Determining the 'in vitro' effect of *Padina pavonica* on the Oestrogen Receptor and Oestrogen Responsive Primary Cell Lines

Sarah Sultana Grixti

A thesis submitted for the degree of Masters of Reproductive Health in the Faculty of Medicine and Surgery at the University of Malta
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knowledge and belief, it contains no material previously published or written by
another person nor material which to a substantial extent has been accepted for the
award of any other degree or diploma of the university or other institute of higher
learning, except where due acknowledgement has been made in text."

Sarah Sultana Grixti

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Abbreviations

A1B1: Amplified in Breast cancer-1
AF-1: Activation Function-1
ALP: Alkaline phosphatase
Anti-ER: Anti-Oestrogen Receptor Antibody
BMD: Bone Mineral Density
BMS: British Menopause Society
CBP: CREB-binding protein
c-DNA: complementary-Deoxyribonucleic Acid
DMSO: Dimethyl Sulfoxide
DNA: Deoxyribonucleic acid
DRESS: Drug Rash with Eosinophilia and Systemic Symptoms
DXA: Dual-energy X-ray Absorptiometry
E2: Oestradiol
EGFR: Epidermal Growth Factor Receptor
EMAS: European Menopause and Andropause Society
EPP: Extract of Padina pavonica
ER: Oestrogen Receptor
ERE: Oestrogen Responsive Elements
ET: Oestrogen Therapy
EU: European Union
Exp: Experiment
FAK: Focal Adhesion Kinase
FBS: Fetal Bovine Serum
FRAX ®: Fracture Risk Assessment
FSH: Follicle Stimulating Hormone
GABA: Gamma-Amino Butyric Acid
GPR30: G-protein coupled receptor 30
HRT: Hormone Replacement Therapy
HSP: Heat-Shock Protein
IGF: Insulin-like Growth Factor
IL: Interleukin
ITAM: Immune Receptor Tyrosine-based Activation Motif
LBD: Ligand-Binding Domain
LXR: Liver X Receptor
MAPK: Mitogen-Activated Protein Kinase
M-CSF: Macrophage Colony Stimulating Factor
MSI: Micro-Satellite Instability
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS: Phosphate-Buffered Saline
PEST: Penicillin and streptomycin (PEST)
pNPP: p-Nitrophenyl Phosphate
PTEN: Phosphate and Tensin Homolog
qPCR: quantitative Polymerase Chain Reaction
Ral: Raloxifene
RANK-L: Receptor Activator for Nuclear factor κ β Ligand
RNA: Ribonucleic Acid
SEM: Standard Error of the Mean
SERM: Selective Oestrogen Receptor Modulator
SHP: Small Heterodimeric Partner
Src: Sclerostin
SRC-1: Steroid Receptor Co-activator 1
ST Dev: Standard Deviation
TFT: Thyroid Function Test
TNF: Tumour Necrosis Factor
TSEC: Tissue Selective Oestrogen Receptor Complex
UPR: UPR-m-TOR-autophagy
VTE: Venous Thrombo-embolism
WHI: Women Health Initiative Study
WHO: World Health Organisation
α-MEM: Alpha Modification of Minimum Essential Medium
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Dedication

Dedicated to my loving husband Daniel, our little one on the way, and my parents - for they have always believed in me and encouraged me to pursue new achievements.
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“Science is but a perversion of itself unless it has as its ultimate goal the betterment of humanity”

- Nikola Tesla
Abstract

Background: Padina pavonica appears to improve the bone mineral density at the lumbar spine and at the hip in post-menopausal women (1). The aims of this project are to compare it to other treatments available on the market, for the treatment of post-menopausal osteoporosis and to shed more light on the mechanism of action of Padina pavonica.

Aims and Objectives: The aim of this research-based experiment is to compare the ability of osteoblasts treated with the extract of Padina pavonica (EPP) to differentiate and fix calcium, with osteoblasts treated with raloxifene and oestradiol. Raloxifene, a selective oestrogen receptor modulator (SERM) and oestradiol in the form of hormone replacement therapy (HRT) are drugs normally used in the management of post-menopausal osteoporosis. A secondary aim is to determine whether the extract exerts its action by modulating the oestrogen receptor. This would imply that the extract of Padina pavonica has a SERM-like activity and is thus potentially prone to the same adverse effects, as other members of this class.

Methodology: This is a research-based experiment in which primary osteoblasts were isolated and cultured from human bone explants. Primary cells obtained using this method were used to objectively assess the extract’s effect on the differentiation of progenitor bone cells to terminally mature osteoblasts. Results were compared to bone cells treated with raloxifene and oestradiol. Alkaline phosphatase activity was measured as an early marker of osteoblast differentiation using spectrophotometry.
An MTT assay was employed as a measure and marker of cellular viability and proliferation. These tests were performed after seven days of incubation. The alkaline phosphatase:MTT ratio was calculated and used as another end point in order to reflect whether any increases in alkaline phosphatase were due to an increase in cell mass or whether this was due to the formation of more terminally differentiated osteoblasts. Cells were also incubated with the drugs for fifteen days. The Alizarin Red assay was then performed. The latter was employed as measure of calcium fixation and bone matrix mineralization. Oestrogen receptor monoclonal IgG antibodies were used in order to try and assess whether the drugs' activity was oestrogen receptor dependent or independent. The results was analysed using multiple-linear regression analysis and the Kruskall-Wallis non-parametric test.

Results: Human primary osteoblasts cells can be easily grown in culture and used as a model for the testing of drugs with potential use in the management of post-menopausal osteoporosis. Cells treated with the extract of *Padina pavonica* expressed alkaline phosphatase activity. This was then found not to be statistically different from oestrogen or raloxifene (p-value: 0.501). A statistically significant difference between drugs was noted in the cell viability assay (MTT) (p-value: 0.002). The highest cell viability was noted in the cells treated with oestradiol. There was no statistically significant difference between cells treated with EPP and cells treated with raloxifene on the cell viability assay (p-value: 0.528). The latter was reflected in the alkaline phosphatase:MTT ratio of the differently treated cultures which revealed a statistically significant difference between groups (p-value 0.034).
Cells treated with the extract of *Padina pavonica* were only slightly inferior to raloxifene in terms of osteoblast differentiation as supported by the second highest average estimated marginal means of the alkaline phosphatase to MTT ratio. The different drugs did not show any statistically significant difference in the bone matrix mineralization assay (p-value: 0.548). The oestrogen receptor antibody tests did not reveal any statistically significant results, but suggest a SERM-like activity of EPP.

Conclusions: Our data supports previous studies, which show a potential role for the extract of *Padina pavonica* in the management of post-menopausal osteoporosis. The mechanism of action of this drug remains to be fully understood. This work indicates a possible direct or indirect modulation (by co-factors) of the oestrogen receptors by EPP and suggests a SERM-like activity of this product. Further Quantitative-Polymerase Chain reaction (q-PCR) studies supported and confirmed by Western-blot protein analysis are key steps in understanding the impact of the extract of *Padina pavonica* on the protein component of bone matrix and to be able to relate differential gene expression signatures to established cell signaling in physiological and pathological pathways.
Introduction

Postmenopausal osteoporosis is a common disease which poses a significant impact on patients and health services. Padina pavonica, a macroalga that grows abundantly in the Mediterranean Sea, was noted to have the ability to fix calcium (2) and has been postulated as having a role to play in the matter. Padina pavonica has been used as an extract in many diets, for a long while, with no reported side effects. A study by Galea et al. (2009) has shown that the extract of Padina pavonica led to an improvement of bone mineral density at the lumbar spine and at the hip of postmenopausal women. Unfortunately this study failed to reach statistical significance, most probably due to the small numbers (1). Numerous animal studies have also been performed, from which the Institute of Cellular Pharmacology, the mother company that produces the extract of Padina pavonica claims positive results. Unfortunately much of this work is not published in peer-reviewed journals (3,4). Marine algae have also been noted to have several other beneficial properties including an anti-diabetogenic, cardio-protective and anti-tumorigenic effect (5–9). The latter adds more interest for the use of this product in clinical practice. Osteoporosis is a disease of the elderly and the elderly are at risk of other diseases. Drugs that are able to result in risk reduction of other diseases that become commoner with increasing age would surely be welcome on the market and clinical practice.

Hormone replacement therapy (HRT) was by many measures a wonder drug in the management of post-menopausal osteoporosis. Its effectiveness at improving the symptoms associated with the climacteric and its ability to improve both protein and
mineralization of bone made hormonal therapies key drugs for use in the climacteric. Unfortunately the sudden decrease in the patient and prescriber confidence that arose after the Women’s Health Initiative (WHI) study led to the challenging search for the ‘ideal drug’ in the management of osteoporosis. In order to take advantage of the presumed effects of oestrogen receptor modulation on bone, selective oestrogen receptor modulators (SERMs) were soon proposed for the treatment of post-menopausal osteoporosis. These were thought to replace HRT by being able to provide its advantageous effects on bone whilst not having its feared risks and being able to reduce the incidence of breast cancer. With tamoxifen (the first generation SERM) being implicated in gynaecological cancers, the development of raloxifene (a second generation SERM) was key in the attempt to exploit of the oestrogen like-effects of tamoxifen and raloxifene on bone. The first line treatment for osteoporosis up till today remains bisphosphonates. However apart than having its own set of serious side effects and issues with administration, it is thought to less effectively cater for the proteinaceous aspects of the extracellular bone matrix. Whilst having the ability to increase the bone tissues’ overall strength by mineralization, and resultant improvement in bone mineral density, the bone tissue would still be prone to fracture, as the bone tissues’ ability to cater for tensile stress remains weak (10).

Controversy still exists on the postulated mechanisms of the extract. However numerous other marine algal compounds are thought to work like phyto-oestrogens (6). Cassar et al. (2013) have postulated a SERM-like conformation for this extract
Whilst surely a SERM-like activity would be an interesting finding from an osteoporosis point of view, there would indeed be a theoretical risk for side effects as with other SERMs. Further studies assessing the molecule's specific tissue oestrogen agonist and antagonist properties on the various tissues would be required.

A primary aim of this study is to identify the effect of three pharmacological agents (oestradiol, raloxifene and EPP) compared to control in regards to osteoblastic differentiation using alkaline phosphate activity and alkaline phosphatase is to MTT ratio, cell viability using MTT and calcium fixation by means of the Alizarin Red assay. A secondary aim of this study is to assess whether the addition of the oestrogen receptor results in any significant differences in osteoblastic differentiation as measured by the previously discussed end points.

This research thus aims to answer the following questions:

- What is the effect of the extract of *Padina pavonica* on osteoblasts and matrix mineralization? How does this compare to the effects of raloxifene and oestrogen?

- Does the addition of oestrogen receptor antibodies have an effect on the treated cells' activities and mineralization?

Another objective of this research is for it to provide data and results to allow for further and more detailed studies that will eventually produce the necessary confidence to perform bigger trials on patients. Since it is the oestrogen receptor
activation in bone, which results in an improvement of both its mineral and
coteinaceous component, this research focuses on the effects of EPP on the oestrogen
receptors. This research aims to address this by comparing EPP’s action to drugs,
which modulate the oestrogen receptors such as oestradiol and raloxifene, and by
culturing primary osteoblast cells with EPP and anti-oestrogen receptor antibodies.
Osteoblasts will be used in this research as oestrogen responsive cell lines.

Soon after a short introduction to cell cultures and bone cell physiology, in which
central terms will be explained, this thesis will include a literature review on
menopause, osteoporosis, the role of marine algae, the molecular mechanisms of
oestrogens, SERMs and endogenous oxysterols and their role in gynaecological
carcinogenesis. A discussion on the ethics, statistical advice and methodology
employed in this dissertation will then ensue. The review of results will be followed
by a discussion and the conclusions of the work performed. Other relevant
information pertaining to this study may be found in the appendix section.
**Cell Cultures**

Individual cells from plant and animal tissues can be made to continue to grow, even in the artificial laboratory environment. The latter is made possible by the addition of the correct nutrients and right conditions. This process is called an *in vitro* process and is different to experiments and processes involving living organisms, including humans, which would otherwise be referred to as *in vivo* processes or experiments. This experiment is an example of animal cell culture.

In the cell culture process, single cells act like independent functional units similar to unicellular organisms. The cells may be isolated from tissue by enzymatic or mechanical methods. They may also be derived from previously established cell strain. The cells’ growth is then dependent on the culture variables such as temperature, pH and available nutrients. Cultures usually would contain a single type of cells but mixed cultures can also be created (12).

**What are Primary Cell Cultures?**

Primary culture is the phase of the cell culture process in which cells are isolated from the index tissue and grown to fill all the available space in the substrate medium. Confluence is the term used to define the state at which all available space in the substrate is occupied. At this stage there is no space for the cells to grow further and thus the cells need to be transferred to a new flask with fresh medium. This provides the cells with the necessary space for further growth. This latter process is also
referred to as a subculture, passaging or splitting. A sub-population of cells can be selected in favour of another, by for example cloning, to become a cell strain.

Soon after the first passage, the primary culture is more appropriately referred to as a cell line or a ‘subclone’. Normal cells lose their ability to proliferate after a small number of passages due to cellular ageing or senescence. These cell lines are thus often referred to as being ‘finite’. As cells with the highest growth capacity predominate, this generally results in a degree of phenotypic or genetic uniformity in the cell population.

A ‘continuous’ cell line is one that can proliferate indefinitely. These cells are the ‘in vitro’ equivalent of cancer cells and have undergone the process of transformation, which can be chemically or virally induced. Continuous cells lines are not equivalent to stem cells. Even though stem cells are also capable of dividing, these occur as part of the natural development of a multi-cellular organism. The HeLa cell line is an example of an immortal human cell line, which originates from naturally occurring cancers. Continuous cells lines are often easier to use and maintain. Continuous cells lines would also have allowed for repeated analysis to take place on genetically identical cells. However, it is important to appreciate that these cells have acquired a number of genetic mutations, which otherwise would not be present in the parent tissue ‘in vivo’. Since this can change the biology of the cells under study, ‘finite’ primary cells were used in this research.
The Culture Environment

Mammalian cells grow best at a pH of about 7.4 and at a temperature between 36 to 37 degrees Celsius.

There are two systems of growing cells in culture. Some cells, like osteoblasts, are dependent on anchorage systems to grow. They are referred to as 'adherent' cells. The other system for growing cells is more suitable for the growth of cells like the haemotopoietic cells. These are 'non-adherent' and are grown in suspension. The lack of anchorage system results in cells free-floating in media. Media that allows for cell-adhesion and dispersion are used in the growth of adherent cells. This results in the formation of a unicellular monolayer of cells. This makes adherent cells easy to visualize under the microscope allowing for the researcher to easily perform cytological analysis if required.

The culture medium provides the necessary nutrients, growth factors and hormones for cell growth. It also regulates the pH and osmotic pressure of the culture. Tissue extracts and body fluids were originally used for cell culture experiments. Present day experiments employ one of the three basic classes of media:

- Basal Media: The majority of cells grow well in this type of media. This contains amino acids, vitamins and inorganic salts as well as a carbon source such as glucose. These formulations need the addition of serum to them. The Alpha Modification of Minimum Essential Medium with Earle’s balanced
salts also known as (α-MEM) was used in this experiment. It was Harry Eagle who developed Minimum Essential Medium (MEM) (13). It is one of the most commonly used of all synthetic cell culture media. MEM includes a relatively high concentration of amino acids as required by mammalian cells. α-MEM also contains non-essential amino acids, sodium pyruvate and additional vitamins as compared to MEM (Appendix) (14).

- Reduced-Serum Media: These require less amount of serum since they are already enriched with some nutrients and animal derived growth factors.
- Serum-Free Media: This does not require the addition of serum. It is cell type specific and requires a higher degree of reagent purity.

Serum is the chief source of growth and adhesion factors, hormone, lipids, and minerals for cells in basal media. Serum regulates cell membrane permeability and serves as a carrier of lipids, enzymes, micronutrients and trace elements into the cell. Serum can unfortunately also have an effect on growth by stimulating or inhibiting cell growth. Fetal bovine serum (FBS) is one of the most widely used serum-supplements for the ‘in vitro’ cell culture of mammalian cells. It has low levels of antibodies and contains the necessary growth factors required by eukaryotic cells. It is made from bovine fetus blood obtained via a closed system of collection at the slaughterhouse. The blood fraction that remains after natural coagulation of blood is the main constituent of FBS. Any remaining red blood cells are removed by centrifugation. In a similar way to animal testing, there are a number of ethical questions surrounding use of FBS because of the blood collection process. This is
weighed against its use in health research (15,16). This product is of course commercially available and being used so often in cell culture experiments, it is readily available in most cell culture laboratories. Aliquots of FBS were judiciously used in this research to create the ‘complete’ medium.

Applications and Limitations of Cell Cultures

There are a number of applications for cell cultures. In a similar way to our experiment it can be used to test the efficacy of drugs on cells types but other applications would include the:

- Investigation of normal physiology and biochemistry of cells, including but not limited to, experiments that aim to study aspects of cell metabolism
- The study of the generation of artificial tissues; from the combination of various tissue types.
- Large-scale cell cultures are also used in the synthesis of proteins or viral products used in clinical practice. These products are known as 'biologicaal'. One of the first commercial products of cultured animal cells was the polio vaccine, which required living cells. This was in fact a milestone for the use of cell cultures on an industrial level (12).

