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PHYTOESTROGENS AS AN ALTERNATIVE TO HRT: AN INVESTIGATION INTO THE MOLECULAR EFFECTS OF PHYTOESTROGENS ON HAEMOSTASIS IN CELL AND ANIMAL MODELS

A thesis submitted for the Degree of Doctor of Philosophy

by

Lynne Kelly

University of Dublin

Trinity College 2011
Declaration

I hereby declare that my thesis is entirely my own work and that it has not been submitted previously for a PhD degree at this or any other university.

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Lynne Kelly
Acknowledgements

I would like to express my thanks to my supervisor Dr. Lucy Norris for giving me the opportunity to carry out this work. Your continuous support over the years is gratefully acknowledged and I have learnt a great deal about this field of research from your constant guidance, enthusiasm and willingness to help. For showing me different ways to approach a research problem and the need to be persistent to accomplish my goals, I will always be in debt to you for making this possible.

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Publications arising from this research

Peer-Reviewed Articles


Abstracts

Summary

There is a direct link between the increased risk of cardiovascular disease and the menopause and this is thought to be due to the progressive lack of endogenous oestrogen in post-menopausal women. Observational studies have provided evidence that hormone therapy reduces the risk of cardiovascular disease but more recent randomised controlled clinical trials have contradicted these results. These trials have shown increases in venous thromboembolism, myocardial infarction and other coronary events in women receiving estradiol in the form of hormone therapy versus placebo. Due to these publications many women chose to use herbal remedies for relief of menopausal symptoms, many of these include phytoestrogens supplements. Phytoestrogens are a group of naturally occurring substances produced from plants with weak oestrogenic and anti-oestrogenic activity and are the main constituents of soy. The aim of this study was to investigate the effects of physiological concentrations of soy phytoestrogens on the regulation of haemostasis gene expression and compare them to the 17β-estradiol and soy free controls. This study has shown that some of these phytoestrogens have a similar, and in some cases a greater effect, than estradiol on haemostasis gene expression, this may have implications in determining the thrombotic risk profile of women taking these supplements.

Initially this study examined an animal model of the menopause, using ovariectomised rats fed on a diet of phytoestrogens for three months in comparison to rats on a soy-free diet. Coagulation and fibrinolysis gene expression was measured using Taqman low density arrays on the RNA extracted and cDNA synthesised from liver biopsies of these rats. Those that were significantly altered were analysed at the protein level. The study showed that these phytoestrogens have the ability to regulate transcription to a significantly greater extent that the control and also estradiol, increasing the levels of factor VII, fibrinogen, prothrombin, tPA and PAI-1 gene expression among others. Oestrogen receptor expression was also analysed in these tissues, only the alpha isoform and the receptor, GPR30 were found which may mediate the transcriptional changes seen.

In order to identify if these results translate to a human model the ability of these phytoestrogens to regulate haemostasis genes in human hepatocyte cell lines was analysed. Using both oestrogen receptor negative (HepG2) and oestrogen receptor alpha-positive cells lines (Hep89), genes that were significantly altered in the animal model were analysed in culture following stimulation with phytoestrogens. The role of the

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inflammatory cytokine IL-1β in mediating any phytoestrogen induced change in expression was also examined. This *in vitro* study displayed a unique differentiation between haemostatic gene expression relative to the oestrogen receptor content of the cells. Overall genistein increased the expression of some of the main markers such as prothrombin, tPA and PAI-1 in Hep89 cells, the opposite observation was found in HepG2 cell culture, implying that oestrogen receptor alpha may be responsible for these changes. The results also showed that IL-1β can regulate the phytoestrogen induced change in gene expression, as the addition of this cytokine to the cells increased PAI-1 expression further in genistein and equol treated cells. These results provide further evidence for the hypothesis that oestrogen receptor alpha regulates the effects of genistein and to a lesser extent equol and daidzein on certain haemostasis genes and showed that the phytoestrogens, genistein, equol and daidzein can regulate the expression of coagulation and fibrinolytic genes expressed in human hepatocyte cell lines.

Vascular endothelium can be both an important anticoagulant and procoagulant surface, and endothelial cells are the site of production of activators and inhibitors of the coagulation and fibrinolytic cascade. For this reason, employing real time PCR, ELISA and high content screening analysis, the final section of this study investigated the effect of phytoestrogens on vascular endothelial cells by analysing genes and proteins from sections of aorta from the rat model and also human endothelial cells. Rat aorta samples were used to measure mRNA expression of endothelial derived genes of the haemostatic system using real time TaqMan PCR. These included genes involved in the key inhibitory pathways of coagulation e.g. APC and TFPI. Genistein gave the most marked response in influencing the expression of endothelial derived coagulation and fibrinolytic factors. Studying these *in vivo* results in an *in vitro* model showed differences in expression of the chosen genes, particularly for the endothelial protein C receptor but there was also a difference in oestrogen receptor expression between these two systems showing the influence of oestrogen receptor levels on the transcriptional control of these markers.

In conclusion this study exemplifies the effects of phytoestrogens on coagulation and fibrinolytic gene and protein expression, in an animal model and in cell culture systems. In comparison to estradiol the results show that these compounds have similar, and in some cases a heightened effect on modulating their expression both at the mRNA and protein levels. This could be important particularly in post-menopausal women at risk of cardiovascular disease who use these ‘natural’ supplements.
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<td>AIB-1</td>
<td>Amplified in breast cancer 1</td>
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<td>ApoA1</td>
<td>Apolipoprotein A1</td>
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<td>AP-1</td>
<td>Activator protein 1</td>
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<td>ATF</td>
<td>Activating transcription factor</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma protein 2</td>
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<td>BSA</td>
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<td>bw</td>
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<td>Cyclic adenosine monophosphate</td>
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<td>conc</td>
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<td>Monkey kidney cell line</td>
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<td>Corner box</td>
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<td>Ct</td>
<td>Threshold time</td>
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<td>dH2O</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Egr-1</td>
<td>Early growth response 1</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
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<td>EtOH</td>
<td>Ethanol</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FOXA2</td>
<td>Forkhead box A2</td>
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<td>g</td>
<td>Gram</td>
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<td>Gla</td>
<td>Gamma-linolenic acid</td>
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<td>gp1b</td>
<td>Glycoprotein Ib</td>
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<tr>
<td>gpIIb/IIIa</td>
<td>Platelet glycoprotein IIb/IIIa</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<td>kg</td>
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<td>MCF-7</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>MTT</td>
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<td>NCoR</td>
<td>Nuclear receptor corepressor 1</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>PCE1</td>
<td>Endoglucanase gene 1</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>P38</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Rip40</td>
<td>Receptor interacting protein 40</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimer partner</td>
</tr>
<tr>
<td>SMAD</td>
<td>Sma and Mad related proteins</td>
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<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoid and thyroid receptors</td>
</tr>
<tr>
<td>Sp-1</td>
<td>Specificity protein 1</td>
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<tr>
<td>TBST</td>
<td>Tris buffered saline-tween</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNS</td>
<td>Trypsin neutralising solution</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine-5- (and 6)-isothiocyanate</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>V</td>
<td>Volt</td>
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<td>VLDL</td>
<td>Very small density lipoprotein</td>
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<td>%</td>
<td>Percent</td>
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<tr>
<td>°C</td>
<td>Degrees centigrade</td>
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**Chapter 3**

