostate cancer cells by phytoestrogens (genistein, daidzein and coumestrol)

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Introduction

Bone is one of the most preferential metastatic target sites for cancers including breast and prostate. Up to 70% of breast or prostate cancer will develop bone metastasis. The consequences of bone metastasis are always devastating. Metastatic process include proliferation and invasion of cancer cells at primary site, intravasation , migration in the circulation and extravasation, arrest in bone marrow, egress from central sinus and attachment to bone surfaces and colonization of cancer cells and bone destruction(Zhang, Ma & Fan, 2010).

Bone metastases may be associated with bone destruction (osteolytic lesions) or alternatively osteosclerosis in which increased osteoblast activity predominates (prostate cancer). Breast cancer patients tend to have a mixed pattern of metastases,40% producing lytic metastases, 40% mixed lytic and sclerotic and 20% sclerotic(Iddon, Byrne & Bundred, 1999).

There is ample evidence that the movement of cancer cells through the body is not random and that different types of cancer cells have different destinations. A specific set of genes that mediate bone metastasis has been described. Cells over-expressing IL-11, MMP-1, connective tissue growth factor (CTGF), CXCR-4 (Fig,1) and osteopontin (OPN), were highly metastatic to the bone in vivo(Sierra, 2005).

Dietary soy has been shown in mice to inhibit prostate tumour growth through inhibition of cell proliferation, increased apoptosis, and reduced microvessel density [3]. Epidemiology studies of Asian women indicate that consumption of a traditional diet high in soy confers significant protection against breast cancer(Hsu et al., 2009).

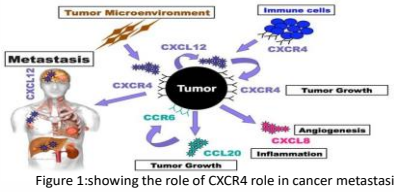

Figure 1:showing the role of CXCR4 role in cancer metastasis

Aim of study

1- see the effect of individual phytoestrogens on breast and prostate cancer viability.
2- see the effective phytoestrogen combinations on cell viability.
3- look at the expression of factors involved in preferential metastasis ,CXCR4, using quantitative real-time PCR.
4- look at the expression of genes involved in osteomimicry or disease progression PTHrP, Runx2 and Collagen I.

Methods

Breast (MCF-7) and prostate (PC3) cancer cells were cultured in 25cc Flask with the phytoestrogens genistein (10^{-5}M and 10^{-6}M) and coumestrol(10^{-5}M, 10^{-6}M and 10^{-7}M) for breast cancer MCF-7 cells and genistein (10^{-6}M and 10^{-7}M) and daidzein (10^{-7}M and 10^{-8}M) for prostate cancer cells (PC3). Each concentration was incubated with the cells individually into two timepoint groups 24 and 72 hours in 37ºC and 5% CO2.

RNA extracted from the cells using GenElute Mammalian total RNA miniprep kit(sigma-aldrich) then cDNA was prepared by M-MLV reverse transcriptase (sigma-aldrich) to be used as a template for quantitation real-time PCR to quantify the CXCR4 gene expression in Breast and prostate cancer treated with phytoestrogens. CXCR4 primers used were Eurofins 5'-GCG CAA GGC CCT CAA GAC CA-3' (Forward) and 5'-GTG CGT GCT GGG CAG AGGTT-3' (Reverse).

Quantitative real-time PCR thermal cycler(Applied Biosystems) used to detect the CXCR4 gene expression in breast and prostate cancer cells using SYBER®Green JumpStart™ Taq ReadyMix™ (Sigma-aldrich) and internal Reference Dye (Sigma-aldrich) in 48 reaction-plate(Applied Biosystems).

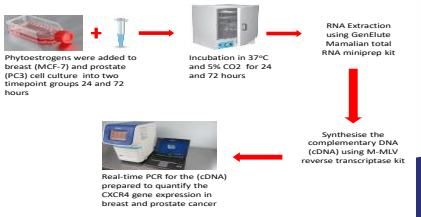

Figure 2: showing the materials and methods used to detect CXCR4 gene expression

Results

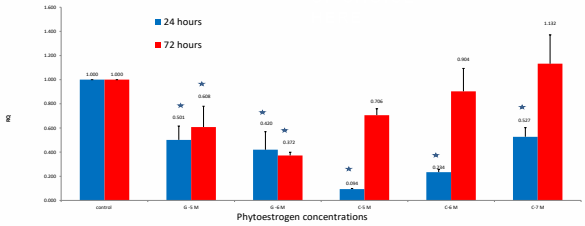

Figure 3:CXCR4 gene expression in MCF-7 cells after treatment with phytoestrogens for 24 and 72 hours showing significant decrease in CXCR4 gene expression for both timepoints 24 and 72 hours but not for coumestrol concentrations after 72 hours. ♦ P<0.05

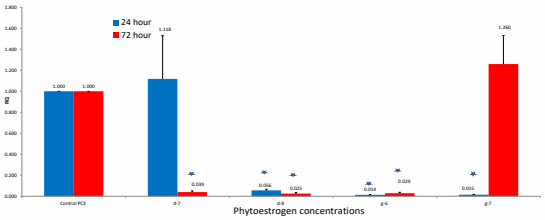

Figure 4: CXCR4 gene expression in PC3 cells after treatment with phytoestrogens for 24 and 72 hour showing significant decrease in CXCR4 gene expression for both timepoints 24 and 72 hours . ♦P<0.05

Effect of phytoestrogens genistein and daidzein on Runx2, Snail and collagen I genes expression in prostate cancer (PC3)

		24h				72 h			
	control	genistein		daidzein		genistein		daidzein	
		10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M	10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M
Runx2	1.00	0.115	0.204	0.139	0.176	1.462	2.819	1.343	1.790
Snail	1.00	0.296	0.252	1.165	0.251	0.360	0.018	0.270	0.178
Collagen I	1.00	0.105	0.197	2.279	0.035	0.131	1.424	0.067	0.086

Effect of phytoestrogens genistein and coumestrol on PTHrP, Snail and collagen I genes expression in prostate cancer (MCF-7)

		24h				72 h			
	control	coumestrol		genistein		coumestrol		genistein	
		10^{-5}M	10^{-6}M	10^{-5}M	10^{-6}M	10^{-5}M	10^{-6}M	10^{-5}M	10^{-6}M
PTHrP	1.00	0.600	1.026	1.802	1.790	1.256	0.777	0.942	1.267	0.113	0.669
Snail	1.00	0.461	1.249	3.050	4.954	3.287	0.852	0.976	1.260	0.343	0.197
Collagen I	1.00	0.190	0.064	0.000	0.000	0.123	0.068	0.159	0.138	0.093	0.119

Figure 5: showing the effect of phytoestrogens on Runx2 and Collagen I genes expression in prostate cancer PC3 cells with PTHrP and Collagen I genes expression in breast cancer (MCF-7) cells

Conclusions

- **In the whole these effects may reduce preferential metastasis and could lead to decreased tumour burden and morbidity as a consequence.**
- **More investigations are required to further understand the functional importance of these and other genes to support the potential therapeutic usage of phytochemicals in breast and prostate cancer.**
- **Looking further into the role of other factors enhance these factors such as growth factors and the role of phytoestrogens on this enhancement.**

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