A main disadvantage of cell culture experiments is that after a period of continuous growth, cell characteristics can change and the cells may exhibit different properties to those of the original parent tissue. In order to try and preserve the characteristics of the tissue from which the cells were attained, primary cells having undergone a relatively small number of splitting processes, ‘passages’, were used in this
experiment. This was indeed possible since bone tissue explants were readily available for osteoblast isolation.

Cell cultures are also at risk of infection and contamination by microorganisms such as bacteria and fungi. The main reason for this is that the growth of animal cell culture, and thus the time taken for incubation of these cells, takes much longer than the time it takes for microorganisms to divide. The history of cell cultures actually dates back to the twentieth century. However, the susceptibility of cell cultures to infection lead to cell culture techniques not becoming a popular laboratory technique until the 1950s. One thus cannot emphasize enough the importance of an aseptic technique when working with cell cultures. The steps for an effective aseptic technique include the use of a sterile work area, such as using a cell culture hood, good personal hygiene, sterile reagents and sterile handling. Antibiotics are sometimes added to culture media in order to prevent infection (12). A combination of Penicillin/Streptomycin was used in this experiment.

**Cell Morphology**

Good cell culture work requires regular review of the cultures by microscopy. Other than allowing for the early detection of contamination, which may otherwise spread to other cultures it may also show signs of cellular strain or deterioration. Such signs would include the presence of granularity around the nucleus, detachment of cells from the substrate and cytoplasmic vacuolation. Common causes for this would be cellular senescence, infection or a need for change of medium.
Mammalian cells also are classified into three basic morphological categories. Osteoblasts are an example of fibroblast-like cells. The other two categories are the epithelial-like and the lymphoblast-like cells (12).
The Osteoblast and Cell Culture

The osteoblast is the cell responsible for the production of bone. It has been defined by histological and morphological criteria. The latter include its cuboidal appearance, its proximity to newly formed bone. It is a differentiated cell and is non-proliferative in its nature (17). The osteoblast originates from mesenchymal stem cells. These are found in large numbers in the periosteum (18).

The Organization, Structure and Function of Bone Cells

A single cell on its own cannot produce functional bone tissue. The functional unit responsible for the latter is in fact called the ‘osteon’. This group of organized osteoblasts is responsible for the production of the functional part of bone also known as the bone matrix. This is entirely extra-cellular and consists of both proteins and minerals. The organic proteinaceous part is primarily collagen type I and is synthesized first with the mineral component being added at a later stage. Non-mineralized bone matrix is known as the osteoid. The protein component of bone matrix has a crucial role to play since it provides the tissue with the property of tensile strength. The mineral component of the bone matrix allows for compressive strength. The protein and mineral component part of bone allow for a tissue that can bend under strain and recover its shape with no damage. Bone fractures happen when the forces that are applied to bone exceed its capacity to behave elastically.
Two types of cells are constantly remodeling bone: osteoblasts and osteoclasts. Osteoblasts have been discussed earlier. They are responsible for the quality of the bone matrix whilst osteoclasts are responsible for bone resorption. Osteocytes are osteoblasts that are buried in matrix. They have a role to play in skeletal maintenance (19).

Local and systemic factors regulate bone homeostasis. Local factors include the autocrine and paracrine factors, produced by bone cells, such as growth factors, cytokines and prostaglandins. Systemic factors include parathyroid hormone, parathyroid-related peptide, calcitonin, vitamin D, glucocorticoids and the sex steroids (20).

Recent work has shown that the importance of bone goes beyond the classical functions of locomotion, support, protection of soft tissues, calcium and phosphate storage and the harboring of bone marrow. Endocrine functions are now being attributed to bone (21). This may partially explain why the maintenance of a healthy skeleton in the post-menopausal period may also lead to an avoidance of disease common with ageing. In fact, the uncarboxylated form of osteocalcin has a much lower affinity for calcium and minerals than its carboxylated form. Acidification of the bone matrix during bone resorption results in this form of osteocalcin being transported to the bloodstream where it reaches other organs. Osteocalcin has been shown to have effects on the pancreas where it acts as a positive regulator of pancreatic insulin secretion. It may also have a potential role in the proliferation of
pancreatic β-cells. Osteocalcin may also stimulate the expression of the adiponectin gene in the adipose layer. This enhances insulin sensitivity in the adipose tissue. The production of the monoamine neurotransmitters in the hippocampus and inhibition of gamma-aminobutyric acid (GABA) production, are also likely to result in an improvement in cognitive skills. The skeletal system has also been noted to have a weighty influence on the immune system, including lymphphoehesis (22–24).

The mechanism of action of oestrogen on bone still elicits many points of controversy and will be explained in detail later on in this thesis. Oestrogen has a pivotal role to play in maintaining bone homeostasis. Oestrogen exerts its effect directly via the oestrogen receptors. Studies have shown that oestrogen may inhibit both osteoblast and osteocyte apoptosis. Oestrogen may also have a direct effect on the longevity of osteoclasts. There is also evidence for a decrease in osteoclast formation, which prevents bone resorption. Osteoblasts and osteocytes are capable of producing osteoclastogenic cytokines such as RANK-L. This cytokine, like many others, allows for the crosstalk between osteoblasts and osteoclasts. Oestrogen seems to decrease the osteoclastogenic cytokine RANKL. It also appears to synthesize osteoprotegrin. Osteoprotegrin is a ‘decoy’ or ‘sink receptor’ of RANK in osteoclasts. By binding to the RANK ligand in osteoclasts it halts the activation of osteoclastogenesis. Other osteoclastogenic factors involved include the interleukins 1, 6, 11, TNF-α, TNF-β and M-CSF (25–27).
Osteoblast and osteoblast-like cultures

Human osteoblast cells were first isolated and cultured from human explanted bone tissue by Mills et al. in 1979 (28). Immortalised continuous cell lines are also available on the market and these usually originate from sarcomas.

The morphological and histological criteria of the typical osteoblast described earlier may not always be applicable to 'in vitro' culture. During 'in vitro' culture proliferation is a necessity and the morphology may be markedly changed. Thus the detection of the osteoblastic phenotype is based on other criteria. One of the most established biochemical markers is alkaline phosphatase. Alkaline phosphatase is also present 'in vivo'.

The osteoblast is the terminally differentiated cell that develops from the periosteal mesenchymal stem cells. The temporal pattern of expression of these biochemical markers represents different stages of differentiation of the culture. The proliferation phase usually coincides with collagen synthesis; this is then followed by the expression of alkaline phosphatase and subsequently osteocalcin secretion as the bone matrix mineralizes (29).

Alkaline Phosphatase (ALP) Assay

The alkaline phosphatase assay is a colorimetric test that is designed to measure the activity of the enzyme alkaline phosphatase in the biological sample. Alkaline
phosphatase is an enzyme that hydrolizes the phosphate esters in an alkaline buffer. It produces an organic radical and an inorganic phosphate. Three main isoforms of this enzyme occur naturally in humans and most other mammals: ALP of intestinal origin, ALP-tissue nonspecific, which is present in liver, bone and kidney, as well as ALP of placental origin. The tissue non-specific form was of particular interest to this study since it is present in bone. Its presence and level of activity was taken as an indication of differentiation of progenitor cells to osteoblasts. It is considered as an early marker of osteoblast differentiation and expression peaks after five to seven days of in vitro culture.

The assay used in this experiment employs p-nitrophenyl phosphate (pNPP) as a phosphatase substrate. The test turns yellow when dephosphorylated by ALP. Colorimetric quantitative analysis should happen at a wavelength of 405nm (30,31).

**Alizarin Red Assay**

Alizarin Red is an organic compound that has been used throughout history as a prominent red dye, principally for the dyeing of textile fabrics. Historically it was obtained from the plants of the madder genus. Cloth dyed with madder root pigment was in fact found in the tomb of the pharaoh Tutankhamun. It became the first natural pigment to be duplicated synthetically.

Its chemical formula is C$_{14}$H$_{8}$O$_{4}$ and it is used in biochemical assays to quantitatively determine by calorimetry the presence of calcium deposition by osteoblasts. It is a
marker of matrix mineralization. Calcium nodules appear as bright red and these may be counted manually using a microscope. Another alternative is to dissolve these nodules with for example dimethyl sulfoxide (DMSO) and perform colorimetric quantitative analysis at wavelengths of 570nm or 590nm. It should not be performed before ten to sixteen days of 'in vitro' culture. A wavelength of 570nm was used in this experiment.

Alizarin Red is commercially available as a water-soluble powder and needs to be reconstituted with distilled water at a concentration of 20mg/ml prior to use. The pH of this solution needs to be adjusted to pH 4.1-4.3 by adding hydrochloric acid or sodium bicarbonate. The solution needs to be filtered and its handling should take place in the dark (32,33).

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay

The MTT assay is a colorimetric assay, which reflects the number of viable cells present in culture. Mitochondrial NAD(P)H-dependent cellular oxidoreductase enzymes are capable of reducing the tetrazolium dye known as MTT to an insoluble compound known as formazan. Tetrazolium dye assays are typically used to measure 'cytotoxicity' implying loss of viable cells or 'cytostatic' activity, which signifies a shift from proliferation to quiescence. MTT is a photosensitive reagent. The reagents and the assays need to be kept and performed in the dark.
MTT is a yellow in colour and when reduced to formazan in turns into a deep purple colour. Formazan needs to be dissolved in DMSO prior to being quantitatively measured with a spectrophotometer at wavelength between 500-700nm. MTT powder is soluble in water and buffer salt solutions, such as PBS. It needs to be reconstituted at a concentration of 5mg/ml for use in the assay (34,35).

**Alkaline Phosphatase:MTT ratio**

The osteoblast is a differentiated cell, and when fully differentiated loses its ability to proliferate. A central assumption of this work is that any drug that encourages osteoblast differentiation will thus result in lower MTT values. It is used in this research as a marker for terminally differentiated osteoblasts and aims to discriminate between changes in alkaline phosphatase activity due to changes in cell numbers or osteoblasts at different stages of differentiation. The alkaline phosphatase to MTT ratio was worked out from the respective calorimetric assays and it aims to show how much alkaline phosphatase is being produced per cell. This research was carried out in biological triplicates. The alkaline phosphatase to MTT ratios were thus obtained by having each of the values from the alkaline phosphatase activity assay divided by the average MTT for that given experiment.
Literature Review

Menopause

Menopause is the term used to define a woman who has stopped having regular menses. It is characterized by the absence of menstruation, a symptom known as amenorrhoea, for more than twelve months, in the absence of other causes such as pregnancy and breast-feeding. It usually happens when a woman is between forty and fifty years of age. The average age is between fifty-one and fifty-two years of age. The physiological process that characterizes menopause sees the termination of menstruation as a reflection of the cessation of ovarian function and the consequent loss of ovarian hormones. Many other causes, including iatrogenic causes exist. Menopausal women are women who have thus lost their functional capacity to reproduce (36).

Premature menopause is usually defined as menopause that occurs before forty years of age. It has more accurately been defined by the British Menopause Society (BMS) as: “... menopause that occurs at an age less than two standard deviations below the mean estimate for the reference population (37).” Premature menopause is also known as premature ovarian failure and can have primary or secondary causes (Table 1).
Table 1: Causes of Premature Menopause (38)

<table>
<thead>
<tr>
<th>Primary Premature Ovarian Failure</th>
<th>Secondary Premature Ovarian Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal abnormalities: including abnormalities of the X-chromosome</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>Auto-immune disease</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>FSH-receptor abnormalities</td>
<td>Bilateral oophorectomy – Surgical menopause</td>
</tr>
<tr>
<td>Enzyme deficiencies leading to inadequate oestrogen synthesis e.g. 18-hydroxylase deficiency</td>
<td>Hysterectomy without oophorectomy</td>
</tr>
<tr>
<td>Galactosaemia</td>
<td>Infections e.g. Tuberculosis and mumps</td>
</tr>
</tbody>
</table>

The climacteric or peri-menopausal period is the term given to the few years surrounding menopause and is characterized by reduced fertility. It may prove to be a difficult time for the patient. Some women may need to present to gynaecology clinics in view of heavy or irregular uterine bleeding. These patients may also experience some menopausal symptoms for example hot flushes (39,40).

Menopause can otherwise be diagnosed clinically. Other than secondary amenorrhea it is characterized by the symptoms shown Table 2.
Table 2: Symptoms of Menopause

<table>
<thead>
<tr>
<th>Vasomotor symptoms:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Hot flushes</td>
</tr>
<tr>
<td>• Night sweats</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vaginal symptoms:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Vaginal dryness</td>
</tr>
<tr>
<td>• Dyspareunia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Urinary symptoms:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Frequency</td>
</tr>
<tr>
<td>• Urgency</td>
</tr>
<tr>
<td>• Incontinence</td>
</tr>
<tr>
<td>• Atrophic cystitis</td>
</tr>
</tbody>
</table>

Other symptoms include:

- Disturbed sleep
- Depression
- Anxiety
- Irritability
- Skin atrophy
- Joint pains

Endocrine assays are generally used to assist one in making the diagnosis of menopause in cases of secondary or premature menopause and when women present with irregular menstrual bleeding in the peri-menopausal period. In menopausal
cases these assays would show raised gonadotrophin levels with normal thyroid function test (TFT) and prolactin. A raised Follicle Stimulating Hormone (FSH) >30mIU/mL with a low oestradiol, confirmed by repeating the assay after three to four weeks is diagnostic of menopause (39–41).

The effects of menopause, including a reduction in bone mass, have been attributed to the relatively dramatic decline in oestrogen levels (42). Untreated premature menopause has been associated with an escalation in the incidence of all cause mortality, osteoporosis, cardiovascular mortality and neurological diseases (38). It is important to emphasize that menopause in itself is not a disease state, but rather, a physiological state. However long-term sex hormone deficiency has been linked and coupled with a number of co-morbidities.

Clinicians and European bodies including the European Menopause and Andropause Society (EMAS) have published on the ‘male menopause’. This is also known as the ‘andropause’ or “Androgen Deficiency of the Aging Male”. This state, which is not recognized by the World Health Organisation (WHO), is characterized by a decrease in the number of Leyding cells leading to a resultant decline in testosterone levels and high gonadotrophin levels. Testosterone has various anabolic functions including bone formation (43,44). Thus, it is postulated that men, in a similar way to women, can suffer from ‘post-andropausal’ symptoms and complications including osteoporosis.
Osteoporosis

Osteoporosis is a disease of both males and females. It has a prevalence of 1:3 in females vs. 1:5 in males. It is in some ways analogous to other 'silent' diseases, like hypertension. Like chronic hypertension it tends to run an asymptomatic course, it is diagnosed using quantitative assessments and its clinical importance lies in the complications that arise when its not treated. It is usually a disease of the elderly, characterized by the gradual and progressive decrease in bone mass and strength (45). Osteoporosis is particularly common in the post-menopausal female. The suggestion that idiopathic osteoporosis in women and menopause were related was first described in 1940. It did not take long, for a specific cause and effect relationship, which saw the loss of 17-β oestradiol linked to bone pathology, to become established (46).

The increased bone fragility makes bone particularly prone to fractures from minimal, if any, trauma. These fractures are thus also notorious as 'fragility' fractures. The prevalence of osteoporosis increases with age, with one in three women over the age of fifty likely to experience a fragility fracture (12). About 50% of these fractures can go undiagnosed, but others can be a catastrophic event in the life of an elderly person. Vertebral fractures are usually asymptomatic but some may lead to chronic pain syndromes. Hip fractures may require surgery and long periods of immobility leading to an increased morbidity and mortality from serious medical events such as deep vein thrombosis, myocardial infarction and sepsis. Most cases of deaths after
hip fractures occur in the three to six months subsequent to the fracture event. About 20-30% appear to be causally related to the fracture event itself (47).

The underlying pathological mechanism of osteoporosis lies in an incongruity between bone reabsorption and bone formation. Hormones namely oestrogen, parathyroid hormone and testosterone are necessary for healthy bone development. Sex hormone binding globulin also plays a supportive role for oestrogen. Receptor Activator for Nuclear factor κ B Ligand (RANK/RANK-L) pathways and Immune Receptor Tyrosine-based Activation Motif (ITAM) pathways are some of the molecular pathways involved in the molecular cross-talk between osteoblasts and osteoclasts. In summary post-menopausal osteoporosis seems to lead to an increase in the number and activity of both pre-osteoblasts and osteoclasts with a resultant and fundamental imbalance in the tight control of osteoclastogenesis (48–52).

The WHO has distinctly defined osteoporosis as: “a disease characterized by low bone mass and micro-architectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture risk (53).” Dual-energy X-ray absorptiometry (DXA) employs small amounts of ionizing radiation in order to measure bone mineral density (BMD). This technique is currently the gold-standard technique used to measure BMD. DXA has its limitations. Osteomalacia will lead to an underestimation of BMD whilst osteoarthritis may result in higher BMD. The machine is very expensive, bulky and employs ionizing radiation (54,55). New innovative techniques, involving ultrasound, could become a useful tool in the
prevention of fractures in the future. Ultrasound techniques have the potential to allow for devices that are cheaper, smaller and more accessible to doctors in primary care. Such techniques would also have the advantage of not posing any risks relating to the use of ionizing radiation. These techniques are unlikely to replace DXA as a diagnostic tool but might have a role to play as a screening tool (56).

Measurements of bone mineral density are used clinically to assess the bone specific fracture risk. It is important to appreciate that the WHO definition of osteoporosis only relates to the bone-specific fracture risk as measured by BMD. These measurements are summarized in Table 3.