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Chapter 1

Introduction
1.1 Cardiovascular disease in post menopausal women

1.1.1 Menopause

Menopause is defined as the permanent cessation of reproductive fertility and is associated with reduced secretion of the ovarian hormones oestrogen and progesterone. Ovarian senescence is a gradual process which occurs over the fourth and fifth decades leading to menopause which occurs at the average age of 51.3 years. Menopause can also occur prematurely through surgical removal of the ovaries or through damage to the ovaries from chemotherapy or radiation. Declining levels of oestrogens produce a variety of unpleasant symptoms, including vasomotor symptoms, effects of sexual function and a decrease in bone density (1). In addition to these symptoms, postmenopausal women are also at increased risk of various age-related diseases. An increased risk of cardiovascular disease (CVD) has been demonstrated in post menopausal women which has been largely attributed to this decline in the hormone oestrogen. Before the menopause, the most prevalent cause of mortality in women is cancer whereas in men it is CVD. In premenopausal women, the age associated risk of CVD lags by 10 years in premenopausal women compared with men. However, by the time women are into their menopausal years the incidence of CVD sharply increases to match that of men (Figure 1.1).

![Figure 1.1: Number of deaths due to cardiovascular disease in men versus women (2).]
Each year, cardiovascular disease (CVD) kills over 4.3 million people in the 53 member states of the World Health Organization European Region and more than 2 million in the 27 member states of the European Union (EU). CVD is responsible for 54% of all deaths in women across Europe and 43% of the deaths in men, killing more people than all cancers combined (3).

The mechanism by which the loss of oestrogen at menopause is associated with increased cardiovascular risk is not fully understood. In postmenopausal women the process of aging and oestrogen withdrawal are interlinked. Age is associated with increased vascular remodelling and endothelial dysfunction and estradiol inhibits these processes and hence the presence of the hormone in premenopausal women may prevent or slow down the age related changes in the vasculature which lead to the development of cardiovascular disease (Figure 1.2).

17β-estradiol is an endogenous oestrogen found mainly in reproductive tissues. Oestrogens have diverse functions in different tissues and cell types in the body. They regulate the growth and development of reproductive systems as well as homeostasis in a variety of tissues. Beyond its essential role in reproduction, estradiol affects the whole organism and plays important roles in bone maintenance, in the central nervous system, and also in the cardiovascular system where it displays certain cardioprotective effects (4). During an ovulatory cycle the serum concentrations of estradiol range from 30-210pg/ml whereas in postmenopausal women the estradiol levels are usually below 20pg/ml (5).

Figure 1.2: Atherosclerosis progression over time (6)
1.1.2 Effect of hormone therapy on CVD

At menopause, lack of oestrogen can bring on symptoms such as hot flushes and vaginal dryness. It also can increase the risk of osteoporosis and heart disease. Because of this, women may choose to take hormone therapy (HT) after menopause. Hormone therapy for postmenopausal women consists of a synthetic oestrogen with or without a synthetic progestin. In comparison to oral contraceptives which require the administration of very high levels of these hormones, e.g. Yasmin contains 30mg of a synthetic oestrogen and 3mg progesterone, menopausal hormone therapy is given at much lower doses. HT preparations can differ regarding the types and dose of oestrogen and progestin present and also the route of administration (Table 1.1). HT can be prescribed in the combined form of oestrogen-progestogen therapy or as unopposed oestrogen for hysterectomized women (7).

<table>
<thead>
<tr>
<th>Oestrogen</th>
<th>Dose</th>
<th>Progestogen</th>
<th>Dose</th>
</tr>
</thead>
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<tr>
<td>Conjugated equine oestrogens</td>
<td>0.3 to 0.625mg</td>
<td>Medroxyprogesterone acetate (MPA)</td>
<td>2.5mg daily or 5mg for 10 to 12 days/month</td>
</tr>
<tr>
<td>Micronized 17β-estradiol</td>
<td>0.5 to 1mg</td>
<td>Micronized progesterone</td>
<td>100mg daily or 200mg for 10 to 12 days/month</td>
</tr>
<tr>
<td>Transdermal estradiol</td>
<td>14 to 100μg</td>
<td>Norethindrone</td>
<td>0.35mg daily or 5mg for 10 to 12 days/month</td>
</tr>
<tr>
<td>Ethinyl estradiol</td>
<td>0.01 to 0.02mg</td>
<td>Levonorgestrel</td>
<td>0.075mg daily</td>
</tr>
<tr>
<td>Vaginal estrogenic preparations e.g. vaginal ring</td>
<td>0.015</td>
<td>Etonogestrel</td>
<td>0.120mg daily</td>
</tr>
<tr>
<td>Other; Oestrogen pellets, gels, creams, intranasal sprays or injections</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: The most common forms and route of delivery of oestrogens and progestogens used in HT.

Although these preparations were used primarily for symptom relief, the replacement of natural oestrogen with a synthetic oestrogen after menopause was thought to have beneficial effects with regard to cardiovascular risk. HT was primarily believed to improve hot flashes, sleeping pattern, genital atrophy and provide protection against osteoporosis and cardiovascular disease until the conflicting publication of observational and randomized controlled trial (RCT) data emerged. The biological plausibility of
cardioprotection by HT is supported by a number of studies demonstrating favourable effects of oestrogen on numerous factors involved in the pathogenesis of atherosclerosis. The largest of the observational studies was the Nurses Health Study which first reported results of the differences in women who used HT compared to those who had never used it. This trial was not randomised and women took one of three doses of oestrogen without a progestin. Women used HT when it possibly had the potential to have the most benefit i.e. after the onset of menopausal symptoms. They reported that postmenopausal use of oestrogen reduces the risk of severe CHD compared with non users and that users had approximately half the risk of developing CHD than non-users (8). The Postmenopausal Estrogen/Progestin Interventions (PEPI) prospective trial, which compared conjugated equine oestrogen (CEE) alone and with three different CEE-progestin combinations and placebo in healthy, postmenopausal women, showed an increase in high density lipoprotein (HDL) and decreases in low density lipoprotein (LDL) and fibrinogen in women receiving oestrogen (9). The other data showing a benefit of HT came from studies in an animal model. Clarkson et al described a ~70% reduction in coronary artery intimal area with HT compared with placebo in ovariectomised cynomolgus monkeys (10). These studies showed that HT may have a cardioprotective benefit.

The first study that suggested otherwise was the Heart and Estrogen/progestin Replacement Study (HERS) in 1998. This trial reported an increase in coronary events in the first year of use leading to a lack of net benefit after two years use of combined HT in women with pre-existing coronary artery disease (CAD) (11). Their follow up study showed no benefit of HT and that the total number of coronary events actually increased in those on HT compared to placebo and stated that HT should not be used for secondary CHD prevention (12). The HERS trial recruited women who were older than the average age of menopause and who had significant heart disease therefore this trial was a secondary prevention study. After publication of this data and the previous Nurses health study data, it was thought that women benefitted from HT early in the menopause transition.

In the Estrogen Replacement and Atherosclerosis (ERA) trial women had documented CHD. They were given either oestrogen alone or in combination with progesterone. The result showed no difference in the progression of coronary atherosclerosis (13). Kronos Early Estrogen Prevention Study (KEEPS) is a secondary trial testing the timing of HT initiation. The hypothesis is that HT will result in cardiovascular protection because of a window of opportunity in early menopause. The objective is to measure carotid intimal
thickness and coronary calcium. The trial ends in 2012 and results should be available soon after (7).

The Women’s Health Initiative (WHI) was a large randomized placebo-controlled clinical trial undertaken to explore the effects of hormone therapy on risk of cardiovascular disease. WHI published preliminary results in 2002 of its oestrogen plus progestin arm. Patients in this arm of the study received conjugated equine estrogens, 0.625mg/day plus medroxyprogesterone acetate, 2.5mg/day. The results stated that the overall risks exceeded the potential benefits and subsequently this arm of the trial was not continued (14). The results from the second arm of the trial, which analysed the use of oestrogen-only hormone therapy in women who had previously undergone a hysterectomy, showed that conjugated equine estrogens, at a concentration of 0.625mg/day, provided no overall protection against myocardial infarction and coronary death (15). The results of these studies prompted the Food and Drug Administration and American Heart Association to conclude that HT should not be initiated or continued for CHD prevention and those that were prescribed the treatment should only be administered it for the shortest time possible (16-17).

The last publication from the Nurses Health Study showed that HT use is associated with an increased risk of stroke (18) which coincides with findings from the combined arm of the large randomised controlled trial Women’s Health Initiative in 2002 (14). Questions arose on this conflicting data as to which study was more correct and what guidelines clinicians and postmenopausal women should follow. Most recently, the WHI study has been re-examined as it became clear that the participants were older than those in the observational studies and could therefore have had some underlying risk for CVD. In 2007, WHI published again, results of reanalyses from both arms of the trial showing that the timing of initiation of HT was influencing its effect on CVD. This publication showed that women who initiated HT closer to the menopause tended to have reduced CHD risk compared with the increase in those women who initiated HT more distant from the menopause. (19)

In the observational studies the women studied were younger in comparison to those in the RCT’s, in which the majority were recruited more than a decade after menopause. There is a possibility that these older women may have underlining cardiovascular risk factors due to their age and the impact on taking hormone therapy is ineffective or may trigger events. Recent reports have examined this and suggested that HT could be atheroprotective when started close to the onset of menopausal symptoms (20-22).
Although the aetiology of the increased risk is not understood, oestrogen can modulate production of several factors known to be involved in the development of atherosclerosis. These include pro-inflammatory cytokines, vasoactive molecules and the activators and inhibitors of the haemostatic system. The early increase in CVD events following therapy would suggest that thrombotic mechanisms are important.

1.1.3 Phytoestrogens as an alternative to HT

As a result of the unfavourable results of the randomised controlled trials, many clinicians and patients have sought alternative therapies, including the use of dietary supplements, to provide relief from menopausal symptoms without the unfavourable side effects. Some of these are food-derived compounds that are known as nutritional supplements or herbal remedies and they are promoted as “natural compounds”, since the products are originally derived from normal food sources. On the other hand the dosage has to be considered as very “unnatural”, i.e. the amount of these compounds in one tablet, often exceeds the amount ingested by normal and “natural” consumption. In most people’s perception any product derived from nature is harmless and an increase in concentration enhances the positive effects. So far, many of these so called “natural” supplements can be obtained from sources which are not government regulated, which raises concerns concerning the possible health risks and benefits associated with their consumption.

Phytoestrogens are widely used as a ‘natural’ alternative to hormone therapy for the relief of menopausal symptoms. Phytoestrogens are a group of naturally occurring substances produced from plants with weak oestrogenic and anti-oestrogenic activity. They are structurally and functionally similar to 17ß-estradiol and are capable of producing estrogenic effects. The three main classes of phytoestrogens are isoflavones, lignans and coumestans (Figure 1.3). The isoflavones are generally restricted to legumes, with the highest concentration found in soybeans and soy products. Lignans are found in almost all cereals and vegetables, particularly linseeds and the coumestans are mainly found in beans, with the highest concentration found in alfalfa.
Central to the absorption and metabolism of isoflavones are intestinal bacteria which hydrolyse conjugated isoflavones to their aglycone form for subsequent bioavailability and metabolism (24-27). Phytoestrogens may also be derived from dietary precursors undergoing bacterial modification in the gut prior to absorption. The major lignans are enterolactone and enterodiol. The main isoflavones are genistein, daidzein and equol. These isoflavones are products of colonic bacterial metabolism which remove a glycoside moiety and confer activity. However, where the aglycone forms are available in foods, these may be absorbed directly. It is apparent there is a variable metabolic response to dietary isoflavones in humans. The metabolites of isoflavone metabolism, equol and O-desmethylandolensin (O-DMA), occur in an inverse relationship suggesting preferred metabolic pathways in individuals. This biotransformation of phytoestrogens may be of particular importance as studies have shown that the ability to metabolise soy varies between individuals. A metabolite of daidzein, equol, has been found to be significantly more oestrogenic than its precursor and O-DMA but the number of people that produce this compound is relatively low, approximately one third (27).

This variation in metabolic pathways may be mediated through the absence of bacteria capable of metabolising phytoestrogens, due to the composition of different subpopulations of microflora present, the intestinal transit time, pH or redox potential (26). Metabolism and absorption play a role in defining the effects of oestrogens in the
body with the first pass liver effect mediating the prothrombotic effects of oestrogens and determining the concentration available for systemic circulation (24).

There may be an important distinction in the clinical response obtained between dietary isoflavones and mammalian derived isoflavones such as equol. The structural similarity between these and the endogenous oestrogens and the presence of a phenolic ring in their chemical structure, a prerequisite for binding to the oestrogen receptor, suggests that they could influence CVD risk in the same manner as conventional HT (24-25). However binding of phytoestrogens to the oestrogen receptor will confer a different 3D structure to the occupied receptor and will affect its ability to interact with coactivators or corepressors which could possibly give rise to changes in downstream cell signalling.