Table 3: Measurements of Bone Mineral Density (53)

| T-Score: defined as the number of standard deviations by which the patient’s BMD differs from the mean peak BMD for young normal subjects of the matching gender. |
|---|---|
| • Normal: A BMD less than 1 standard deviation below the young normal mean |
| • Osteopaenia: A BMD between 1 and 2.5 standard deviations below the young normal mean |
| • Osteoporosis: A BMD more than 2.5 standard deviations below the young normal mean |
| • Severe Osteoporosis: A BMD more than 2.5 standard deviations below the young normal mean with fragility fractures |

| Z-score: defined as the number of standard deviations by which the patient’s BMD differs from the mean BMD for subjects with the identical age. |
Falls and a previous history of fragility fracture are highly predictive of future fracture risk (53,57). Risk factors for osteoporosis are shown in Table 4.

Table 4: Risk Factors of Osteoporosis (58–62)

<table>
<thead>
<tr>
<th>Non modifiable risk factors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Age: BMD decreases with age</td>
</tr>
<tr>
<td>• Sex: Females have a smaller bone mass, and loose bone more quickly following menopause</td>
</tr>
<tr>
<td>• Ethnicity: Afro-Caribbean have a higher bone mass and a slower rate of loss</td>
</tr>
<tr>
<td>• Reproductive Factors: A late menopause is associated with a higher BMD.</td>
</tr>
<tr>
<td>• Family history of osteoporosis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modifiable risk factors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Weight</td>
</tr>
<tr>
<td>• Alcohol</td>
</tr>
<tr>
<td>• Smoking</td>
</tr>
<tr>
<td>• Diet</td>
</tr>
</tbody>
</table>

Osteoporosis is not just a *de novo* condition. It could be due to some clinical conditions and drugs. Table 5 summaries the conditions associated with secondary osteoporosis.
### Table 5: Causes of Secondary Osteoporosis (63)

- Anorexia nervosa
- Renal disease
- Long term corticosteroid use (>3 months)
- Chronic liver disease
- Coeliac disease
- Hyperparathyroidism
- Rheumatoid arthritis
- Inflammatory bowel disease
- Vitamin D Deficiency
- Male hypogonadism

Fracture risk calculators, such as the Fracture Risk Assessment (FRAX®) score is a computer based algorithm which has now become validated for use in many countries. The FRAX® score can be used to identify individuals who would thus benefit from DXA scan or calculate the ten-year probability of a major fracture with improved sensitivity when used together with BMD (64,65).

In a recent report, published in 2010, the European Union (EU) has given an overview of its current burden of osteoporosis. Within the EU, in 2010, there were 22,000,000 women and 5,500,000 men living with osteoporosis. The economic burden of incident and previous fragility fractures was estimated at thirty-seven billion euros. The latter has also translated into 1,180,000 quality-adjusted years lost.
in 2010. These costs are expected to increase by 25% in 2025 (66). This data suggests that the development of better, cost-effective treatment for osteoporosis is likely to remain topical in the years to come.

**Interventions in Osteoporosis**

**Global Strategies for the Prevention and Management of Osteoporosis**

Some non-pharmacological interventions have been associated with a decreased fragility fracture risk. Many of these recommendations are now part of a population strategy that aims to decrease the impact of osteoporosis on patients and health service systems. Table 6 summarizes these non-pharmacological interventions:

**Table 6: Non-pharmacological interventions associated with an improvement in BMD (57,67-69)**

<table>
<thead>
<tr>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-intensity strength and low impact weigh bearing exercise</td>
</tr>
<tr>
<td>Calcium (+/- Vitamin D) Supplements</td>
</tr>
<tr>
<td>Smoking cessation</td>
</tr>
<tr>
<td>Reduced alcohol consumption</td>
</tr>
<tr>
<td>Fall prevention programs</td>
</tr>
<tr>
<td>Hip protectors</td>
</tr>
</tbody>
</table>
Pharmacologic interventions for patients at high risk of fragility fractures

Patients at high risk for osteoporotic fractures (T-score < -2.5) would benefit from pharmacological therapy. Table 7 summarizes the drugs currently available for the prevention and treatment of osteoporotic fractures. Table 8 compares the efficacies of the commonest drugs in use.

Table 7: Drugs used in the treatment of Osteoporosis

<table>
<thead>
<tr>
<th>Drug Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium and Vitamin D supplements</td>
</tr>
<tr>
<td>Bisphosphonates e.g. Alendronic acid</td>
</tr>
<tr>
<td>Selective Oestrogen Receptor Modulators (SERMs) e.g. Raloxifene</td>
</tr>
<tr>
<td>Hormone Replacement Therapy (HRT)</td>
</tr>
<tr>
<td>Calcitonin</td>
</tr>
<tr>
<td>Denosumab</td>
</tr>
<tr>
<td>Strontium Renalate</td>
</tr>
<tr>
<td>Teriparatide - Parathyroid Hormone Analogue</td>
</tr>
</tbody>
</table>
Future: Anti-sclerostin, Cathepsin K inhibitors, Tissue Selective Oestrogen Complexes

Figure 1: Milestones in the discovery of anti-osteoporotic therapy (70)

Table 8: Efficacy of various drugs on fracture prevention and BMD gain (71)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Spine BMD gain (%)</th>
<th>Fracture reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisphosphonates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alendronate</td>
<td>5-7</td>
<td>30-45</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>4-6</td>
<td>32-43</td>
</tr>
<tr>
<td>Zoledronate</td>
<td>6-9</td>
<td>70</td>
</tr>
<tr>
<td>Oestrogen</td>
<td>3-5</td>
<td>35</td>
</tr>
<tr>
<td>SERM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raloxifene</td>
<td>1.2-3</td>
<td>30-40</td>
</tr>
<tr>
<td>Denosumab</td>
<td>3-6</td>
<td>55-70</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>1-1.5</td>
<td>20-30</td>
</tr>
<tr>
<td>Anabolic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Teriparatide</td>
<td>10-15</td>
<td>50-65</td>
</tr>
<tr>
<td>Strontium Ranelate</td>
<td>2-4</td>
<td>20-35</td>
</tr>
</tbody>
</table>
Oestrogen Therapy (ET) and Hormone Replacement Therapy (HRT) in Post-Menopausal Osteoporosis

ET/HRT is used in the women experiencing severe post-menopausal symptoms. Both oestrogen only and oestrogen combined with a progestogen, in HRT, are valid options in the prevention and treatment of osteoporosis. Both are capable to normalize bone remodeling. In patients with an intact uterus unopposed oestrogen therapy has been shown to increase the risk of endometrial hyperplasia and cancer. This risk becomes significant after five years of therapy (72). Cyclical progesterone and oestrogen therapy (HRT) rather than ET is advised in order to reduce this risk (73). ET/HRT is usually reserved for short durations in the early post-menopausal period for a maximum of five years. The duration of therapy is limited by the increased breast cancer risk after three to five years of use. This implies that in clinical practice it is usually used at a time when the age associated fragility risk is relatively low (72).

Results from the Women’s Health Initiative Study (WHI) have shown that HRT has been associated with a reduced fragility risk. The bone-mineral density of the subjects taking part in WHI study was however not known, since the latter was a secondary end-point measurement. Thus, the WHI study makes a strong case for the use of HRT in the primary prevention of osteoporosis and osteoporotic fractures. When it comes to the use of HRT in the treatment of osteoporosis, or in patients who have already suffered fragility fractures, results are more limited and results are less conclusive. It is generally assumed that the use of HRT in women with a low BMD
will also result in a decreased fracture risk, in a similar fashion as to women with a normal BMD (74).

Studies have shown that bone quality and overall bone strength, is dependent on multiple factors other than bone mineral density. Such factors include: bone geometry; bone mass distribution and trabecular bone micro-architecture. HRT, through its complex mechanism of action, decreases fracture risk by improving BMD and bone quality (10). Sex hormones exert their mechanism of action by influencing genetic expression. HRT has an effect on the bone matrix by improving the protein and collagen content of bone. The latter is the scaffolding onto which calcium sits. This leads to an improved bone matrix-mineral content ratio (75).

The outcomes of the WHI study on the increased risks of breast cancer, cardiovascular disease, cognitive decline and strokes (74) greatly reduced prescribed and patient confidence with ET/HRT. The Cochrane review on the matter is based on two big studies from the WHI and supports similar findings to the WHI (76). The WHI has been widely criticized for many reasons such as the average of age 63. This is much higher than the age at which ET/HRT is usually started. A recent review has shown that the use of HRT in women under the age of sixty, or within ten years of onset of menopause, has benefits that generally outweigh the risks. Age and time since menopause effect the benefit-risk profile for ET/HRT use (72). Clinical practice guidelines currently recommend that HRT is used for the prevention of fragility fractures in young post-menopausal women, especially if they have
menopausal symptoms. A thorough assessment for cardiovascular risks is recommended especially if the patient is more than 60 years of age and that ET/HRT is used for the shortest possible to control symptoms (63).

Tibolone is a synthetic steroid drug, which has been used as hormone replacement therapy in post-menopausal women. In a similar fashion to HRT it has been shown to be effective at improving bone mineral density as well as vasomotor symptoms. Unfortunately it has also been associated with post-menopausal vaginal bleeding, endometrial cancer, VTEs and breast cancer (77).

**First-Line Drugs in Osteoporosis**

**Calcium and Vitamin D Supplementation:**
Calcium and Vitamin D supplementation have been shown to decrease the risk of hip fractures and other non-vertebral fractures among elderly patients. Vitamin D supplementation has the added benefit of improving muscle strength (78). Bisphosphonates have been linked with hypocalcemia. The different mechanisms of Vitamin D and bisphosphonates make combination therapy an effective method of improving calcium haemostasis and bone mass in patients with osteoporosis (79).
**Bisphosphonates**

Bisphosphonates are inorganic pyrophosphates that have been shown to decrease bone reabsorption by inducing apoptosis in osteoclasts (80). They are the drugs used as first-line management in most patients at high risk of fragility fractures.

Bisphosphonates have been shown to have a beneficial effect on fracture risk and BMD. The residual fracture benefits seem to persist even after treatment is discontinued (81). Bisphosphonates have however notoriously been associated with poor compliance because of their adverse effects and the difficulty with administration of the oral formulations. Patients taking oral bisphosphonates have to remain upright for at least thirty minutes after their administration. This is thought to prevent the upper gastro-intestinal upset and inflammation that may lead to ulcers in the oesophagus. Oral bisphosphonates have also been associated with an increased risk of Barrett’s oesophagus and an increased risk for oesophageal carcinoma (82,83).

High dose intra-venous bisphosphonates have been linked with a risk for osteonecrosis of the jaw. This has been mainly reported in cancer patients (84). Bisphosphonates have recently been associated with atrial fibrillation. Bisphosphonates are thus best avoided in patients at risk of adverse events from atrial fibrillation and should be used with caution in patients with ischaemic heart disease, heart failure and diabetes mellitus (85).
Selective Oestrogen Receptor Modulators (SERMs)

In the chase for the ideal drug in the treatment of osteoporosis, SERMs have surely picked up a lot of interest. Their interesting mechanism of action could in theory allow one to get the positive effects of oestrogen, whilst avoiding unwanted tissue-specific negative effects (86). Different compounds of SERMs have different agonist or antagonist effects on specific tissues. The ratio of co-repressor and co-activator proteins in different cell types and the changes in conformation of the estrogen receptor induced by drug binding are the main contributors to this interesting outcome (87).

Raloxifene can be considered as a first-line option in post-menopausal women with contra-indications to other therapies or if deemed at high risk for breast cancer (77). It has the ideal properties of being an oestrogen agonist on bone tissue, thus decreasing the risk of bone fractures and being and oestrogen antagonistic on breast and endometrial tissue. Raloxifene could in theory decrease the risk of breast cancer in women, who because of their age, are at a higher risk of developing breast cancer. Lasofoxifene and ospemifene are drugs with similar properties. These drugs contrast the SERM tamoxifen that is clinically only used in the management of breast cancer. Tamoxifen is an oestrogen agonist on bone and uterus and an oestrogen antagonist on breast. Tamoxifen can in fact result in vaginal bleeding and an increased endometrial lining, due to endometrial pathology, in the post-menopausal. Pre-menopausal women may still experience bone loss with tamoxifen (86).
Despite these potential advantages, SERMs have been associated with ‘class’ side effects. Patients being treated with SERMs may complain of hot flushes and are increased risk of venous thrombo-embolism (VTE) (86). The risk seems to be at least as high as for patients taking HRT (88). These side effects are likely to be related to the activation and modulation of the oestrogen receptor.

**Other Drugs used in Osteoporosis**

**Denosumab**

Denosumab is a human monoclonal antibody that inhibits RANK-L (Receptor activator of nuclear factor-kappa Ligand). Osteoprotegrin is the physiological inhibitor of RANK-L. Denosumab mimics the action of endogenous osteoprotegrin. RANK-L is one of the most important protein signals for osteoclastogenesis and bone removal. It becomes overexpressed in conditions characterized by bone loss. Denosumab is a fully human monoclonal antibody that specifically inhibits RANK-L. Since RANK-L is also expressed in T-lymphocyte cells it comes to no surprise that the use of denosumab has been associated with a risk of infections, rashes and eczema. Denosumab like bisphosphonates has also been associated with osteonecrosis of the jaw (89–92).

**Strontium Ranelate**

Strontium renalate is a salt of strontium. Strontium is an element very similar to calcium. It is incorporated in bone with ease and without disrupting mineralization.
It is often thought of as having a dual-action since it works by stimulating the calcium sensing receptors leading to differentiation of precursor cells to osteoblasts and it also stimulates osteoblasts to secrete osteoprotegrin. This inhibits mature osteoclast formation (70,93). This results in an increase in bone formation by osteoblasts and a reduction in bone re-absorption from a reduction in the number of osteoclasts. A meta-analysis by Kanis et al (2011) showed a 31% decrease in fractures (94). This treatment is reserved for the treatment of severe osteoporosis since it was noted to increase the risk for mycardial infarction and VTE. Its use in patients with ischamic heart disease (IHD), peripheral artery disease, cerebrovascular disease and uncontrolled hypertension is contra-indicated (95,96). It is also associated with severe allergic reactions including the Drug Rash with Eosinophilia and Systemic Symptoms (DRESS) (97).

**Calcitonin**

Calcitonin is a polypeptide hormone that is produced in humans primarily by the parafollicular cells of the thyroid gland. It is manufactured synthetically by recombinant DNA technology or by chemical peptide synthesis and used in the treatment of hypercalcemia and osteoporosis. Salmon calcitonin closely resembles human calcitonin but is more active. It is rapidly absorbed and eliminated. Calcitonin results in lowering of blood calcium levels by inhibiting calcium absorption from the intestine, inhibiting osteoclastic activity, stimulating osteoblastic activity and inhibiting renal tubular cell re-absorption of calcium. The inhibition of osteoclastic activity and stimulation of osteoblastic activity results in an improvement
in BMD (70,71). Salmon calcitonin has historically been used as an injectable, however an intra-nasal formulation is also available which is associated with less nausea, vomiting and flushing (98,99). As a desirable additional effect, calcitonin has been also noted to decrease the pain associated with fractures (100). Salmon calcitonin has been recently under scrutiny in view of a potential association with liver cancer (101).

**Teriparatide [Human Recombinant Parathyroid Hormone (1-34)]**

Teriparatide is a parathyroid hormone analog and anabolic agent approved for the treatment of osteoporosis. Its intermittent use induces activity within osteoblasts more than it does in osteoclasts. It is generally safe, but common side effects include headache, nausea, dizziness and limb pain. Teriparatide poses a theoretical risk for osteosarcoma and should not be prescribed to patients at risk for osteosarcoma, for example patients suffering from Paget’s disease. Teriparatide is administered as an injectable once daily in the abdomen or thigh. It cannot be prescribed for more than two years since the long-term safety and efficacy of the drug has not been evaluated for longer (70,71,102).

**Drugs in Clinical Development**

Cathepsin K plays a key role in normal bone reabsorption by osteoclasts. Cathepsin K inhibitors, like balicatib and odanacatib are currently being developed for use in
osteoporosis (103). Sclerostin (Src) kinases are a family of tyrosine kinases that play a role in the activity and survival of osteoclasts. Src kinase inhibitors, namely saracatinib and future compounds similar to it, have the potential to become pharmacologic agents used for the treatment of osteoporosis (104). Tissue Selective Oestrogen Receptor Complexes (TSECs) combine a SERM and an oestrogen. The bezadoxifene and conjugated equine oestrogen combination has been shown to treat the vasomotor symptoms associated with menopause and prevent bone loss. No apparent negative side effects on breast and endometrium were noted (105).

The Marine Environment: Full of Unexploited Resources?

Marine Algae and their Potential Uses

Theories about how life originated on earth are vast in number. Abiogenesis is the natural process of life arising from non-living matter. One of the most strongly held notions is the idea that the first living cell arose from the sea. In support of the latter is the fact that our oceans comprise more than ninety percent of the world’s living biomass. In the recent years, marine organisms, have become recognized as prospective reservoirs of potential candidate sources for remarkable molecules. Marine algae have been identified as a major under exploited resource (6,106,107).

Marine macro-algae have been broadly classified in three classes: phaeophyta (brown alga), rhodophyta (red alga) and chlorophyta (green alga). More than 15,000 primary and secondary metabolites have been reported to be of marine algal origin (108).
Compounds originating from marine alga have been shown to display varied biological properties, including but not limited to: anti-inflammatory, anti-mutagenic, anti-tumorigenic, anti-diabetic, anti-hypertensive, anti-coagulant, anti-viral, anti-oxidant, anti-allergic, anti-inflammatory and anti-obesity properties. Other than that, marine algal products are hepatoprotectors and have the ability to inhibit lipoxygenase, aldose reductase and cholinesterases. A number of studies have shown a potential neuro-protective effect; suggesting a potential role for these agents in neurodegenerative diseases such as Alzheimer’s disease (9,106).