1.1.4 Health effects of phytoestrogens in postmenopausal women

The majority of data relating to the beneficial effects of phytoestrogens is based on comparisons between Asian populations, where the intake of soy which is rich in phytoestrogens is high and Western populations. The prevalence of menopausal symptoms varies considerably around the world. In Asia only 10-20% of postmenopausal women experience hot flashes compared to 70-80% in Western countries and the incidence of cardiovascular disease is also less in the Far East. There is also a lower incidence of hormone-dependant diseases such as breast cancer, cardiovascular disease, prostate cancer and osteoporosis in countries like China and Japan where soy foods are staples of their diet. This benefit was not observed in Asian women living in the west suggesting that a dietary rather than a genetic factor was involved (23, 25). Animal studies have shown a reduction in the size and number of atherosclerotic plaques in soy treated rabbits (28). Van der Schouw investigated the relationship between phytoestrogen intake and cardiovascular risk in a Dutch population and failed to show a protective effect however dietary intake was low compared with Asian populations (29).

Reviews of the effects of phytoestrogens on climacteric symptoms are conflicting, some reporting the efficacy of soybean preparations for example, Huntley et al reported on a systematic review of thirteen randomised controlled trials. The results were conflicting with only four trials reporting the beneficial effects of soy preparations (30). Others do not support these findings, a second meta analysis of twenty-five trials reported that soy preparations do not improve menopausal symptoms (31).
The rapidly changing eating trends in Japan or China now make it difficult to make an accurate determination of the intake of isoflavones in these countries in which soy is traditionally a staple. Recent estimates from the Asian diet indicate intakes of 25-40mg/day soy (27, 32) compared to 1mg/day in postmenopausal women in the US (33). The great diversity of phytoestrogens makes it difficult to make general conclusions about their health effects, since different members of these plant-derived substances may have different activities, pharmacokinetic properties and metabolic fate. This highlights the difficulties of studying the clinical effects of bioactive mixtures over the pharmaceuticals available and which lead to us looking further at specific aspects of these compounds. However due to the concerns regarding the risk of cardiovascular disease in post menopausal women using HT, the role of phytoestrogens on CVD risk needs to be further examined.

The most common of the phytoestrogens are found in soybean products. Of these genistein, equol and daidzein are the most important with regard to studies on CVD, Figure 1.4.

Genistein is an isoflavone that occurs naturally in the diet and is found in a wide variety of plant-derived foods, although predominantly in soy products. Its structure resembles that of endogenous oestrogens and it is capable of binding to both oestrogen receptors, with a higher binding affinity for the beta isoform (ERβ) (34-35). Genistein binds to these oestrogen receptors with an affinity of 100 to 10,000-fold less than that of estradiol but it can also compete with estradiol and displace this endogenous oestrogen from its binding sites (36). Genistein has shown to increase oestrogen synthesis via an extragonadal pathway by stimulating aromatase activity in hepatocytes which may be of importance in the use of this type of phytoestrogen in postmenopausal women (37). Genistein has also shown effects in 1) adipocytes which may implicate its possible role in the prevention of obesity (38), 2) a cardioprotective role in postmenopausal women (39-40) and 3) similar effects to estradiol on the uterus and mammary gland (41).

Equol is a heterocyclic phenol that was first isolated and identified from pregnant mares’ urine (42). It is a chiral molecule and exists as R- and S-enantiomeric forms (43). In humans equol is a metabolite of the isoflavone daidzein produced in the gastrointestinal (GI) tract by gut microflora. This aglycone form is more readily absorbed from the GI tract than its glycoside precursor. It is structurally similar to 17β-estradiol and it
possesses oestrogenic activity, having affinity for both oestrogen receptors (44). It has been suggested to have uterotrophic effects; it increases vaginal epithelial thickness in ovariectomised mice (45) and also plays a positive role in mood-related symptoms of postmenopausal women (46). It has also been shown to exert mild oestrogenic effects in the pituitary, mammary gland, liver and fat tissues in an ovariectomised rat model of menopause (47-50). Despite its oestrogenic potential there is little known of the possible thrombotic effects this metabolite.

Daidzein is one of the main isoflavones found in soy products. After ingestion of this phytoestrogen, it is hydrolysed to equol and O-DMA by the bacteria in the large intestine and then absorbed.

Figure 1.4: Chemical structure of the phytoestrogens genistein, daidzein, equol and the endogenous oestrogen 17β-estradiol (51).
1.1.5 Selective oestrogen receptor modulators (SERMs)

Selective oestrogen receptor modulators (SERMs) have been proposed as an alternative regimen for HT. SERMs are a group of synthetic drugs that are clinically used to reverse mainly the growth promoting effects of oestrogens in reproductive cancers. The term SERM describes synthetic oestrogen ligands which through displaying tissue selective pharmacology can act as either an oestrogen receptor agonist or antagonist. As an oestrogen antagonist, they oppose the action of oestrogen in certain tissues while mimicking the effects in others as an agonist. A new approach being tested for the use of SERMs is those which are combined with oestrogen, also known as tissue selective oestrogen complexes (TSECs) for prevention of endometrial hyperplasia and possibly other diseases (52). SERMs and selective agonists for oestrogen receptor alpha (ERα), such as propyl-pyrazole triol (PPT), ERβ such as diarylpropionitrile (DPN), and G-coupled protein receptor (GPR30) such as G1 are being evaluated (53). An ideal SERM would act as an ER agonist on the cardiovascular system, bone, vagina and bladder while acting as an antagonist on the endometrium and breast (Figure 1.5).

Figure 1.5: Profile for an ideal selective oestrogen receptor modulator (SERM) (54)
1.2 Thrombus formation in the arterial and venous systems

The vascular endothelium is a continuous layer of endothelial cells which separates blood from the vessel wall. This layer acts as a semipermeable barrier which regulates the transfer of molecules and also controls functions in vascular haemostasis. Haemostasis maintains the integrity of the circulatory system after vascular damage. It is composed of four major events that occur in a set order following the loss of vascular integrity. The initial phase of the process is vascular constriction. This limits the flow of blood to the area of injury. Next platelets, which are irregularly shaped anuclear cells found in the bone marrow, become activated by thrombin and are recruited to the site of injury, forming a temporary, loose platelet plug where they become the major component of the developing thrombus. The protein fibrinogen is primarily responsible for stimulating platelet clumping. Platelets clump by binding to collagen that becomes exposed following rupture of the endothelial lining of vessels. In addition to induced secretion, activated platelets change their shape to accommodate the formation of the plug. To insure stability of the initially loose platelet plug, a fibrin mesh (also called a thrombus) forms and entraps the plug, this thrombus must be dissolved in order for normal blood flow to resume following tissue repair. The dissolution of the thrombus occurs through the action of plasmin a proteolytic enzyme which is responsible for breakdown of fibrin (55).

The endothelium can sense changes in blood vessels and respond by releasing vasodilatory or vasoconstrictive substances therefore regulating vascular tone. These substances include endothelium-derived relaxing factors such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor and vasoconstrictors like endothelin-1 and prostaglandins (56). The endothelium also synthesises endothelial protein C receptor (EPCR), tissue plasminogen activator (tPA) and tissue factor pathway inhibitor (TFPI) which act as anticoagulants and plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (VWF) and protease-activated receptors (PARs) as procoagulants (57). There is an association between the endothelium, inflammation, lipids and thrombosis. Activation of the immune system leads to the production of pro-inflammatory cytokines which can influence thrombus formation (58), e.g. EPCR and thrombomodulin key proteins involved in anticoagulation are down regulated by tumour necrosis factor alpha (TNFα) (59).
Lipids can modulate thrombotic factors, for example low density lipoprotein (LDL) promotes platelet activation while a variant form of LDL, lipoprotein a (Lp(a)) is known to promote thrombogenesis (8), HDL inhibits platelet aggregation (60) while inflammatory markers C-reactive protein (CRP), interleukins and selectins (E- and P-selectin) also play a role in influencing haemostasis and potential tissue damage resulting from vascular injury. For example interleukin-1 (IL-1) stimulates endothelial cells to produce intercellular adhesion molecule-1 (ICAM-1) which binds leukocytes to the endothelium and interleukin-6 (IL-6) from endothelial cells enhances plaque growth (61). Also, on activated endothelial cells, E and P selectins bind to receptors on leukocytes and increase tissue factor mediated initiation of the coagulation cascade (3, 6). Haemostasis is controlled by a balance between procoagulant and anticoagulant factors which maintains the process of blood clotting, and then the subsequent dissolution of the clot, following repair of the injured tissue. An alteration in endothelial function can contribute to a loss of this tightly regulated balance and lead to various pathological states. On disturbing the regulatory mechanisms of haemostasis excessive quantities of thrombin form, which at a local level can initiate thrombosis (Figure 1.6).

1.2.1 Arterial thrombosis

Arterial thrombosis mainly occurs when an atherosclerotic plaque ruptures. Atherosclerosis is a complex process that begins early in childhood and progresses with age. This systemic arterial disease preferentially affects the intima/lining of large- and medium-sized systemic vessels, including the carotid aorta, coronary and peripheral arteries. An atherosclerotic plaque mainly consists of collagen, proteoglycans, cholesterol, phospholipids, cells including macrophages, T-lymphocytes, and smooth-muscle cells, alongside thrombotic material with platelets and fibrin deposition (62). This plaque develops through the accumulation of lipid deposits and lipid-laden macrophages in the wall of the artery. Lipoproteins are retained in the subendothelium, macrophages ingest these lipoproteins and form foam cells, inflammation causes smooth muscle cells to infiltrate the area and leads to collagen synthesis (63). A critical process in the progression from these subclinical atherosclerotic lesions to clinically relevant lesions is the breakdown of these plaques. The exact mechanism of plaque disruption is unknown. One method suggested is the thinning of the fibrous cap of the plaque by matrix metalloproteinases (MMPs), protease-mediated digestion and decreased synthesis of collagen (64). The processes of plaque disruption trigger inflammatory mechanisms and
promote thrombosis and coagulation (63). The thrombus that forms at a ruptured plaque is rich in platelets. These cells circulate in the blood and are involved in haemostasis; rapidly forming a primary haemostatic plug at the site of vascular injury. Following plaque rupture, these platelets are rapidly recruited to the site through the interaction of specific cell-surface platelet receptors with collagen and von Willebrand factor (VWF). After adhesion to the vessel wall, platelet aggregation results in the growth of the thrombus. The protease of the coagulation cascade, thrombin (factor II) cleaves and subsequently activates the platelet/thrombin receptor (PAR1). The activated platelets then release their contents which further promotes the recruitment, adhesion, aggregation and activation of more platelets to the site of injury (55). There is evidence that interactions between platelets, VWF and extracellular matrix (e.g. collagen) play a primary role in initiating an arterial clot (57). Arterial thrombosis may also occur in the absence of atherosclerosis as shown in animal models (65) and a specific subset of clinical patients (66).
Figure 1.6: The formation of arterial and venous thrombosis. a: Artery. The initial trigger of arterial thrombosis is atherosclerotic plaque rupture leading to disruption of the endothelium and the release of the contents of the plaque into the lumen of the artery. b: Vein. Unlike in the artery, when thrombosis occurs in a vein, the endothelium remains intact and Virchows triad becomes the causing factor (55).
1.2.2 Venous Thrombosis (VTE)

Venous thromboembolism is the term given to collectively describe deep vein thrombosis (DVT) and pulmonary embolism (PE), which is the third leading cause of death, after myocardial infarction and stroke. DVT mainly occurs in the legs whereas PE is a complication of DVT that occurs when part of the thrombus breaks away from the vein in the leg and travels up to the lungs, lodging in the pulmonary artery resulting in the obstruction of normal blood flow (55). The incidence of VTE is about 1 in 1000 in the western world and this incidence rises with increasing age to as much as 1 in 100 (67).

The pathogenesis of venous thromboembolism is centred on three key factors known as Virchow’s triad, (a) the thrombogenicity of circulating blood, (b) changes in the vessel wall and (c) stasis (68) (Figure 1.7). More recently stasis is thought to be a more permissive factor and blood constituents, including inflammatory mediators and changes in the vascular endothelium are considered more important (69-70). Unlike in arterial thrombosis where platelets are the core component, in venous thrombi the main constituent is fibrin which facilitates the thrombus attaching to the vessel wall. The processes that initiate the formation of such thrombi are uncertain but inflammation and stasis play a major role. Inflammation activates the endothelium which in turn causes the release of Weibel-Palade bodies containing VWF and p-selectin which can facilitate the binding of leucocytes to the area. Inflammation also causes the release of inflammatory mediators which can down regulate anticoagulant pathways thereby promoting thrombus formation at a site of endothelial damage. Stasis can also activate the endothelium but in a different manner. For example, the risk for DVT is increased in bedridden patients and also those on long haul flights (71). This occurs as a result of low flow rate of blood causing a build up of prothrombotic components such as thrombin that would normally be inactivated by regular flowing blood (72). Venous thrombosis is believed to be initiated at the venous valves (57), stasis and hypoxia may occur at these valves increasing the thrombotic risk. Hypoxia can also lead to a hypercoagulable state in the blood by increasing the availability of tissue factor and P-selectin (69).