Marine algal substances have been in use by human beings for a very long time. They have been used as a food additive and for their pharmacological properties in Eastern countries, Europe and America. Marine algal products can be a source of vitamins, trace-minerals, lipids, amino-acids and dietary fibers. A case can be made for the re-introduction of macro-algal products as part of our diet since there has been an association with an improvement in cardiovascular health and with maintaining a normal body weight (6,106,107).

A main conclusion from epidemiological studies has been that Asian women are less at risk of osteoporosis than Caucasian ladies. A potential reason behind this phenomenon could be a difference in diet. With Asians having a higher intake of algal-derived foodstuffs. Some plants have selective oestrogenic properties and are called phyto-oestrogens, also known as isoflavones. Marine algal may improve BMD and bone strength by targeting both genomic and non-genomic pathways (107).
Padina pavonica and its extract Maltadienol

Padina pavonica is one of the most researched algae in the field of osteoporosis. It is a member of the Pheophyta family in the order of Dictyales. The alga grows abundantly all year round in the southern Mediterranean Sea, at about sixty metres below sea level. In its natural environment the alga has a whitish-crystal appearance. Its morphology has been attributed to the deposition of calcium carbonate outside its body in the form of aragonite crystals. Aragonite crystals are formed under very specific temperature and pressure conditions. Such conditions are not present in the Mediterranean Sea. It is thus thought that the plant produces a substance that fixes calcium. The extract has been shown to be non-mutagenic in rats but more than that this macro-alga, like many others, has been a part of Mediterranean and Asian diets for a very long time (107).

The ability of this alga to fix calcium stirred a lot of interest and led to it being farmed on an industrial level. The past years have seen the development of underwater farms and the creation of industrial units for its extraction. Malta is a small archipelago of islands in the south of the Mediterranean Sea. Padina pavonica grows all year round in its surrounding waters and has proven to be an ideal place for the establishment of underwater farms. In northern areas of the Mediterranean Sea the alga only grows for three to four months a year (2).

The main extract of the macro-alga Padina pavonica has been registered as Maltadienol \( (C_{28}H_{44}O_2) \). It has a symmetrical structure with two six carbon structures
leading to a conformation comparable to a primitive steroidal molecule. The distinctive central symmetry is typical of a number of primeval compounds such as the tetraterpenes and the carotenoids (11).

A number of studies have shown that the Extract of *Padina Pavonica* (EPP) increases the uptake and fixation of calcium by osteoblasts in culture. EPP seems to augment the fixation of calcium by osteoblasts even when cultured in the presence of calcium channel inhibitors like Interleukin-1 (IL-1). Other drugs for example oestradiol, calcitonin, vitamin D and bisphosphonates do not demonstrate any activity in osteoblasts when cultured with calcium channel inhibitors or IL-1. It thus seems that the mechanism of action of the active metabolite in EPP is not related to an effect on the movement of calcium through osteoblast calcium channels. This data suggests that fixation of calcium could also happen in inflammatory conditions (2,107). The mechanism of action of EPP is however still unclear. A study by Cassar et al. suggests that the maltadienol scaffold could be used in order to develop drugs with the potential of modulating the oestrogen receptor. This would make its mechanism of action very similar to SERM-like compounds (11).

The biological activities of *Padina pavonica* studied have been summarized in Table 9:
Table 9: Biological Activities of *Padina pavonica*

| Treatment of osteoporosis and musculoskeletal health | • Increase in bone density noted in a small clinical study with no visible effects on the endometrium and vagina of post-menopausal women (1)  
• Increased calcium fixation in osteoblasts treated with EPP noted ‘in vitro’ (2)  
Some results from animal studies may also be a direct result of improved calcium fixation in animals given EPP  
• Improvement in tendon health of chickens. This resulted in an overall reduction in mortality due to ‘slipped tendons’ (3).  
• Improved egg quality in chickens treated with EPP (4). |
| Anti-diabetogenic effect | An animal study has shown that *P. pavonica* may have this effect by:  
• Preventing B-cell mass in pancreas  
• Improvement of insulin secreting capacity  
• Improvement in insulin sensitivity  
• Enhanced adipose tissue adiponectin mRNA expression |
| Cardioprotective and anti-hyperlipidaemic | • Increased serum adiponectin (109)  
| Enhanced adipose tissue adiponectin mRNA expression  
| Increased serum adiponectin (109) |
| Anti-tumour activity | Cytotoxic effects against the following cell lines was shown in the following in vitro studies:  
| • RPMI-7951 Human melanoma cell lines (110)  
| • HeLa Human Cervix cell lines (111)  
| • MDA-MB-453 Breast cancer cell lines (111)  
| • H460 Lung Carcinoma cell lines (112)  
| • HepG2 Liver Carcinoma cell lines (112)  
| • KB cells (7) |
| Anti-viral | *Herpes simplex* virus  
Hepatitis A virus (113) |
| Anti-bacterial | *Staphylococcus aureus*  
*Salmonella typhimurium* (8) |
| Anti-fungal | *Aspergillus niger* (8) |
| Neuro-protective | Protective against Parkinson’s disease  
Prevents synaptic mitochondrial damage preventing neurodegeneration |
The Clinical Implications from the Mechanisms of Action of Oestrogens and SERMs

As mentioned earlier in this literature review, oestrogen signaling has been implicated in various disease processes. Cessation of ovarian oestrogen production at menopause leads to increased risks for osteoporosis, cardiovascular disease and vasomotor instability. On the other hand therapy involving the oestrogen receptor has been associated with an increased risk for breast and endometrial cancers as well as a risk for thrombo-embolic diseases such as stroke and deep vein thrombosis. This highlights that oestrogen signaling results in different effects on different cells. An understanding of any treatment that may potentially involve oestrogen signaling requires an understanding of the actions of oestrogens and their receptors in normal physiology and in disease (114)

The intricate cellular targets of 17-β oestradiol in bone

TNF-α producing T-cells are a potential target for oestrogen action in bone. It has been postulated that an increase in TNF-α producing T-cells leads to an increase in TNF-α and RANK-L in post-menopausal women RANK-L is a major osteoclast-differentiating factor (115). Whether TNF-α is a direct target of the ER-α action or not is still a matter of controversy.
The role of 17-β oestradiol in osteoblasts has also been a matter of debate. Some ‘in vitro’ studies indicate that 17-β oestradiol increases the pre-osteoblast leading to a larger cells mass committed towards osteoblast differentiation. Other ‘in vitro’ studies have shown no effect or even an inhibiting effect on the osteoblast (114,116–119). In mice a significant increase in the number of osteoblast progenitor cells was observed. The latter appears to be similar to what happens in post-menopausal women (118). There are also multiple studies which shows that 17-β oestradiol leads to an increase in alkaline phosphatase activity and expression of genes that leads to an increased osteoblastic activity such as: IGF-1 and IL-6 and hormone receptors including the progesterone and Vitamin D receptor (117). Activated oestrogen receptor-α (ER-α) is an important inhibitor of Nuclear factor-κB (NF-κB) which in turn inhibits bone formation (120). As described earlier 17-β oestradiol also has the very important role of inducing the expression of osteoprotegrin which inactivates RANK-L inhibiting osteoclastogenesis (121). Sclerostin is another ostoeclastic factor, which is inhibited by oestrogen (122). 17-β oestradiol suppresses osteoblastic and osteocyte apoptosis. This appears to be very important since osteocytes more than osteoblasts are the major source for RANK-L (123,124).

The role of 17-β oestradiol in progenitor osteoblast cells appears to be different to that in the mature differentiated osteoblast cell. Studies have shown that whilst ablation of ER-α in the early osteoblast progenitor cells resulted loss of cortical bone, knockout of ER-α in the differentiated osteoblast did not translate into a loss of bone
mass. A decreased longevity was however noted in the osteoblasts unable to express ER-α (114,125).

The Oestrogen Receptors

Oestrogens have a variety of clinical functions including the regulation of menstrual cycle, reproduction, modulation of bone density, brain function and cholesterol mobilization. There are two main receptors for oestrogen: ER-α and ER-β. These two receptors are both members of the nuclear receptor superfamily of ligand-inducible transcription factors (126-128). ER-α and ER-β have a similar DNA binding domain being about 96% homologous. They also share a moderate sequence identity of their ligand-binding domains (LBDs). Both of these receptors are expressed in bone tissue. ER-α is the commoner receptor of the two and seems to be the most important when it comes to the effects of oestrogens on bone. ER-β is commoner than ER-α in the ovary, lung and prostate. It is the different hormone-independent transcriptional activation function (AF-1) domains of their N-terminus, which characterizes each receptor. ER-α AF1 domain is active in reporter-mediated gene expression whilst a negligible activity is noted with ER-β (129).

Heat-Shock Proteins (HSPs) hold the oestrogen receptor in the ‘off’ state when not being stimulated by the sex hormone. The binding of oestradiol leads to a conformational change in the receptor which results in disaggregation of the HSPs from the receptors. The oestrogen receptors then undergo homo or heterodimerization (129,130). The dimers then translocate to the cell’s nucleus where they
affect the regulatory region of the target genes. This occurs either directly via ‘zinc fingers’ that ‘recognise’ specific oestrogen response elements (ERE) or indirectly by interacting with transcription factors such as NF-κB on DNA (50). Co-regulators, which can be ‘activators or repressors’, result in a large protein complex on the DNA bound receptor. It is this complex that determines whether the target gene is expressed. More than three hundred proteins can act as co-regulator and this helps one understand why the same ligand-receptor complex in the same cell can have different effects on gene transcription (131). Non-transcriptional functions of the ER in the cytoplasm are fast and transient signals that could lead to a convergence of oestrogen signaling pathways (132). These pathways may also be involved in carcinogenesis (133).

**Molecular Pharmacology of Oestrogen Receptors**

With the emergence of SERMs, the true nature and complexity of oestrogen receptor signaling started to unfold. It became obvious that the unassuming belief that binding of an agonist molecule to the oestrogen receptor, resulted in conformational changes compatible with activation, whilst the binding of an antagonist resulted in conformational changes that led to an inhibitory signal, was too simplistic. Studies relating to tamoxifen showed how the perceived ‘anti-oestrogen’ was capable of increasing the bone mineral density of vertebral spines in post-menopausal women and acting as a partial-agonist on the uterine endometrium. It had become clear that its original description of being an ‘anti-oestrogen’ was no longer appropriate. It was hence reclassified as a selective oestrogen receptor modulator (SERM). Data from
studies with SERM compounds soon started revealing how subtle differences in the conformation of the ER-ligands led to different biological activities. This further reinforced the SERM concept and the notion that the corresponding oestrogen receptor activity was determined by the bound ligands.

A change in the expression of the SRC-1 protein in cells results in a different agonist-antagonist profile of tamoxifen. It is the disruption of the transcription co-repressor complex switches tamoxifen from an antagonist to an agonist. Drugs, that manipulate the interaction of the ER with its co-regulators, can be key drugs for the future since they are likely to show an improved safety profile. The identification of the SRC-1 protein is thus likely to be a milestone in the development and use of SERMs in clinical practice (134–136).

In summary, the function of an ER-modulator is dependent on:

- The tissue expression of ER-α and ER-β and the molecule’s binding affinity to the receptors
- The type of co-regulators it engages on the receptor complex and the conformational changes it causes to the ER receptor
- The activity of ER-coregulator complexes as determined by the influence of existing signaling pathways
- The sequence of the ERE
- The transcription factor milieu close to the DNA bound receptor complex (114,130).
Clinical Relevance of Endogenous SERMs

The action of SERMs is indeed very complex. This has steered scientists to a search for endogenous compounds that employ the same mechanism of action of SERM compounds. Umetani et al. (2007) discovered that 27-hydroxycholesterol could modulate the ERs (137,138). 27-hydroxycholesterol is an oxysterol. It is a 'shunt' product of cholesterol metabolism. It has been regarded as a potential key ancestral ER ligand. This has increased the interest on its actions and effects in humans (139). It has been shown to have an ER-antagonist effect on the cardiovascular system by reducing the effectiveness of the cardiovascular protection offered by oestradiol, whilst having an ER agonist action on breast cancer (137). 27-hydroxycholesterol has also been shown to have a negative effect on bone physiology. The latter mechanism is likely to be partially related to the disruption of ER action (140).

Patients who suffer from hypercholesterolaemia and cardiovascular disease are at a higher risk for osteoporotic fractures (141). The use of statins in these patients has been associated with an improvement in bone mineral density and a decreased fracture risk (142–145). The mechanism for the latter is still somewhat controversial. The theory that statins have a direct effect on osteoblast differentiation secondary to their ability to inhibit protein prenylation has fallen out of favour as the drugs undergo significant first pass metabolism in the liver. This implies that statins would be unlikely to reach significant concentration levels to exert this action on bone. It is thus more likely that a reduction in the cholesterol levels itself is the cause for an improved bone mineral density profile (114). Animal studies, supporting the latter,
include murine studies. In these studies mice developed an osteoporotic phenotype when fed a high cholesterol diet. Other than that increases in 27-hydroxycholesterol by injection or by disruption of CYP7B1 locus which codes for the enzyme responsible for the catabolism of 27-hydroxycholesterol resulted in a significantly decreased bone mass. Administration of 17-β oestradiol results in a partial reversal of this effect in the mice whose CYP7B1 gene was knocked out. Mice with high levels of 27-hydroxycholesterol experienced a thinning of cortical bone thickness as compared to mice in which oophorectomy was performed (146). This suggests that the ER is one of the target action of 27-hydroxycholesterol and that post-menopausal women with elevated cholesterol are at increased risk of cortical bone loss and thus have a higher risk for fractures.

The study of 27-hydroxycholesterol has recognized new players in oestrogen signaling within bone tissue. The Liver X Receptors (LXR), LXRα and LXRβ are two genetically discrete members of the nuclear receptor superfamily involved in cholesterol, fatty acid and glucose metabolism. Their transcription is increased in the presence of 27-hydroxycholesterol. This may explain why 17-β oestradiol is incapable of completely reversing the effect of 27-hydroxycholesterol in bone, as its mechanism of action involves more than just the oestrogen receptor (147,148). Treatment of female mice with an LXR agonist resulted in a thinning of trabecular and cortical bone. This result was similar to mice treated with 27-hydroxycholesterol. Both LXR agonists and 27-hydroxycholesterol resulted in an inhibition of osteoblast proliferation, a down-regulation of genes associated with osteoblast differentiation.
such as RUNX2, collagen 1A1 and osteocalcin as well as an inhibition of terminal differentiation and mineral deposition. Gene expression studies confirmed that 27-hydroxycholesterol has an effect on both osteoblasts and osteoclasts (114,149).

Proof that the nuclear receptors LXR and ER have a convergent action to bring about their effect on bone lies in the fact that 27-hydroxycholesterol induces the expression of TNFα and RANK-L. Thus, current data supports the notion that 27-hydroxycholesterol mediates its effect on bone by activating LXR and inhibiting ER and hindering the ability of the ER to completely inhibit the stimulation of LXR (114,150). Other than that, there appears to be a role for the orphan nuclear receptor Small Heterodimeric Partner (SHP). SHP is a repressor of LXR transcription. Animal studies have revealed a decrease in the expression of the latter is induced by 17-β oestradiol, and decreased by 27-hydroxycholesterol. The decreased expression of SHP thus appears to be another mechanism by which 27-hydroxycholesterol results on a negative influence on bone biology (151).

**Inferences from the Molecular Pharmacology of Bone Homeostasis**

It is well known that soon after menopause women generally experience LDL raises and a decrease in HDL. Conclusions from current literature reveal that this is likely to result from an increase in the oxysterol, 27-hydroxycholesterol. This also has a negative effect on bone tissue. Thus, hypercholesteroaemia in the post-menopausal period has a more deleterious effect as compared to the pre-menopausal state. The latter are protected by oestrogens, which increase HDL and suppress LDL. Since
HMG-CoA reductase inhibitors are widely used in the treatment of hypercholesteraemia their use together with lifestyle modification would be expected to result in improved bone health (114).

Oestrogens in the context of HRT exhibit a very favourable anti-resorptive activity in post-menopausal women suffering from osteoporosis. The clinical response of SERMs seems to be somewhat less. Data seems to suggest that oestrogen therapy re-induces the expression SHP in bone and future SERMs may be screened for their activity on SHP expression in bone tissue. Another key factor to determine why SERMs are less clinically effective compared to oestrogen therapy should include an assessment for concomitant treatment with statins (114).

One of the most promising developments, brought about by the study of ER, is the development of Tissue Selective Oestrogen Receptor Complexes (TSECs). These interesting drugs combine a SERM and an oestrogen such as: raloxifene/17-B oestradiol and bazedoxifene/conjugated equine oestrogens. The latter complex has been shown to improve vasomotor symptoms and prevent bone loss without any negative impact on breast or endometrium (105). No progestins would be required since the SERM compound would act as an oestrogen antagonist on the endometrium. When it comes to TSECs it appears to be likely that each SERM/Oestrogen preparation will have its own properties and requires a separate study of its mechanism of action. Their favourable profile is thought to be brought about by the: pharmacology of the constituent SERM/oestrogen preparation, penetration of the
blood brain barriers and the response of the target tissues to oestrogens. There is also literature in favour of the idea that the exposure of a cell to a SERM influences the environment in which oestrogen acts and vice versa. Heterodimerisation of the ER monomers bound by different ligands leading to different gene expression profiles may also play a role (152).
The Molecular Mechanisms of Oestrogens and SERMs in Endometrial Cancer

Endometrial malignancy is the most common gynaecological cancer in European and North American women. It ranks as the seventh most common cause of malignancy in women worldwide (153,154). The incidence of endometrial cancer is increasing and the latter has been correlated with the obesity epidemic (155). In Europe nearly 10,000 women die of endometrial cancer each year (156).