There are other factors that can increase the risk of venous thrombosis, both genetic and environmental. Genetic influences include a range of thrombophilias while the environmental factors range from taking oral contraceptive pills (OCP), hormone
replacement therapy, old age, surgery, obesity, pregnancy and immobilization (73). In the mid Nineties, oral contraception use came under scrutiny with the publication of papers discussing the effects of the progestin component of the pill. The reports suggested that OCPs containing the newer third-generation progestin increased the risk of VTE (74-77). The process of aging modifies several factors of the haemostatic system which could increase the risk of VTE. HT is by the most part prescribed to patients in older age, coupled with the effects of HT shown on the haemostatic system, the risk of a cardiovascular event is increased (78). Surgery and immobilisation can be combined for risk of VTE. Surgery can increase VTE risk particularly in patients undergoing surgery on the lower half of the body such knee or hip replacement surgery. Also the recovery time for these surgeries may be long periods of time and this amount of immobilisation can lead to deep vein thrombosis (79). Obesity may also be linked these to risk factors for VTE. Increased lipid levels are usually seen in these patients leading to vascular damage and alteration in haemostasis, surgery such a gastric bypass could lead these patients to develop deep vein thrombosis or a pulmonary embolism (80). In a review of hospital records, the risk of DVT and PE was 2.2 and 2.5 times higher in patients who were obese, respectively. This review also showed that age was a factor in the patients also, those under 40 years had a five times higher risk for developing a thrombus (81). Pregnancy is also a significant risk factor for VTE with a two- to five-fold increased incidence compared to non-pregnant women (82).

Figure 1.7: Components of Virchow's triad for thrombogenesis: alterations in blood flow producing stasis, damage to the vascular endothelium, and changes in blood constituents resulting in hypercoagulability.
1.2.3 Coagulation and Fibrinolysis

In a healthy individual blood circulates as a liquid. When required, blood can gel rapidly to form a fibrin clot. Fibrin clot formation in response to tissue injury is the most clinically relevant event of haemostasis under normal physiological conditions. The explosive activation of the haemostatic system occurs as a result of the cascade system of coagulation in which inactive zymogens and cofactors are sequentially activated by proteolytic cleavage. The resulting fibrin produced stimulates the fibrinolytic system, limiting fibrin deposition to the site of injury and a system of naturally occurring anticoagulants feedback and prevent further activation of the coagulation pathway. Therefore coagulation is a complex system of soluble enzymes and substrates which ultimately lead to the conversion of fibrinogen to fibrin. Fibrin acts locally at the site of injury to stabilise a clot before it is naturally dissolved by the process of fibrinolysis.

Coagulation

Coagulation involves a complex series of enzymes that are sequentially activated leading to the formation of a thrombus. Each enzyme is activated in sequence in what is known as the cascade system (Figure 1.8). It was previously believed that this cascade was organised into separate pathways, the intrinsic and the extrinsic pathway. Coagulation was intitiated by factor XII (FXII) of the intrinsic pathway and activated factor VII/tissue factor (FVIIa/TF) complex of the extrinsic pathway. These pathways then converged at the prothrombinase complex (FXa/FVa). Recent publications have changed this view to one that suggests there is no exclusion between the two and they work synergistically (83-85). The process of clot formation occurs in distinct but overlapping steps; initiation, amplification and propagation. The initiation step involves mainly factor VII (FVII), which is found circulating in plasma, binding to tissue factor (TF). FVII is a vitamin-K-dependent plasma protein produced in the liver (86). Factor VII possesses a serine protease active site and has poor catalytic activity until it binds with TF following injury to the vasculature (87). TF complexed with FVII leads to the formation of a fibrin clot and research has shown that under pathological conditions this can be the main route for arterial thrombosis (88). FVIIa/TF activates factor IX (FIX) and factor X (FX) by limited
proteolytic cleavage. FX can also be converted to its active form by activated factor VIII (FVIIIa). Factor VIII is a protein found in plasma mostly complexed to von Willebrand factor (VWF) which becomes activated when released from VWF. Thrombin can also convert FVIII to its activated form FVIIIa via a feedback mechanism. FVIIIa forms a complex with activated FIX, the tenase complex, the result of which substantially increases the amount of FXa generated. FXa is a poor enzyme without association with its cofactor active factor V (FVa), the prothrombinase complex, and through the action of this complex, alongside calcium and phospholipids, converts its substrate prothrombin to active thrombin which leads to an explosive generation of thrombin and formation of a fibrin clot. The large-scale thrombin generation through the interaction of FV, FVIII, FXI and the action of platelets, is part of the amplification and propagation steps of blood coagulation (83). Thrombin generation occurs through a number of steps guided by the tenase complex.

The end product, active enzyme α-thrombin, leads the conversion of fibrinogen to fibrin. Fibrinogen is a plasma protein synthesised in the liver. Converted by thrombin to fibrin, it forms an insoluble polymer that seals the site of injury by forming a haemostatic plug.
Figure 1.8: The blood coagulation cascade (89)
1.2.3 (i) Proteins of the coagulation system

**Factor VII (FVII)**

FVII is a vitamin-K-dependant plasma protein produced in the liver (86) which circulates in an inactive form known as a zymogen. Deficiency in FVII is a rare autosomal recessive bleeding disorder, approx 1 in 500,000 (90). FVII polymorphisms have been associated with an increased risk of cardiovascular diseases particularly polymorphisms in the promoter region of the gene (91). The protein structure of factor VII consists of a Gla site, EGF1 and EGF2 sites (92). FVII gene is regulated in a similar manner to other vitamin K dependant serine proteases expressed in the liver. In addition to binding sites for Sp-1, a ubiquitous transcription factor of the zinc-finger family, reporter gene analysis of the human FVII gene showed that it contained an oestrogen response element which was responsive to ligand bound ERα (93). Hepatocyte nuclear factor-4 (HNF-4), a tissue-restricted orphan receptor, is also important for expression of the factor VII gene (94).

**Tissue Factor (TF)**

Tissue factor is expressed by adventitial cells surrounding blood vessels, vascular smooth muscle cells, pericytes and leukocytes such as monocytes and neutrophils (95-96). TF can also be found within platelets and also within microparticles, which are small membrane fragments released from activated or apoptotic cells (97). TF is also expressed in many tissue types such as brain, lung, kidney, heart, testis and placenta. Mutations in the gene encoding TF have been predicted to lead to a prothrombotic phenotype. Humans with a deficiency in TF have not been identified. TF expression can be induced in many ways, for example by LPS, IL-1, TNF and agents such as estradiol, thrombin and hypoxia (92). Its molecular structure involves a Gla domain that is involved in its binding to a phospholipid surface and an EGF1 domain involved in the binding to FVII and the formation of the FVIIa/TF complex. TF can, depending on its source, play a role in both venous and arterial thrombosis (95). TF expression is regulated by Sp1 which controls basal TF gene expression. AP-1, NFκB and Egr-1 also mediate induction of the human TF promoter in many different cell types. (98)
Factor VIII (FVIII)

FVIII is a plasma glycoprotein expressed in the liver that functions as an essential cofactor in the blood coagulation cascade. Qualitative or quantitative deficiencies of FVIII cause the bleeding tendencies characteristic of the inherited bleeding disorder hemophilia A (99). It is noncovalently bound to VWF until activated to form its role as a cofactor in the coagulation cascade through the activation of FX (100). When there is tissue injury, the FVIII/VWF complex can bind to platelets on a phospholipid surface via glycoprotein IIb/IIIa (gpIIb/IIIa), VWF becomes dissociated and FVIII is activated to FVIIIa. The promoter region of FVIII gene contains binding sites for hepatocyte nuclear factors 1, 3 and 4 (HNF1, HNF3, HNF4), CCAAT enhancer binding protein (C/EBP) and NFκB. The presence of C/EBPβ, C/EBPδ and NFκB which are involved in the acute phase response suggest that FVIII may be influenced by inflammatory cytokines (101).