Endometrial carcinoma is generally classified according to the biological and histopathological variables of the tumour. This ‘dualisatic model’ has been commonly accepted to describe the pathogenesis of endometrial cancer. Type I endometrial cancers are the commoner of the two types and account for 80% of cases. These are characteristic of unopposed oestrogen overstimulation or obesity associated hyper-oestrogenic. They tend to be well-differentiated and endometriod in histology. They endometrial tumour cell thus tends to retain a similar morphology to a normal endometrial cell (156). These tumours have a tendency to be low-grade tumours and usually have a favourable prognosis. Type I endometrial tumours are associated with abnormalities in DNA repair genes such as KRAS, PTEN and β-catenin (133). On the other hand type II endometrial cancers are characterised by non-endometriod histology, such as serous adenocarcinoma and are not associated with hyper-oestrogenic states. They are frequently associated with p53 mutations and are more likely to be aggressive and have a greater metastatic potential (157).
In a similar way to other cancers, the progressive accumulation of genetic abnormalities and epigenetic changes leads to a disruption of cellular signaling that leads to abnormal cell proliferation, cell death processes and angiogenesis (133). This results in the transformation of normal endometrium to a cancerous one. Most endometrial carcinomas are type I cancers and thus by definition oestrogen-associated. Insights on the roles for oestrogen and selective oestrogen-receptor in endometrial carcinogenesis remain key to understanding the risk these drugs impose to our patients.

The first reports that post-menopausal women with an intact uterus and having unopposed oestrogen therapy had a 20-35% increased incidence of endometrial cancer were published in 1980 (158). Tamoxifen was introduced for the treatment of breast cancer in postmenopausal women and subsequently used as adjuvant therapy in pre- and post-menopausal women having surgery for breast cancer. It was later in the late 1980s that the first associations between endometrial cancer and tamoxifen use was made (159).

**The Expression of Oestrogen Receptors in the Normal and Pathological Endometrium**

The uterus mainly expresses ER-\(\alpha\). Data from murine studies has shown that ER-\(\beta\) may have an anti-proliferative function and exert a regulatory role on the activity of ER-\(\alpha\). In fact mice with a knocked-out ER-\(\beta\) experienced an exaggerated response to
oestrogen (160,161). ER-α expression was also decreased in grade III endometrial tumours samples as compared to grade I endometrial tumours. ER-β on the other hand was not related to tumour grade. This results in an altered ER-α ER-β ratio in tumour tissue. This supports the idea that an imbalance in ER-α and ER-β ratio may be involved in the pathogenesis and development of endometrial cancer (162,163).

Splicing errors, which result in ER-α and ER-β variants, may also play a role in carcinogenesis. The ER-α exon splice variant (Δ5ERα) is expressed in endometrial carcinomas. This variant leads to hormone-independent activation of transcription giving endometrial carcinomatous cells a proliferative advantage (164,165). An ER-β exon 8 splice variant ER-βcx may have a role in breast cancer progression. It remains controversial whether it has a role, if any, in the development and progression of endometrial carcinoma (166–168).

**SERM-associated Endometrial Cancer**

SERMs are commonly used in the management of osteoporosis and breast cancer. Raloxifene is a second generation SERM and as mentioned earlier in this literature review it is a key drug in the management of osteoporosis. Raloxifene, like the other notorious SERM tamoxifen, is effective at reducing the risk of developing breast cancer. Other than being beneficial in reducing the risk for breast cancer (in women who because of their age are at high risk for this disease) and its agonistic properties on bone, it’s widespread use stems from the fact that unlike tamoxifen it has not been associated with an increase in the incidence of endometrial cancer (169).
Tamoxifen is the most prescribed anti-neoplastic drug worldwide. It is prescribed to patients in the treatment of, or the prevention of ER-α breast cancer in patients who are deemed high risk for breast cancer. It works by blocking signal transduction in breast cancer cells. Tamoxifen use for five years has been noted to significantly reduce the breast cancer relapse rate within a ten-year period. The risk reduction is age dependent with females aged less than sixty having a risk reduction of 37% and patients aged between sixty and seventy years of age, having a risk reduction of 54%. Continuing tamoxifen for a period of 10 years has been shown to reduce breast cancer recurrence even further (170). Tamoxifen has been associated with multiple uterine pathologies including endometrial hyperplasia, endometrial polyps, carcinomas and uterine sarcomas (171). The risk for endometrial cancer is associated with the duration of use and body mass index (172,173). The increased risk for endometrial carcinoma varies across different studies and ranges from a 1.5 to 6.9-fold increase in incidence (174). Breast cancer in itself already imparts a higher risk for endometrial pathology. In fact in a study which assessed patients prior to starting tamoxifen treatment showed a 31.3% increase in endometrial pathology, including endometrial hyperplasia (175,176).

SERM-associated endometrial cancer was previously associated with a favourable prognosis. Unfortunately, quite the contrary was noted on more recent studies (170). The three year endometrial cancer specific survival significantly decreased from 94% to 76% in patients requiring tamoxifen therapy for more than 5 years (172). Long-
term tamoxifen users are also more likely to have stage III and IV cancers. They also have a higher risk of developing malignant mixed mesodermal tumours (177).

**Mechanisms involved in Tamoxifen-Associated Endometrial Cancer**

A number of mechanisms have been postulated in the process of tamoxifen-associated endometrial cancer. The SERM tamoxifen appears to upregulate endometrial signaling pathways involved in cell proliferation such as mitogen-activated protein kinase (MAPK) pathways, c-MYC and insulin-like growth factor (IGF-1) and to promote cytoskeletal remodeling and migration of endometrial cancer cells which encourages invasion of these cells (178). Endometrial cell migration appears to be dependent upon oestrogen signaling and is likely to be mediated by tamoxifen induced phosphorylation of focal adhesion kinase (FAK) (179,180). The oestrogen responsive endometrial stromal cells are able to promote epithelial proliferation through the release of paracrine factors. There is a strong chance that tamoxifen promotes endometrial cancer from its postulated effects on the endometrial stromal cells (181).

The products of metabolism of tamoxifen also have oestrogenic effects and have been postulated to react with proteins or DNA adducts to cause DNA damage. This mechanism is referred to as genotoxicity. However, recent literature does not support the hypothesis that genotoxicity is a major pathway for tamoxifen-induced endometrial cancer. In fact in a study that compared genetic changes in patients receiving long-term tamoxifen to non-tamoxifen users the genomic profiles were
indistinguishable. The genomic profiles were however noted to correlate significantly with the endometrial tumour subtype (182).

As mentioned earlier a number of DNA mismatch repair genes have been associated with sporadic Type I endometrial cancer. These include aberrations in the phosphate and tensin homolog (PTEN) gene and other DNA alterations such as increased microsatellite instability (MSI). Patients on tamoxifen appear to have similar rates of genetic mutations as the non-exposed group with endometrial cancer (183–185). It appears that tamoxifen when used long-term may promote carcinogenesis mainly using non-genomic alterations. Tamoxifen may offer endometrial cells with genetic aberrations a growth advantage via epigenetic changes and oestrogenic pathways (186).

The important roles of co-regulators in oestrogen signal transduction have already been outlined elsewhere in this literature review. In the endometrium, tamoxifen recruits the co-activators steroid receptor co-activator (SRC-1), amplified in breast cancer-1 (AIB1) and the CREB-binding protein (CBP) whilst co-suppressors are recruited in breast. This allows tamoxifen to exert its anti-oestrogen activity on the breast, and have an antagonistic effect at the endometrium. Tissue dependent expression of co-regulators is unlikely to wholly explain the differences in oestrogen signal transduction at the different tissues. As mentioned earlier on, molecular mechanisms including the splice variants of the ERα are likely to have an important role to play in carcinogenesis. The mutated ER-α is thought to encourage tamoxifen
mediated endometrial cell growth via the MAPK phosphorylation and Akt pathways (187).

The G-protein coupled receptor 30 (GPR30) is a membrane-bound oestrogen receptor, which has been implicated in the rapid non-genomic effects of oestrogen. The presence of GPR30 has been noted in high-grade and advanced stage tumours and is thus associated with a poor prognosis (188,189). Tamoxifen is an agonist for GPR30. GPR30 activation releases heparin-bound growth factor, which stimulates the human epidermal growth factor receptor (EGFR)/MAPK transduction pathway (188). This phosphorylation pathway brings about stimulatory effects on endometrial cancer cells. The expression of GPR30 correlates well with the time between the introduction of tamoxifen therapy and the development of a pathological endometrium (190).

A review of current available literature has shown that although there is an overlap between tamoxifen and oestradiol-mediated gene expression, tamoxifen has the ability to interact with its own specific genes in endometrial cells. In a study by Tamm-Rosenstien et al. (2013) only 22% of the tamoxifen altered gene expression in endometrial cells overlapped with the genes expression in endometrial cells treated with oestradiol (191).

Another pathway that has been implicated in tamoxifen-associated endometrial cancer is the UPR-mTOR-autophagy (UPR) signaling pathway. Endocrine resistant
breast cancers escape hormonal treatment through upregulation of this pathway. Whilst the pro-survival UPR components such as XBP1 and GRP78 are there to protect healthy cells against oxidative stress, this pathway often remains upregulated in cancer cells. The UPR signaling pathway is highly expressed in endocrine-resistant breast cancer and is likely to be present in endometrial cell after prolonged tamoxifen use. The endoplasmic reticulum stress and UPR pathways are also involved in glucose and lipid metabolism (192,193). Future drugs that block the UPR pathway may thus benefit tamoxifen users directly, by allowing for the treatment of endocrine resistant breast cancer, preventing tamoxifen-induced breast cancer and indirectly through the inhibition of obesity (186).
The ‘Ideal Drug’ for Osteoporosis

Osteoporosis is a big financial burden on national health care systems and imposes a significant degree of morbidity and mortality in our elderly population. The incidence of patients at risk of osteoporotic fractures is likely to increase because of our ageing populations and sedentary lifestyles. These factors seem to be making an already significant health-care related problem an even bigger in the coming future. It thus comes to no surprise that a number of actions have been taken on a global level. Countries have met and discussed population strategies. Funding has also been made available in order to improve our therapeutic options.

A thorough review of the available literature on the treatment of osteoporosis can help one appreciate that there are currently many therapies available for the treatment of this condition. Unfortunately the existing therapeutic options all seem to have issues relating to their administration, efficacy or long-term safety issues. The search for an even better alternative drug in the prevention of osteoporotic fragility fractures and treatment of osteoporosis is still on.

It is clear that modulation of the oestrogen receptors remains a valid option in the management of osteoporosis. However the latter modulation may also result in negative effects as outlined. A thorough understanding of these molecular mechanisms is required when performing research and evaluating molecules that have the potential of modulating the oestrogen receptors.
Methodology

Reagents

The following materials were used: Alpha-modification of Eagle’s medium (α-MEM), Penicillin and streptomycin (PEST), Phosphate-buffered saline (PBS), 10% FBS, Trypsin-EDTA, dimethyl sulfoxide (DMSO), alkaline phosphatase, MTT, Alizarin Red, extract of padina pavonica (EPP), 17-β oestradiol, raloxifene and anti-oestrogen receptor monoclonal antibodies (Anti-ER).

The first part of this research saw the isolation of osteoblasts and the establishment of human primary osteoblast cell lines.

Cell isolation technique

Human non-infected bone specimens were obtained from orthopaedic surgery and transferred in saline solution to the research laboratory. The bone specimens were briefly rinsed in PBS and cut into small pieces (1-2mm in diameter). The cells were rinsed again in α-MEM. Isolated cells were incubated with α-MEM supplemented with FBS and PEST. The medium was left undisturbed for four days before the first medium was changed. Thereafter the medium was changed once every week. When the primary culture had become confluent, the cells were detached using Trypsin/EDTA and pelleted. The cells were then either split into two flasks or used for experimentation. The cells were reconstituted in 10mL of α-MEM and carefully
counted in a haemocytometer to ensure that an equal number of cells are seeded in the various experimental cultures. The latter was repeated as necessary during the course of the research. All of the experiments took place in biological triplicates and the cells were re-suspended and counted for each experiment such that an equal number of cells were seeded in each well.

Comparing the effectiveness of the extract of Padina pavonica (EPP) to raloxifene and oestradiol

In the first part of this experiment the dry power of EPP as provided by Institute of Cellular Pharmacology was dissolved and different concentrations prepared in order to determine which concentration is most efficacious at improving osteoblast differentiation and increasing bone mineralization 'in vitro'. A main aim of this study was to compare the 'in vitro' effect of the extract to key drugs in the management of osteoporosis namely: oestradiol and raloxifene. To this attempt dimethyl sulfoxide (DMSO) was used as a vehicle for the dry extract at a concentration of 2μl/ml of culture medium (0.2%). A large range of concentrations of EPP: 1, 2, 5, 10 and 20ug/Ml of culture medium were prepared. The following concentrations were used for both oestradiol and raloxifene (0.001nM, 0.01nM, 0.1nM, 1nM, 10nM). 6,000 cells/0.5ml/well (96 well plates) in medium were seeded in three different 96 well plates and left to settle for 24 hours. The supernatant was then discarded and the mineralized medium with EPP, oestradiol and raloxifene were added. Alkaline phosphatase activity was tested for the in the 96 well plate after seven days of
incubation. The alkaline phosphatase was stored in the refrigerator and left to settle and reach room temperature before use. The supernatant in the plate was removed and the plate washed twice with PBS. 50μl of alkaline phosphatase was added to each well. The plate was replaced in the incubator for forty-five minutes. Spectroscopy at a wavelength of 405nm was then performed and results noted. MTT was performed on the second 96 well-plate. This was performed in a dimly lit room. 20μl of MTT was placed in each well. The supernatant was not discarded. This was left to incubate for four hours. The supernatant was then discarded and DMSO added to each well. The plate was shaken in a shaker prior to being read in the spectrophotometer at a wavelength of 570nm and results noted. Incubations were continued over fifteen days for the bone mineralization assay. The supernatant was removed and the cells were carefully washed with PBS whilst being careful not to disrupt the cell monolayer. The PBS was aspirated. The cells were then transferred to a fume cupboard where 4% paraldehyde was added to cover the cellular monolayer for thirty minutes. The fixed cells then had the para-aldehyde removed and the cells were washed with distilled water. The distilled water was removed and Alizarin Red was added to cover the monolayer. The cells were incubated with Alizarin Red for forty-five minutes to allow for them to stain. The Alizarin red solution was again aspirated and the cells were washed four times with 1ml distilled water. The distilled water was aspirated and PBS was added. The PBS was again removed and DMSO was used to dissolve the bound stain. Spectroscopy was performed at 570nm and results noted.
Comparing the effectiveness of the extract of Padina pavonica (EPP) to raloxifene and oestradiol when combined with the oestrogen receptor antibodies

In an attempt to assess whether the effect of EPP is oestrogen dependent or independent, cells of equal number were again seeded in three 96 well plates. The supernatant was again discarded. The mineralized medium with one of the drugs (10nM oestrogen or 10nM raloxifene or 10ug/ml EPP) was added. The monoclonal antibodies (1mcg/ml) were added and incubations continued for seven days for the alkaline phosphatase assay and MTT assays. Incubations were continued for fifteen days for the bone mineralization assay. The above calorimetric assays were done and prepared as explained above.
Ethics

The Examination Board is hereby assured that the submitted work conforms to normal ethical practice. The University Research and Ethics Committee (UREC) granted approval for this work. No reference is hereby being made to any patients. A copy of the letter of approval given by UREC can be found in the appendix section within this dissertation.

The chairperson of the Orthopaedics Department at Mater Dei Hospital, Mr. Ray Gatt, also gave the necessary approval for this study. Female patients admitted for elective total knee and total hip replacements at Mater Dei Hospital were recruited in this study. Patients were asked to sign a consent form. A copy of this consent form is available within the appendix section of this dissertation.

Samples from bone specimens that would have been routinely discarded during these surgeries and otherwise discarded as medical waste were obtained and used in this research. Very few patients were recruited and these were selected at random. The data was completely anonymised. The subjects were not directly affected in any way. The surgical operation these patients had was not altered in any way. The participation of subjects who were minor, mentally infirm and not legally competent to consent was beyond the scope of this project. Subjects only participated by giving consent to use otherwise discarded bone specimens. This was thus a one off participation. No further investigations or information was asked from the patient. The patients were not deceived in any way, and participation in this research did not lead to any direct benefits.
Statistical Advice

In this study the effect of several variables namely: the drugs (oestradiol, raloxifene and EPP), their respective doses and the response of the cell cultures to the oestrogen receptor antibody were taken into account. The outcome variables considered were the following: alkaline phosphatase activity, MTT values, Alizarin Red staining and alkaline phosphatase to MTT ratio.