Factor IX (FIX)

FIX is a vitamin K dependant serine protease expressed in the liver. FIX plays a key role in the intrinsic pathway of blood coagulation and is activated in the presence of calcium ions by the TF:FVIIa complex or by FXIa. FIX plays a role in the development of venous thromboembolism (102). The protein contains a calcium binding EGF domain and a vitamin K dependant Gla domain. A deficiency in FIX leads to haemophilia. Haemophilia B is an inherited, X-linked bleeding disorder caused by mutations in the gene for FIX. Binding sites on the promoter include a region for the transcription factor nuclear factor-1 liver (NF1-L) and C/EBP binding (103).

Factor X (FX)

Factor X is a vitamin-K-dependant coagulation factor which may be activated by the FVII/TF complex or the tenase complex. FX protein is in the form of two chains bound together with 1 or more disulphide bonds; the light chain contains 2 EGF-like domains, while the heavy chain contains the catalytic domain which is structurally homologous to those of the other haemostatic serine proteases. The gene for FX has a highly homologous domain structure to that of the other vitamin K dependant coagulation factors. It contains an SP-1, nuclear factor gamma (NF-γ) and HNF-4 binding site, HNF-4 has been identified as a regulator of ER-mediated transcription (104) and SP-1 binding sites have also shown to interact with oestrogen receptors (105).
**Factor V (FV)**

FV is expressed in the liver as a serine protease. As FV plays such a pivotal role in the conversion of prothrombin to active thrombin and the formation of a fibrin clot, mutations in the gene encoding FV could lead to a state of hypercoagulability. One such mutation is the widely known and most common factor V Leiden mutation or G1691A mutation. Factor V Leiden causes the inability of protein C to inactivate FV which normally regulates the amount of thrombin produced (106) and therefore APC resistance, see section 1.2.3 (ii). In this disorder the excessive clotting usually occurs in the veins leading to deep vein thrombosis (107). The lack of inactivation of FVa results in a predisposition to thrombosis, which is caused by the hypercoagulable condition. One copy of the mutated gene appears to be present in approximately 17–20% of patients with venous thromboembolic events in the general Caucasian population and heterozygous carriers of factor V Leiden have a three- to seven-fold increased risk of VTE, while homozygous subjects have a 50- to 100-fold increased risk (108-109). Women carrying this mutation and using HT have an increased risk of VTE which may be explained by the fact that oestrogens can induce an increase in acquired resistance to APC, leading to higher levels of activated FV (110).

**Von Willebrand Factor (VWF)**

The glycoprotein VWF is produced by Weibel-Palade bodies in the endothelium and megakaryocytes. VWF facilitates in platelet adhesion and aggregation and is normally found circulating bound to coagulation FVIII. VWF can also bind collagen after blood vessel injury. VWF deficiency, dysfunction or absence manifests as von Willebrand disease (VWD) which leads to excessive bleeding. VWD is the most common inherited bleeding disorder, found in 1 in 100 women (111). VWF gene is located at the short arm of the human chromosome 12 (12p13.2), contains 52 exons, and spans about 180 kb. Various binding functions of VWF are localized to different domains of this molecule including binding to platelet glycoprotein Ib (GP1b), heparin, and minor binding sites for collagen types I and III.6 (112).

**Prothrombin**

Prothrombin is a plasma glycoprotein that is vitamin-K-dependant produced by the liver. It is the precursor to the serine protease thrombin (FIIa), a key enzyme in haemostasis and thrombosis. Prothrombin is converted to thrombin in a number of steps. The initial
cleavage site at Arg320 produces meizothrombin which is followed by cleavage at Arg271 to produce the active enzyme α-thrombin and the by-product, prothrombin fragment 1.2 (86). The prothrombin G20210A mutation has been associated with increased levels of prothrombin and is clearly associated with an increased risk of venous thrombosis. This variation in the prothrombin gene is the second most common cause of inherited thrombophilia. The mutation involves a single base-pair substitution, from guanine to adenine, at nucleotide 20210 in the 3’ untranslated region of the gene (113). Carriers of this mutation have a higher risk of thrombosis due to the increased plasma levels of prothrombin, approximately 2-5 fold (114). This risk is increased 25 fold in women using HT as shown in the Estrogen and Thromboembolism Risk (ESTHER) study (115). Transcription factor binding sites in the enhancer region of the prothrombin gene contribute to the transcriptional activity of the promoter which includes sites for hepatocyte nuclear factor HNF4, HNF1-α, HNF3-β and Sp1 and Sp3. The prothrombin enhancer plays a major role in regulation of prothrombin expression. The transcription factors HNF4-α, HNF3-β and Sp1/Sp3, have also been found to play an important role in the regulation of prothrombin expression (116).

Fibrinogen

Soluble fibrinogen is converted into an insoluble fibrin polymer, a process which occurs over several stages. The first stage of this fibrin formation involves the cleavage of fibrinogen to fibrin monomers, fibrinopeptide A and fibrinopeptide B. These monomers interact non-covalently to form a fibrin mesh to form strands, the basis of the fibrin polymer which is then cross-linked by FXIIIa and calcium to form the fibrin clot. Inherited disorders of fibrinogen are rare and those with abnormalities in fibrinogen have shown to have both haemorrhagic and thrombotic events (117). Fibrinogen acts as a signalling molecule and is important in adhesive processes required for the trafficking of immune cells required during wound healing. In addition, both fibrinogen and fibrin play a role in tumour genesis by promoting angiogenesis essential for tumour growth (86). Fibrinogen is a glycoprotein containing 2 copies each of 3 polypeptide chains (α, β and γ) each encoded by a separate gene (Figure 1.9). Fibrinogen β is an important regulator of synthesis of the fibrinogen molecule and polymorphisms in this gene which lead to elevated fibrinogen levels have been linked to arterial thrombosis (118). Increased plasma fibrinogen levels have been directly associated with an increase in risk of cardiovascular disease, including ischemic heart disease and stroke (119), however
debate persists whether this is a direct effect or whether it is secondary to other changes related to the atherosclerotic process. A common G/A polymorphism in the gene for the fibrinogen beta-chain (FGB G-455A) is associated with elevated fibrinogen levels (120). The β-chain fibrinogen gene contains several regulatory sites including IL-6 responsive elements and HNF-1 site (121-122). The IL-6 sites are considered to be important in mediating raised levels as a result of an acute phase response (123).