Since some of the variables, could potentially be inter-related, it was deemed best not to look at specifics effects in isolation, but simultaneously take into account the results from other variables. A multiple linear regression allows one to account for these seemingly independent variables even when they may in fact be related. A multiple linear regression was thus employed to determine to which extent is linearly dependent on other variables. Results from the experiment were converted to categorical explanatory binary variables. These were introduced in the statistical package SPSS® version 20. Results will be expressed in a tables and graphs showing 95% confidence interval. Both will be given in 3 decimal places. A p-value of <0.05 was used were applicable.

Multiple linear regression analysis requires that some assumptions are made in order to be employed in the analysis of data. The following assumptions were thus made:

- there was a linear relationship between the study and outcome variables
- the observations of the samples were independent
for each value of the variables under study there was a normal distribution of outcome variables.

the variability of the distribution of the outcome variables was the same for all values under study i.e. the variance was constant.

the study variables could be measured without error (194).

In order to evaluate the suspicion that the action of Anti-ERα or Anti-ERβ modulates the effect of the different drugs, interaction terms were also introduced into the multiple linear regression model.

The Kruskall-Wallis test is a non-parametric test which allows one to test for any differences between groups of results relating to the same drug. Since in this experiment there were only three biological repeats it was best to opt for non-parametric testing when statistical analysis was required to ascertain whether the groups of results originated from the same distribution (194). The statistical package Minitab Express ® was employed.
Results

Human primary osteoblast cells were used in this study as an example of oestrogen responsive cell line.

After the isolation procedure described above the cells were harvested until all the cultures had reached confluence. The primary human osteoblast cells required an average of 43 days to reach confluence and be used for further testing and experimentation.

Figure 2: Human primary osteoblast cells in culture (x10 magnification)
Figure 2 and Figure 3 clearly show that human explants can be successfully used to isolate primary human bone cells in culture to reach confluence. The osteoblast typical morphological appearance is hereby clearly shown. The osteoblasts can be seen to grow out of the small fragments of human bone explants to reach confluence.

As discussed earlier in the methodology, in this study specific end points have been considered in order to determine and gain better insight on the effect and molecular mechanism of action of the extract of *Padina pavonica*. 
The following end points will be discussed:

- Alkaline phosphatase activity
- MTT
- Alkaline phosphatase activity: MTT ratio
- Matrix mineralization assay (Alizarin Red assay)

Each experiment was repeated three times. Hence each experiment took place in biological triplicates. The average, standard deviation, standard error of the mean and 95% confidence intervals were calculated for each end point. Results will be shown in the form of tables and graphs. Since the experiment took place in biological triplicates, the graphs show the average value for each of the end points plotted against the dose and drug for that experiment.

Results comparing the effectiveness of EPP to oestradiol and raloxifene will be shown first. Results from the antibody tests will be presented in the final part of the results section.

The last part of the results section will feature the results from statistical analysis of the raw data.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.955</td>
<td>2.645</td>
<td>2.696</td>
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<td>0.166</td>
<td>0.096</td>
<td>2.577</td>
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<td>3.201</td>
<td>2.597</td>
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<td>0.232</td>
<td>2.291</td>
<td>3.201</td>
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<td>3.446</td>
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<td>0.013</td>
<td>3.400</td>
<td>3.450</td>
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<tr>
<td>EPP 5ug/ml</td>
<td>3.492</td>
<td>3.500</td>
<td>3.500</td>
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<td>0.003</td>
<td>3.492</td>
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</tr>
<tr>
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<td>3.500</td>
<td>3.500</td>
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<td>0.000</td>
<td>3.500</td>
<td>3.500</td>
</tr>
<tr>
<td>EPP 20ug/ml</td>
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<td>3.500</td>
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<td>0.139</td>
<td>0.080</td>
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<td>3.500</td>
<td>3.477</td>
<td>0.040</td>
<td>0.023</td>
<td>3.431</td>
<td>3.522</td>
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<td>E2 1nM</td>
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<td>0.000</td>
<td>0.000</td>
<td>3.500</td>
<td>3.500</td>
</tr>
<tr>
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<td>3.500</td>
<td>3.500</td>
<td>3.500</td>
<td>0.000</td>
<td>0.000</td>
<td>3.500</td>
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<td>3.500</td>
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<td>0.040</td>
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<td>3.414</td>
<td>3.388</td>
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<td>0.069</td>
<td>3.252</td>
<td>3.523</td>
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<td>0.000</td>
<td>0.000</td>
<td>3.500</td>
<td>3.500</td>
</tr>
<tr>
<td>Ral 1nM</td>
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<td>3.500</td>
<td>3.500</td>
<td>3.500</td>
<td>0.000</td>
<td>0.000</td>
<td>3.500</td>
<td>3.500</td>
</tr>
<tr>
<td>Ral 10nM</td>
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<td>3.500</td>
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<td>0.190</td>
<td>0.110</td>
<td>3.175</td>
<td>3.605</td>
</tr>
</tbody>
</table>

EPP = Extract of Padina Pavonica; E2 = Oestradiol; Ral = Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean.
Graph 1: Average Alkaline Phosphatase Activity vs. Drug

Treatment of cells with any drug resulted in an improvement in the average of alkaline phosphatase activity. The extract of *Padina pavonica* requires an ‘in vitro’ concentration of 2ug/ml in order to start having an interesting effect compared to the osteoblasts treated with the complete medium only. The extract of *Padina pavonica* obtained its best response in alkaline phosphatase activity response at concentrations of 10ug/ml and 20ug/ml.

EPP = Extract of *Padina pavonica*; E2 = Oestradiol; Ral = Raloxifene
Table 11: MTT activity vs. Drug

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.962</td>
<td>0.703</td>
<td>0.648</td>
<td>0.771</td>
<td>0.168</td>
<td>0.097</td>
<td>0.581</td>
<td>0.961</td>
</tr>
<tr>
<td>EPP 1ug/ml</td>
<td>0.595</td>
<td>0.585</td>
<td>0.575</td>
<td>0.585</td>
<td>0.010</td>
<td>0.006</td>
<td>0.574</td>
<td>0.596</td>
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<tr>
<td>EPP 2ug/ml</td>
<td>0.646</td>
<td>0.656</td>
<td>0.699</td>
<td>0.667</td>
<td>0.028</td>
<td>0.016</td>
<td>0.635</td>
<td>0.699</td>
</tr>
<tr>
<td>EPP 5ug/ml</td>
<td>0.697</td>
<td>0.678</td>
<td>0.606</td>
<td>0.660</td>
<td>0.048</td>
<td>0.028</td>
<td>0.606</td>
<td>0.715</td>
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<tr>
<td>EPP 10ug/ml</td>
<td>0.735</td>
<td>0.756</td>
<td>0.717</td>
<td>0.736</td>
<td>0.020</td>
<td>0.011</td>
<td>0.736</td>
<td>0.736</td>
</tr>
<tr>
<td>EPP 20ug/ml</td>
<td>0.768</td>
<td>0.613</td>
<td>0.755</td>
<td>0.712</td>
<td>0.086</td>
<td>0.050</td>
<td>0.712</td>
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<tr>
<td>E2 0.001nM</td>
<td>0.863</td>
<td>0.811</td>
<td>0.825</td>
<td>0.833</td>
<td>0.027</td>
<td>0.016</td>
<td>0.803</td>
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<tr>
<td>E2 0.01nM</td>
<td>0.831</td>
<td>0.797</td>
<td>0.754</td>
<td>0.794</td>
<td>0.039</td>
<td>0.022</td>
<td>0.750</td>
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<tr>
<td>E2 0.1nM</td>
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<td>0.008</td>
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<td>E2 10nM</td>
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<td>0.760</td>
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<td>0.020</td>
<td>0.800</td>
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<tr>
<td>Ral 0.001nM</td>
<td>0.748</td>
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<td>0.753</td>
<td>0.005</td>
<td>0.003</td>
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<td>Ral 0.01nM</td>
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<td>0.661</td>
<td>0.668</td>
<td>0.669</td>
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<td>0.005</td>
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<td>Ral 0.1nM</td>
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<td>0.652</td>
<td>0.649</td>
<td>0.018</td>
<td>0.011</td>
<td>0.649</td>
<td>0.649</td>
</tr>
<tr>
<td>Ral 1nM</td>
<td>0.675</td>
<td>0.714</td>
<td>0.627</td>
<td>0.672</td>
<td>0.044</td>
<td>0.025</td>
<td>0.672</td>
<td>0.672</td>
</tr>
<tr>
<td>Ral 10nM</td>
<td>0.720</td>
<td>0.681</td>
<td>0.744</td>
<td>0.715</td>
<td>0.032</td>
<td>0.018</td>
<td>0.679</td>
<td>0.751</td>
</tr>
</tbody>
</table>

EPP = Extract of *Padina Pavonica*; E2 = Oestradiol; Ral = Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean
Graph 2: Average MTT activity vs Drug

The cell numbers appear to decrease as compared to control when the cells are treated with mid-range oestradiol and raloxifene doses. With higher concentrations of oestradiol and raloxifene this effect appears to get somewhat reversed. The extract of Padina pavonica too appears to have an effect on cell numbers leading to a lowering of the MTT assay result. The higher concentrations of the extract resulted in a higher MTT result as compared to mid-range doses.

EPP = Extract of Padina pavonica; E2 = Oestradiol; Ral = Raloxifene
Table 12: Alkaline Phosphatase:MTT ratio vs. Drug

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>Control</td>
<td>3.833</td>
<td>3.431</td>
<td>3.497</td>
<td>3.587</td>
<td>0.216</td>
<td>0.124</td>
<td>3.343</td>
</tr>
<tr>
<td>EPP 1ug/ml</td>
<td>4.169</td>
<td>5.472</td>
<td>4.439</td>
<td>4.693</td>
<td>0.687</td>
<td>0.397</td>
<td>3.916</td>
</tr>
<tr>
<td>EPP 2ug/ml</td>
<td>5.100</td>
<td>5.136</td>
<td>5.166</td>
<td>5.134</td>
<td>0.033</td>
<td>0.019</td>
<td>5.097</td>
</tr>
<tr>
<td>EPP 5ug/ml</td>
<td>5.288</td>
<td>5.300</td>
<td>5.300</td>
<td>5.296</td>
<td>0.007</td>
<td>0.004</td>
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</tr>
<tr>
<td>EPP 10ug/ml</td>
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<td>4.755</td>
<td>4.755</td>
<td>4.755</td>
<td>0.000</td>
<td>0.000</td>
<td>4.755</td>
</tr>
<tr>
<td>EPP 20ug/ml</td>
<td>4.916</td>
<td>4.916</td>
<td>4.916</td>
<td>4.916</td>
<td>0.000</td>
<td>0.000</td>
<td>4.916</td>
</tr>
<tr>
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<td>4.083</td>
<td>4.000</td>
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<td>4.095</td>
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<td>4.213</td>
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<td>4.648</td>
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<td>0.266</td>
<td>0.153</td>
<td>4.441</td>
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</table>

EPP = Extract of Padina pavonica; E2 = Oestradiol; Ral = Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean
Graph 3: Alkaline Phosphatase: MTT vs Drug

The extract of *Padina pavonica* showed a similar dose-dependent effect to osteoblasts treated with oestradiol and raloxifene. The extract of *Padina pavonica* showed a promising second best alkaline phophatase to MTT ratio at a dose of 5ug/ml, after raloxifene at a dose 0.1nM.

EPP = Extract of *Padina pavonica*; E2 = Oestradiol; Ral = Raloxifene
Table 13: Alizarin Red Activity vs Drug

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.260</td>
<td>0.328</td>
<td>0.300</td>
<td>0.296</td>
<td>0.034</td>
<td>0.020</td>
<td>0.257</td>
<td>0.335</td>
</tr>
<tr>
<td>EPP 1ug/ml</td>
<td>0.302</td>
<td>0.329</td>
<td>0.198</td>
<td>0.276</td>
<td>0.069</td>
<td>0.040</td>
<td>0.198</td>
<td>0.355</td>
</tr>
<tr>
<td>EPP 2ug/ml</td>
<td>0.472</td>
<td>0.317</td>
<td>0.286</td>
<td>0.358</td>
<td>0.100</td>
<td>0.058</td>
<td>0.246</td>
<td>0.471</td>
</tr>
<tr>
<td>EPP 5ug/ml</td>
<td>0.393</td>
<td>0.415</td>
<td>0.361</td>
<td>0.390</td>
<td>0.027</td>
<td>0.016</td>
<td>0.359</td>
<td>0.420</td>
</tr>
<tr>
<td>EPP 10ug/ml</td>
<td>0.517</td>
<td>0.385</td>
<td>0.306</td>
<td>0.403</td>
<td>0.107</td>
<td>0.062</td>
<td>0.282</td>
<td>0.523</td>
</tr>
<tr>
<td>EPP 20ug/ml</td>
<td>0.400</td>
<td>0.395</td>
<td>0.271</td>
<td>0.355</td>
<td>0.073</td>
<td>0.042</td>
<td>0.355</td>
<td>0.355</td>
</tr>
<tr>
<td>E2 0.001nM</td>
<td>0.326</td>
<td>0.331</td>
<td>0.315</td>
<td>0.324</td>
<td>0.008</td>
<td>0.005</td>
<td>0.324</td>
<td>0.324</td>
</tr>
<tr>
<td>E2 0.01nM</td>
<td>0.308</td>
<td>0.316</td>
<td>0.342</td>
<td>0.322</td>
<td>0.018</td>
<td>0.010</td>
<td>0.302</td>
<td>0.342</td>
</tr>
<tr>
<td>E2 0.1nM</td>
<td>0.323</td>
<td>0.356</td>
<td>0.324</td>
<td>0.334</td>
<td>0.019</td>
<td>0.011</td>
<td>0.313</td>
<td>0.356</td>
</tr>
<tr>
<td>E2 1nM</td>
<td>0.287</td>
<td>0.335</td>
<td>0.376</td>
<td>0.333</td>
<td>0.045</td>
<td>0.026</td>
<td>0.282</td>
<td>0.383</td>
</tr>
<tr>
<td>E2 10nM</td>
<td>0.273</td>
<td>0.384</td>
<td>0.430</td>
<td>0.362</td>
<td>0.081</td>
<td>0.047</td>
<td>0.362</td>
<td>0.362</td>
</tr>
<tr>
<td>Ral 0.001nM</td>
<td>0.339</td>
<td>0.318</td>
<td>0.372</td>
<td>0.343</td>
<td>0.027</td>
<td>0.016</td>
<td>0.343</td>
<td>0.343</td>
</tr>
<tr>
<td>Ral 0.01nM</td>
<td>0.359</td>
<td>0.341</td>
<td>0.417</td>
<td>0.372</td>
<td>0.040</td>
<td>0.023</td>
<td>0.327</td>
<td>0.417</td>
</tr>
<tr>
<td>Ral 0.1nM</td>
<td>0.342</td>
<td>0.385</td>
<td>0.412</td>
<td>0.380</td>
<td>0.035</td>
<td>0.020</td>
<td>0.340</td>
<td>0.420</td>
</tr>
<tr>
<td>Ral 1nM</td>
<td>0.364</td>
<td>0.367</td>
<td>0.388</td>
<td>0.373</td>
<td>0.013</td>
<td>0.008</td>
<td>0.373</td>
<td>0.373</td>
</tr>
<tr>
<td>Ral 10nM</td>
<td>0.384</td>
<td>0.295</td>
<td>0.387</td>
<td>0.355</td>
<td>0.052</td>
<td>0.030</td>
<td>0.355</td>
<td>0.355</td>
</tr>
</tbody>
</table>

EPP = Extract of Padina pavonica; E2 = Oestradiol; Ral = Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean
The extract of *Padina pavonica* appears to improve bone matrix mineralization compared to control in a similar fashion to oestradiol and raloxifene. The best improvement in bone matrix mineralization seems to have been brought about by the 10ug/ml dose.

EPP = Extract of *Padina pavonica*; E2 = Oestradiol; Ral = Raloxifene
Table 14: Alkaline Phosphatase Activity vs. Drug +/- Anti-Oestrogen Receptor Antibodies

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>EPP</td>
<td>3.160</td>
<td>2.966</td>
<td>3.327</td>
<td><strong>3.151</strong></td>
<td>0.181</td>
<td>0.104</td>
<td>2.947</td>
</tr>
<tr>
<td>EPP + Anti- Erα</td>
<td>2.992</td>
<td>2.484</td>
<td>2.463</td>
<td><strong>2.646</strong></td>
<td>0.300</td>
<td>0.173</td>
<td>2.307</td>
</tr>
<tr>
<td>EPP + Anti-Erβ</td>
<td>2.983</td>
<td>2.927</td>
<td>2.252</td>
<td><strong>2.721</strong></td>
<td>0.407</td>
<td>0.235</td>
<td>2.721</td>
</tr>
<tr>
<td>EPP + Anti-ERα+β</td>
<td>2.480</td>
<td>2.840</td>
<td>2.118</td>
<td><strong>2.479</strong></td>
<td>0.361</td>
<td>0.208</td>
<td>2.479</td>
</tr>
<tr>
<td>E2</td>
<td>2.811</td>
<td>2.974</td>
<td>2.915</td>
<td><strong>2.900</strong></td>
<td>0.083</td>
<td>0.048</td>
<td>2.807</td>
</tr>
<tr>
<td>E2 + Anti-Erα</td>
<td>2.752</td>
<td>3.077</td>
<td>2.729</td>
<td><strong>2.853</strong></td>
<td>0.195</td>
<td>0.112</td>
<td>2.632</td>
</tr>
<tr>
<td>E2 + Anti-Erβ</td>
<td>2.569</td>
<td>2.883</td>
<td>2.735</td>
<td><strong>2.729</strong></td>
<td>0.157</td>
<td>0.091</td>
<td>2.551</td>
</tr>
<tr>
<td>E2 + Anti-ERα+β</td>
<td>2.440</td>
<td>2.716</td>
<td>2.555</td>
<td><strong>2.570</strong></td>
<td>0.139</td>
<td>0.080</td>
<td>2.413</td>
</tr>
<tr>
<td>Ral</td>
<td>2.831</td>
<td>3.180</td>
<td>2.931</td>
<td><strong>2.981</strong></td>
<td>0.180</td>
<td>0.104</td>
<td>2.777</td>
</tr>
<tr>
<td>Ral + Anti-Erα</td>
<td>3.050</td>
<td>2.895</td>
<td>2.625</td>
<td><strong>2.857</strong></td>
<td>0.215</td>
<td>0.124</td>
<td>2.857</td>
</tr>
<tr>
<td>Ral + Anti-Erβ</td>
<td>2.759</td>
<td>2.686</td>
<td>2.929</td>
<td><strong>2.791</strong></td>
<td>0.125</td>
<td>0.072</td>
<td>2.791</td>
</tr>
<tr>
<td>Ral + Anti-ERα+β</td>
<td>2.400</td>
<td>2.823</td>
<td>2.167</td>
<td><strong>2.463</strong></td>
<td>0.333</td>
<td>0.192</td>
<td>2.087</td>
</tr>
<tr>
<td>Control</td>
<td>2.893</td>
<td>3.034</td>
<td>3.117</td>
<td><strong>3.015</strong></td>
<td>0.113</td>
<td>0.065</td>
<td>2.887</td>
</tr>
<tr>
<td>Anti-ERα</td>
<td>2.733</td>
<td>2.943</td>
<td>2.531</td>
<td><strong>2.736</strong></td>
<td>0.206</td>
<td>0.119</td>
<td>2.503</td>
</tr>
<tr>
<td>Anti-ERβ</td>
<td>2.697</td>
<td>2.542</td>
<td>2.596</td>
<td><strong>2.612</strong></td>
<td>0.079</td>
<td>0.045</td>
<td>2.612</td>
</tr>
<tr>
<td>Anti-ERα+β</td>
<td>2.568</td>
<td>2.378</td>
<td>2.413</td>
<td><strong>2.453</strong></td>
<td>0.101</td>
<td>0.058</td>
<td>2.453</td>
</tr>
</tbody>
</table>

EPP = 10μg/ml Extract of Padina pavonica; E2 = 10nM Oestradiol; Ral = 10nMRaloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean; Anti-ERα = Anti-Oestrogen Receptor – alpha; Anti-ERβ = Anti-Oestrogen Receptor - Beta
Graph 5: Alkaline Phosphatase Activity vs. Drug +/- Anti-Oestrogen Receptor Antibody

Incubation with both anti-oestrogen receptors resulted in the biggest decrease in alkaline phosphatase activity in all the three different drugs. This graph seems to suggest a similar yet different mechanism of action of the extract of *Padina Pavonica* to oestradiol and raloxifene. Blockade by the anti-oestrogen receptor alpha resulted in a more prominent decrease in the alkaline phosphatase assay as compared to the other two drugs.

EPP = 10μg/ml Extract of *Padina pavonica*; E2 = 10nM Oestradiol; Ral = 10nM Raloxifene; Anti-ERα = Anti-Oestrogen Receptor – alpha; Anti-ERβ = Anti-Oestrogen Receptor - Beta
Table 15: MTT activity vs. Drug +/- Anti-Oestrogen Receptor Antibodies

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP</td>
<td>0.628</td>
<td>0.662</td>
<td>0.695</td>
<td>0.662</td>
<td>0.034</td>
<td>0.019</td>
<td>0.624</td>
<td>0.700</td>
</tr>
<tr>
<td>EPP + Anti- Era</td>
<td>0.654</td>
<td>0.6</td>
<td>0.641</td>
<td>0.632</td>
<td>0.028</td>
<td>0.016</td>
<td>0.600</td>
<td>0.664</td>
</tr>
<tr>
<td>EPP + Anti-ERβ</td>
<td>0.687</td>
<td>0.687</td>
<td>0.704</td>
<td>0.693</td>
<td>0.010</td>
<td>0.006</td>
<td>0.693</td>
<td>0.693</td>
</tr>
<tr>
<td>EPP + Anti-ERα+β</td>
<td>0.624</td>
<td>0.721</td>
<td>0.778</td>
<td>0.708</td>
<td>0.078</td>
<td>0.045</td>
<td>0.708</td>
<td>0.708</td>
</tr>
<tr>
<td>E2</td>
<td>0.614</td>
<td>0.665</td>
<td>0.761</td>
<td>0.680</td>
<td>0.075</td>
<td>0.043</td>
<td>0.596</td>
<td>0.764</td>
</tr>
<tr>
<td>E2 + Anti-Era</td>
<td>0.649</td>
<td>0.67</td>
<td>0.613</td>
<td>0.644</td>
<td>0.029</td>
<td>0.017</td>
<td>0.611</td>
<td>0.677</td>
</tr>
<tr>
<td>E2 + Anti-ERβ</td>
<td>0.724</td>
<td>0.599</td>
<td>0.617</td>
<td>0.647</td>
<td>0.068</td>
<td>0.039</td>
<td>0.570</td>
<td>0.723</td>
</tr>
<tr>
<td>E2+ Anti-ERα+β</td>
<td>0.747</td>
<td>0.699</td>
<td>0.732</td>
<td>0.726</td>
<td>0.025</td>
<td>0.014</td>
<td>0.698</td>
<td>0.754</td>
</tr>
<tr>
<td>Ral</td>
<td>0.667</td>
<td>0.709</td>
<td>0.688</td>
<td>0.688</td>
<td>0.021</td>
<td>0.012</td>
<td>0.664</td>
<td>0.712</td>
</tr>
<tr>
<td>Ral + Anti-Era</td>
<td>0.596</td>
<td>0.631</td>
<td>0.652</td>
<td>0.626</td>
<td>0.028</td>
<td>0.016</td>
<td>0.626</td>
<td>0.626</td>
</tr>
<tr>
<td>Ral + Anti-ERβ</td>
<td>0.558</td>
<td>0.686</td>
<td>0.729</td>
<td>0.658</td>
<td>0.089</td>
<td>0.051</td>
<td>0.658</td>
<td>0.658</td>
</tr>
<tr>
<td>Ral + Anti-ERα+β</td>
<td>0.634</td>
<td>0.859</td>
<td>0.663</td>
<td>0.719</td>
<td>0.122</td>
<td>0.071</td>
<td>0.580</td>
<td>0.857</td>
</tr>
<tr>
<td>Control</td>
<td>0.786</td>
<td>0.594</td>
<td>0.664</td>
<td>0.684</td>
<td>0.097</td>
<td>0.065</td>
<td>0.571</td>
<td>0.791</td>
</tr>
<tr>
<td>Anti-ERα</td>
<td>0.753</td>
<td>0.679</td>
<td>0.685</td>
<td>0.706</td>
<td>0.041</td>
<td>0.024</td>
<td>0.659</td>
<td>0.752</td>
</tr>
<tr>
<td>Anti-ERβ</td>
<td>0.759</td>
<td>0.729</td>
<td>0.705</td>
<td>0.731</td>
<td>0.027</td>
<td>0.016</td>
<td>0.731</td>
<td>0.731</td>
</tr>
<tr>
<td>Anti-ERα+β</td>
<td>0.714</td>
<td>0.898</td>
<td>0.841</td>
<td>0.818</td>
<td>0.094</td>
<td>0.054</td>
<td>0.818</td>
<td>0.818</td>
</tr>
</tbody>
</table>

EPP = 10μg/ml Extract of Padina pavonica; E2 = 10nM Oestradiol; Ral = 10nM Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean; Anti-ERα = Anti-Oestrogen Receptor - alpha; Anti-ERβ = Anti-Oestrogen Receptor - Beta
Graph 6: MTT vs Drug +/- Oestrogen Receptor Antibodies

Irrespective of drug used, an increase in cell numbers was noted when cultures were treated with both anti-oestrogen receptors. This could signify a lower number of terminally differentiated osteoblasts in culture.

EPP = 10μg/ml Extract of *Padina pavonica*; E2 = 10nM Oestradiol; Ral = 10nM Raloxifene; Anti-ERα = Anti-Oestrogen Receptor - alpha; Anti-ERβ = Anti-Oestrogen Receptor - Beta
Table 16: Alkaline Phosphatase: MTT vs. Drug +/- Anti-Oestrogen Receptor Antibody

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP</td>
<td>4.776</td>
<td>4.483</td>
<td>5.028</td>
<td>4.762</td>
<td>0.273</td>
<td>0.158</td>
<td>4.453 - 5.071</td>
</tr>
<tr>
<td>EPP + Anti-Era</td>
<td>4.737</td>
<td>3.932</td>
<td>3.899</td>
<td>4.189</td>
<td>0.474</td>
<td>0.274</td>
<td>3.653 - 4.726</td>
</tr>
<tr>
<td>EPP + Anti-Erβ</td>
<td>4.307</td>
<td>4.226</td>
<td>3.251</td>
<td>3.928</td>
<td>0.587</td>
<td>0.339</td>
<td>3.928 - 3.928</td>
</tr>
<tr>
<td>EPP + Anti-ERα+β</td>
<td>3.504</td>
<td>4.013</td>
<td>2.993</td>
<td>3.504</td>
<td>0.510</td>
<td>0.295</td>
<td>3.504 - 3.504</td>
</tr>
<tr>
<td>E2</td>
<td>4.134</td>
<td>4.374</td>
<td>4.287</td>
<td>4.265</td>
<td>0.121</td>
<td>0.070</td>
<td>4.127 - 4.402</td>
</tr>
<tr>
<td>E2 + Anti-Era</td>
<td>4.273</td>
<td>4.778</td>
<td>4.238</td>
<td>4.430</td>
<td>0.302</td>
<td>0.174</td>
<td>4.088 - 4.772</td>
</tr>
<tr>
<td>E2 + Anti-Erβ</td>
<td>3.973</td>
<td>4.458</td>
<td>4.229</td>
<td>4.220</td>
<td>0.243</td>
<td>0.140</td>
<td>3.945 - 4.495</td>
</tr>
<tr>
<td>E2 + Anti-ERα+β</td>
<td>3.361</td>
<td>3.741</td>
<td>3.519</td>
<td>3.540</td>
<td>0.191</td>
<td>0.110</td>
<td>3.324 - 3.756</td>
</tr>
<tr>
<td>Ral</td>
<td>4.115</td>
<td>4.622</td>
<td>4.260</td>
<td>4.332</td>
<td>0.261</td>
<td>0.151</td>
<td>4.037 - 4.628</td>
</tr>
<tr>
<td>Ral + Anti-Era</td>
<td>4.870</td>
<td>4.622</td>
<td>4.191</td>
<td>4.561</td>
<td>0.343</td>
<td>0.198</td>
<td>4.561 - 4.561</td>
</tr>
<tr>
<td>Ral + Anti-Erβ</td>
<td>4.195</td>
<td>4.084</td>
<td>4.454</td>
<td>4.244</td>
<td>0.190</td>
<td>0.109</td>
<td>4.244 - 4.244</td>
</tr>
<tr>
<td>Ral + Anti-ERα+β</td>
<td>3.340</td>
<td>3.928</td>
<td>3.015</td>
<td>3.428</td>
<td>0.463</td>
<td>0.267</td>
<td>2.904 - 3.951</td>
</tr>
<tr>
<td>Control</td>
<td>4.246</td>
<td>4.453</td>
<td>4.575</td>
<td>4.425</td>
<td>0.166</td>
<td>0.096</td>
<td>4.237 - 4.613</td>
</tr>
<tr>
<td>Anti-ERα</td>
<td>3.873</td>
<td>4.171</td>
<td>3.587</td>
<td>3.877</td>
<td>0.292</td>
<td>0.169</td>
<td>3.546 - 4.207</td>
</tr>
<tr>
<td>Anti-ERβ</td>
<td>3.689</td>
<td>3.477</td>
<td>3.551</td>
<td>3.573</td>
<td>0.108</td>
<td>0.062</td>
<td>3.573 - 3.573</td>
</tr>
<tr>
<td>Anti-ERα+β</td>
<td>3.141</td>
<td>2.908</td>
<td>2.951</td>
<td>3.000</td>
<td>0.124</td>
<td>0.071</td>
<td>3.000 - 3.000</td>
</tr>
</tbody>
</table>

EPP = 10ug/ml Extract of Padina pavonica; E2 = 10nM Oestradiol; Ral = 10nM Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean; Anti-ERα = Anti-Oestrogen Receptor – alpha; Anti-ERβ = Anti-Oestrogen Receptor - Beta
Graph 7: Average Alkaline Phosphatase:MTT vs Drug +/- Oestrogen Receptor Antibodies

As expected blockade with both anti-oestrogen receptors resulted in the biggest effect on the Alkaline Phosphatase:MTT ratio of cells treated with oestrogen and raloxifene. A similar effect was noted in the cells treated with the extract of *Padina pavonica*. This may be due to a decrease in the formation of terminally differentiated osteoblasts and may imply a SERM-like action for the extract.

EPP = 10ug/ml Extract of *Padina pavonica*; E2 = 10nM Oestradiol; Ral = 10nM Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean; Anti-ERα = Anti-Oestrogen Receptor – alpha; Anti-ERβ = Anti-Oestrogen Receptor - Beta
Table 17: Average Alizarin Red Activity vs. Drug +/- Anti-Oestrogen Receptor Antibody

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
<th>Average</th>
<th>ST Dev</th>
<th>SEM</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
</tr>
<tr>
<td>EPP</td>
<td>0.229</td>
<td>0.306</td>
<td>0.190</td>
<td>0.242</td>
<td>0.059</td>
<td>0.034</td>
<td>0.175 - 0.308</td>
</tr>
<tr>
<td>EPP + Anti-Era</td>
<td>0.125</td>
<td>0.203</td>
<td>0.121</td>
<td>0.150</td>
<td>0.046</td>
<td>0.027</td>
<td>0.097 - 0.202</td>
</tr>
<tr>
<td>EPP + Anti-Erb</td>
<td>0.113</td>
<td>0.112</td>
<td>0.117</td>
<td>0.114</td>
<td>0.003</td>
<td>0.002</td>
<td>0.114 - 0.114</td>
</tr>
<tr>
<td>EPP + Anti-ERa+β</td>
<td>0.112</td>
<td>0.140</td>
<td>0.138</td>
<td>0.130</td>
<td>0.016</td>
<td>0.009</td>
<td>0.130 - 0.130</td>
</tr>
<tr>
<td>E2</td>
<td>0.229</td>
<td>0.213</td>
<td>0.300</td>
<td>0.247</td>
<td>0.046</td>
<td>0.027</td>
<td>0.195 - 0.300</td>
</tr>
<tr>
<td>E2 + Anti-Era</td>
<td>0.113</td>
<td>0.157</td>
<td>0.128</td>
<td>0.133</td>
<td>0.022</td>
<td>0.013</td>
<td>0.107 - 0.158</td>
</tr>
<tr>
<td>E2 + Anti-Erb</td>
<td>0.122</td>
<td>0.109</td>
<td>0.142</td>
<td>0.124</td>
<td>0.017</td>
<td>0.010</td>
<td>0.106 - 0.143</td>
</tr>
<tr>
<td>E2+ Anti-ERa+β</td>
<td>0.123</td>
<td>0.107</td>
<td>0.110</td>
<td>0.113</td>
<td>0.009</td>
<td>0.005</td>
<td>0.104 - 0.123</td>
</tr>
<tr>
<td>Ral</td>
<td>0.271</td>
<td>0.210</td>
<td>0.280</td>
<td>0.254</td>
<td>0.038</td>
<td>0.022</td>
<td>0.211 - 0.297</td>
</tr>
<tr>
<td>Ral + Anti-Era</td>
<td>0.124</td>
<td>0.136</td>
<td>0.138</td>
<td>0.133</td>
<td>0.008</td>
<td>0.004</td>
<td>0.133 - 0.133</td>
</tr>
<tr>
<td>Ral + Anti-Erb</td>
<td>0.103</td>
<td>0.148</td>
<td>0.117</td>
<td>0.123</td>
<td>0.023</td>
<td>0.013</td>
<td>0.123 - 0.123</td>
</tr>
<tr>
<td>Ral + Anti-ERa+β</td>
<td>0.146</td>
<td>0.116</td>
<td>0.102</td>
<td>0.121</td>
<td>0.022</td>
<td>0.013</td>
<td>0.096 - 0.147</td>
</tr>
<tr>
<td>Control</td>
<td>0.218</td>
<td>0.220</td>
<td>0.217</td>
<td>0.218</td>
<td>0.002</td>
<td>0.001</td>
<td>0.217 - 0.220</td>
</tr>
<tr>
<td>Anti-ERa</td>
<td>0.173</td>
<td>0.165</td>
<td>0.136</td>
<td>0.158</td>
<td>0.019</td>
<td>0.011</td>
<td>0.136 - 0.180</td>
</tr>
<tr>
<td>Anti-ERβ</td>
<td>0.150</td>
<td>0.112</td>
<td>0.111</td>
<td>0.124</td>
<td>0.022</td>
<td>0.013</td>
<td>0.124 - 0.124</td>
</tr>
<tr>
<td>Anti-ERa+β</td>
<td>0.106</td>
<td>0.122</td>
<td>0.117</td>
<td>0.115</td>
<td>0.008</td>
<td>0.005</td>
<td>0.115 - 0.115</td>
</tr>
</tbody>
</table>

EPP = 10μg/ml Extract of Padina pavonica; E2 = 10nM Oestradiol; Ral = 10nM Raloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean; Anti-ERα = Anti-Oestrogen Receptor – alpha; Anti-ERβ = Anti-Oestrogen Receptor – beta.
Graph 8: Average Alizaren Red Assay vs Drug +/- Anti-Oestrogen Receptor Antibody

Incubation with both anti-oestrogen receptors resulted in the biggest decrease in calcium fixation by osteoblasts. A similar scenario was noted in the cells treated with the extract of *Padina pavonica*.

EPP = 10μg/ml Extract of *Padina pavonica*; E2 = 10nM Oestradiol; Ral = 10nMRaloxifene; Exp #1 = Biological Triplicate #1; Exp #2 = Biological Triplicate #2; Exp #3 = Biological Triplicate #3; ST Dev = Standard Deviation; SEM = Standard Error of the Mean; Anti-ERα = Anti-Oestrogen Receptor - alpha; Anti-ERβ = Anti-Oestrogen Receptor - Beta

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Statistical Analysis of Results

A summary of statistical analysis of the results using multi-linear regression is hereby shown.

Table 18: Statistical analysis of the effect of drug on the experiments' end-points

<table>
<thead>
<tr>
<th>Effect of treatment drugs: EPP, oestradiol or raloxifene on the experiments' end-points</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase Activity vs Drug</td>
<td>0.501</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>MTT Activity vs Drug</td>
<td>0.002</td>
<td>Statistically significant</td>
</tr>
<tr>
<td>Alkaline Phosphatase:MTT activity vs Drug</td>
<td>0.034</td>
<td>Statistically significant</td>
</tr>
<tr>
<td>Alizarin Red vs Drug</td>
<td>0.548</td>
<td>Not statistically significant</td>
</tr>
</tbody>
</table>

Alkaline phosphatase activity, as described earlier in this dissertation, was used as a measure of early osteoblast differentiation. The lack of statistical significance, between drugs, is also reflected in the similar estimated marginal means for alkaline phosphatase activity; when the data analyzed using multi-linear regression.

Table 19: Estimated Marginal Means - Alkaline Phosphatase Assay

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Error</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP</td>
<td>2.873</td>
<td>0.115</td>
<td>2.645 - 3.102</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>2.990</td>
<td>0.115</td>
<td>2.762 - 3.219</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>2.995</td>
<td>0.115</td>
<td>2.786 - 3.223</td>
</tr>
</tbody>
</table>
MTT activity, as described earlier in this dissertation, was used as a measure of cell viability. It is a reflection of the cultures’ longevity and proliferation of progenitor osteoblast cells. When raloxifene is used as the comparator drug, to assess how the drugs differ between one another on their effect on MTT activity, statistical results are as follows:

<table>
<thead>
<tr>
<th>Raloxifene vs</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP</td>
<td>0.528</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.06</td>
<td>Statistically significant</td>
</tr>
</tbody>
</table>

The latter is reflected in the estimated marginal means for MTT activity when the data is analyzed using multi-linear regression.

<table>
<thead>
<tr>
<th>Mean</th>
<th>Std. Error</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP</td>
<td>0.684</td>
<td>0.016</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.742</td>
<td>0.016</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>0.695</td>
<td>0.016</td>
</tr>
</tbody>
</table>

The alkaline phosphatase:MTT ratio, as described earlier in this dissertation was taken as a marker of osteoblast differentiation. The terminally differentiated osteoblast is by non-
definition a non-proliferative cell. When raloxifene is taken as the comparator drug, in order to assess how the drugs differ between one another, on their effect on the alkaline phosphatase to MTT ratio, statistical results are as follows:

Table 22: Statistical analysis of the effect of oestradiol and EPP, as compared to raloxifene, on alkaline phosphatase:MTT ratio

<table>
<thead>
<tr>
<th>Raloxifene vs</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP</td>
<td>0.301</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.009</td>
<td>Statistically significant</td>
</tr>
</tbody>
</table>

The estimated marginal means for the alkaline phosphatase:MTT ratios, as obtained from multi-linear regression analysis, are as follows:

Table 23: Estimated Marginal Means - Alkaline Phosphatase:MTT

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Error</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Padina</td>
<td>4.005</td>
<td>0.100</td>
<td>3.806</td>
<td>4.205</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>3.865</td>
<td>0.100</td>
<td>3.666</td>
<td>4.065</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>4.137</td>
<td>0.100</td>
<td>3.937</td>
<td>4.336</td>
</tr>
</tbody>
</table>

Raloxifene had the highest estimated marginal means for the alkaline phosphatase:MTT ratio. Oestradiol cultures ranked third after the cultures treated with EPP. This interesting finding may imply that more differentiated osteoblasts were present in the raloxifene and EPP cultures as compared to oestradiol. The higher MTT in oestradiol is likely to reflect higher cell numbers due to progenitor cells which as less effective at expressing alkaline phosphatase activity.
The Alizarin Red assay, as described earlier in this dissertation was taken as a marker of calcium fixation and thus bone matrix mineralization. The estimated marginal means for the Alizarin Red assay as obtained during multi-linear regression analysis, is as follows:

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Error</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Padina</td>
<td>0.233</td>
<td>0.017</td>
<td>0.199</td>
<td>0.266</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.219</td>
<td>0.017</td>
<td>0.185</td>
<td>0.252</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>0.237</td>
<td>0.017</td>
<td>0.203</td>
<td>0.270</td>
</tr>
</tbody>
</table>

In order to evaluate the suspicion that the action of Anti-ER-α or Anti-ER-β modulates the effect of the different drugs, interaction terms were used into the multi-linear regression model to assess whether the reaction to ER-α or ER-β antibody is influence by the presence or absence of specific drugs. None of the interaction terms had a significant influence suggesting that their action is not synergistic.

In order to determine whether the antibodies had a statistically significant effect in changing each of the drugs population means for the end-points of our study the Kruskall-Wallis test by ranks was employed. This was done in order to assess whether the samples originated from the same distribution as the cultures treated with the drug without any antibodies added to it. None of these tests were statistically significant.
Table 25: Statistical analysis of the different treatment drugs: EPP, oestradiol and raloxifene with or without ER-antibodies on the experimental end-points

<table>
<thead>
<tr>
<th>End-Point</th>
<th>Drug</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase Activity vs Drug +/- ER-α, ER-β and ER-α+β</td>
<td>EPP</td>
<td>0.147</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>oestradiol</td>
<td>0.075</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>raloxifene</td>
<td>0.147</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>MTT Activity vs Drug +/- ER-α, ER-β and ER-α+β</td>
<td>EPP</td>
<td>0.268</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>oestradiol</td>
<td>0.270</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>raloxifene</td>
<td>0.312</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>Alkaline Phosphatase:MTT activity vs Drug +/- ER-α, ER-β and ER-α+β</td>
<td>EPP</td>
<td>0.09</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>oestradiol</td>
<td>0.082</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>raloxifene</td>
<td>0.067</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>Alizarin Red vs Drug +/- ER-α, ER-β and ER-α+β</td>
<td>EPP</td>
<td>0.06</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>oestradiol</td>
<td>0.07</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td></td>
<td>raloxifene</td>
<td>0.08</td>
<td>Not statistically significant</td>
</tr>
</tbody>
</table>
Discussion

This research has shown that primary osteoblast cells can be readily isolated from human bone explants and grown to reach confluence. The latter set-up can thus be considered with confidence for use in 'in vitro' testing, especially when genetic work is planned. As discussed earlier in this dissertation whilst continuous cell lines, are less labour intensive and more fragile to culture, they have undergone the process of transformation. This implies that gene expression may be altered in these cells and whilst allowing for a homogenous expression of genetic material amongst the different cells they are more likely to have a different genetic profile to that found in normal tissues.

A main concept of cell physiology and biology is that cells that are terminally differentiated are non proliferative. Terminally differentiated cells tend to redirect their energies to protein synthesis. In the case of osteoblasts terminally differentiated cells would be expected to produce collagen the main proteinaceous component of the extracellular bone matrix. The osteoblast would also be capable of producing enzymes such as alkaline phosphatase. The activity of the latter was measured in this work. As the osteoblast progenitor cells become more specialized in producing proteins they thus lose their ability to divide.

This work supports available literature that the extract of Padina pavonica may have a role to play in the management of post-menopausal osteoporosis. The results appear to suggest that EPP at 10ug/ml has similar effects as the other agents on osteoblastic differentiation and is better than control. EPP also shows better calcium fixation.
properties than controls and oestradiol and raloxifene. Whilst analysis carried out between the three agents shows no differences, there appears to be a difference to the control value.

The results, as pictorially represented by the graphs in the results section, seem to show that the extract of Padina pavonica was comparable to raloxifene. The extract of Padina pavonica has shown a similar dose dependent effect on all the end points measured. In summary, Padina pavonica appears to encourage the formation of the terminally mature osteoblast. An increase in alkaline phosphatase activity and decrease in MTT assay was reflected in the alkaline phosphatase is to MTT ratio. The fact that there was no statistically significant difference on the alkaline phosphatase is to MTT ratio between the extract of Padina pavonica and raloxifene, is also very promising and interesting for future work. This is especially so, when one considers that raloxifene had the best estimated marginal means for the alkaline phosphatase is to MTT ratio as calculated by multiple linear regression analysis.

The second arm of the study aimed to attempt to identify a relationship between the three pharmacological agents and oestrogen receptor antibodies. The experiments with the antibodies did not achieve any statistical significance. This was a recurring scenario in the three treatment arms of this study. The graphical reduction in the overall alkaline phosphatase activity, an increase in MTT activity and thus lower alkaline phosphatase activity in all the three arms of the experiment seems to suggest that the extract of Padina pavonica exerts its action at least in part via the oestrogen receptor. We hereby postulate
that the extract of *Padina pavonica* could modulate the oestrogen receptor either directly or indirectly via co-factors and may have an effect on the oestrogen responsive primary cell-lines represented in this research by osteoblasts. This modulation of the oestrogen receptor implies a SERM-like activity as supported by available literature on the compound (1,11).

With more biological repeats statistical significance could have potentially been achieved. It may also be that the monoclonal antibodies were being displaced in the presence of either drug. Another limitation of this experiment is the fact that the monoclonal IgG antibodies used against the oestrogen receptors are generally used in research for detection of the receptors by for example immunohistochemistry. The dose to use for this experiment was obtained by inference form the concentrations used in the immunohistochemistry. No literature was available on whether these drugs have any effect on the transcription of cells.

An important limitation of this work is that the protein content of the matrix was not assessed. This study only assessed the mineral component as measured by the Alizarin Red assay. As outlined in the results section, this experiment showed that the extract of *Padina pavonica* had the second best estimated marginal means after raloxifene. The multiple liner regression analysis also showed that there was no statistically significant difference in the results of the Alizarin Red Assay between the extract, raloxifene and oestrogen. Future work including genetic studies and protein analysis will be key in reliably determining the effect of the extract on collagen synthesis.
As discussed earlier in the literature review, serum in the form of FBS may have had an effect on this work by promoting cell growth. An improvement for future work would include using a lower concentration of FBS and the use of a negative control on osteoblast growth. Cell culture work is also at risk of infection and pipetting errors.
Conclusion: Is there a role for EPP in the prevention of osteoporotic fractures?

The hormone deficiency that characterizes the post-menopausal periods is a leading cause of osteoporosis. Osteoporosis is thus likely to co-exist with other co-morbidities, or be diagnosed in patients at risk of developing other co-morbid chronic conditions. Such conditions could include diabetes, hyperlipidemia, ischaemic heart disease and cancer. A sub-optimal immune response may also make these patients more prone to infectious agents and neoplasia. Surely the ‘ideal drug’ in this scenario would be one that improves bone strength, reduces the incidence of osteoporotic fragility fractures and prevents or decreases the incidence and severity of these other co-morbid conditions that in themselves increase the morbidity, mortality and financial burden on patients and on national health services.

The extract of Padina pavonica seems to be a strong candidate in the prevention of osteoporotic fractures. Studies so far have shown an improvement in calcium fixation of cells receiving treatment with EPP leading to an amelioration in bone mineral density (1). EPP is well tolerated and may have a role to play in reducing the burden imposed by other medical conditions. This study supports available work. The actions of the extract of Padina pavonica on the end-points of our study, which reflect osteoblast differentiation and bone matrix mineralization, suggest that its ‘in vitro’ action is not inferior to key drugs in the management of osteoporosis.
SERMs, the drugs that have become popular in the past decades still pose a high risk for VTEs. There is also a need for further data on the safety profile of these products. As mentioned earlier in this dissertation any SERM molecule needs to be evaluated in itself, because of tissue dependent factors that may determine whether it is agonistic or antagonistic at specific tissues including the breast and endometrium. More studies are needed to determine whether the effects of EPP on calcium fixation are oestrogen dependent or independent.

Future work should aim to elucidate the ‘in-vitro’ transcriptional effects of the extract of Padina pavonica not only on bone but also on human breast, endometrial and endothelial cell lines using gene expression micro-arrays or RNA-sequencing. This would allow one to relate differential gene expression signatures to established cell signaling and disease pathways, thereby providing an innovative, objective and unbiased insight into the cellular mechanism of the extract of Padina pavonica.

Such a set-up is likely to involve the cell culture of human breast, bone, endometrial and endothelial cells. Since the extract of Padina pavonica is expected to have a SERM-like effect, results could potentially be compared to cell-lines treated with raloxifene, which is a key role player in the field. RNA extraction and c-DNA synthesis should be followed by gene analysis using gene expression micro-arrays or RNA sequencing of cell-lines treated with the extract of Padina pavonica. The gene expression network should then be analyzed in order to relate the observed transcript changes to the physiologically meaningful pathways that describe the action of the extract of Padina pavonica. The
gene expression dataset should be validated using qPCR and protein analysis by Western blots.

To conclude this research has shown promising results and supports the need for more work in the field. The extract of *Padina pavonica* may exert its effects via the oestrogen receptor either directly or indirectly. Whilst a SERM-like effect has potential in the management of osteoporosis, the fact that the extract of *Padina pavonica* may indeed modulate the oestrogen receptor could potentially imply that the latter may have SERM-associated side-effects. It is strongly hoped that further studies that lead to a clear insight on the mechanism of action of EPP, can instill the necessary confidence to conduct further clinical studies on this very interesting molecule.
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Appendix
Dr Sarah Sultana Grixti
FIt G10, Belmonte Heights
St. Ignatius’ Street
Sliema SLM2023

Dear Dr Sarah Sultana Grixti,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

**Determining the ‘in vitro’ effect of Padina pavonica on the oestrogen receptor and oestrogen responsive primary cell lines**

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

[Signature]

Dr. Mario Vassallo
Chairman
Research Ethics Committee
**Patient Information Sheet**

We are doing a study looking into the treatments for brittle bones. This disease is also known as osteoporosis. A locally grown plant may provide a substance that may help with this disease.

Your surgery routinely sees the removal of bone fragments. These are usually discarded as medical waste. We are hereby requesting your permission to collect these bone fragments. This will allow us to grown bone cells; which will then be treated with different medicines currently on the market and the plant extract that has been mentioned earlier.

The researchers will not be collecting any data about you at all. Your kind help with this study will thus remain completely anonymous. We hope that in the future many may benefit from this study, but no direct benefit, financial or otherwise, will be derived from this study.

We are happy to answer any questions you might have! Thanks for your help.

Dr. S. Sultana Grixti
Principal Investigator
Mob: 79882112

Prof. M. P. Brincat & Mr. R. Galea
Project Supervisors

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**Fuljett ta' Informazzjoni ghall-Pazjent**


Ir-ricekaturi mhux ha jkunu qed jigbru l-ebda informazzjoni fuqek. L-ghajnuna prezzjuza tiehek f’dan l-istudju hija kompletament anonima. Fil-waqt li nisperaw li hafna nies jibbenefikaw minn dan l-istudju fil-futur, nispecifikaw li inti mhux ha tkun qed tircievi l-ebda beneficcju diret, finanzjarju u mhux, ghal-ghajnuna tiehek f’dan l-istudju.

Tidejjjaqx tistaqsina jekk ghandek xi mistoqsijiet! Grazzi mill-qalb.

Dr. S. Sultana Grixti
Ricerkatrici Principali
Mob: 79882112

Prof. M. P. Brincat u Mr. R. Galea
Supervizuri fuq dan l-istudju
**Consent Form**

I, ________________________________ hereby give my consent to participate in this study freely according to the information given above. I have also been given the opportunity to ask questions and any queries have been clarified.

Donor’s Signature & ID Card No: ________________________________

Researcher's Name, Signature & ID Card No: ________________________________

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**Formula ta' Kunsens**

Jiena, ________________________________ hawnhekk qed/qeghda naghti il-kunsens tiegħi biex nippartecipa f’dan l-istudja minghajr sfurzar u kundizzjonijiet u skond l-informazzjoni li nghatajat. Gejt moghtijmoghtija l-opportunita’ biex naghmel il-mistoqsijiet u kull dubju gie ikklarifikat.

Firma u ID tad-Donatur: ________________________________

Isem, firma u ID tar-ricerkatur/ricerkatrici: ________________________________
**Specifications for Alpha -Minimum Essential Medium Eagle (MEM)**
Source: Sigma-Aldrich®

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