Figure 1.9: The structure of Fibrinogen (124)
1.2.3 (ii) Inhibitors of the coagulation pathway

The coagulation system is regulated by a series of anticoagulant pathways that ensure a localized response. This is necessary to avoid unwanted activation of the coagulation pathway and therefore excess fibrin generation and deposition and vessel occlusion.

**TFPI pathway**

![TFPI pathway](image)

The initiation of coagulation through TF/FVIIa is shut down through the action of tissue factor pathway inhibitor (TFPI) (Figure 1.10). TFPI is predominantly produced from the microvascular endothelium and is found in three distinct regions. The first is in the circulation and contains both free TFPI and TFPI that is bound to plasma lipoproteins. The second is found within the cytoplasm of platelets, and the largest 80% is bound to the endothelium. Ten percent of the total TFPI is contained within platelets and is released in response to thrombin and other stimulants (126). TFPI is a 276-aa Kunitz-type inhibitor (Mr=42 kDa) consisting of a negatively charged N terminus, 3 consecutive Kunitz domains, and a positively charged C-terminal tail. The first Kunitz domain binds and inhibits FVIIa, the second FXa and the third is proposed to bind to the vessel wall (127).
TFPI inhibits the FXa/TF/FVIIa complex in a 2-stage process. First, TFPI binds and inactivates FXa. Secondly, TFPI/FXa forms a quaternary complex with TF/FVIIa, thus inhibiting thrombin generation. TFPI also causes monocytes to degrade and internalize TF/FVIIa complexes on the cell surface (128).

Activated protein C pathway

Thrombin is not only procoagulant as described above but it can also activate an anticoagulant pathway, the protein C pathway (Figure 1.11). When thrombin binds to the cellular receptor thrombomodulin, then FV, FVIII, FXI and fibrin are no longer preferred substrates. Thrombomodulin can be found on the surface of endothelial cells and its preferred substrate is protein C which generates activated protein C (APC). The cleavage of protein C to APC is accelerated by the endothelial protein C receptor (EPCR) on the surface of endothelial cells. APC becomes dissociated from EPCR and APC binds to its cofactor protein S. Protein S is a vitamin-K-dependant plasma protein that is inactive until bound to APC. APC along with its cofactor protein S can inactivate further thrombin generation by inactivating the cofactors FVa and FVIIIa, therefore shutting down the prothrombinase and tenase complexes. APC cleaves two sites on FVIIIa to inactive tenase activity. APC cleaves FVa in a two-phase process which in patients with the factor V Leiden mutation cannot be completely accomplished leaving FVa active, an increased risk of thrombosis and these patient phenotypes being APC resistant (129).
1.2.3 (iii) Proteins of the APC pathway

**Protein C**

Protein C is a vitamin-K-dependent plasma protein which circulates as an inactive zymogen. It is synthesized predominantly in the liver, but recent evidence suggests that in mice, the epididymus, kidney, brain, lung, and cells from male reproductive tissues are also sites of its synthesis (130). It becomes activated to APC through binding via its Gla domain to the endothelial protein C receptor (EPCR) and through thrombin bound to thrombomodulin. Once activated, APC binds protein S and inhibits coagulation. Protein C deficiency is a rare autosomal recessive disorder that increases the risk to VTE (131). The promoter region of the protein C gene contains regulatory sites for HNF-1 and two overlapping binding sites for HNF-3, an Sp-1 site and a unique regulatory element (designated PCE1). HNF1-α is reported to be an activator of the protein C promoter. Transactivation of the gene via HNF3 is relatively low and the binding of this transcription factor is thought to facilitate the binding of HNF1 to the promoter thereby increasing the rate of transcription. A deficiency of protein C combined with factor V Leiden mutation is associated with an increase in thrombotic disease (132).

**Protein S**

Protein S is a vitamin-K-dependant protein produced in the liver that inhibits blood coagulation by serving as a non-enzymatic cofactor for activated protein C in the protein C anticoagulant pathway. It circulates in two forms, unbound protein S that acts as a cofactor for protein C, and protein S that is bound to C4b-binding protein and has no function in the APC pathway. Protein S acts as a cofactor of TFPI in the down regulation of factor X activation, which provides a mechanistic basis for the APC-independent anticoagulant activity of protein S in plasma (133). Low levels of protein S are a risk factor for the development of deep venous thrombosis. The regulation of protein S levels through transcriptional regulation of the gene involves a pivotal role for Sp1. At least four Sp-binding sites have been identified. Binding sites for the hepatocyte-specific forkhead transcription factor FOXA2, nuclear factor Y, and the cAMP-response element-binding protein/activating transcription factor (CREB/ATF) have also been identified (134). In general, the prevalence of protein S deficiency is ~1 in 500. Approximately 1-2% of patients that present with DVT are heterozygous for protein S deficiency. Although the genetic hereditability of protein S deficiency is relatively rare, many other
conditions lead to acquired protein S deficiency such as hormonal state as illustrated by decreased protein S levels during pregnancy and oral contraceptive use (135-136).

**Thrombomodulin**

Thrombomodulin is a non-enzymatic endothelial cell surface receptor. It is an integral membrane glycoprotein consisting of a single 559-residue polypeptide, with five domains (137). Thrombomodulin binds thrombin, changes thrombin conformation and allows thrombin to activate protein C and thrombin-activatable fibrinolysis inhibitor (TAFI). Activated protein C and TAFI inhibit coagulation and fibrinolysis, respectively. Thrombomodulin plays an important role in thromboresistance. Thrombomodulin expression levels are influenced by multiple gene polymorphisms. Several of the polymorphisms are possibly associated with coronary heart disease (138).

**Endothelial protein C receptor (EPCR)**

EPCR is a transmembrane receptor for activated protein C. It is expressed by endothelial cells and is structurally similar to the major histocompatibility complex class 1/CD1 family of proteins (139). The encoded protein is an N-glycosylated type I membrane protein that enhances the activation of protein C. When protein C becomes activated, it remains bound to EPCR before associating with protein S on the surface of platelets or endothelium. Mutations in this gene have been associated with venous thromboembolism and myocardial infarction, as well as with late foetal loss during pregnancy (140). The EPCR gene contains many regulatory sites in the promoter region (141), since it does not contain the core promoter TATA sequence it has various transcription initiation sites particularly Sp1 sites, an Ap-1 site, NF-1 site and two Ap-2 sites which initiate its transcription and expression (142-143). A soluble form of EPCR has been identified (sEPCR) that may be procoagulant (144), other forms have also been identified that include point mutations and polymorphisms of the gene. A 23 base-pair insertion in exon 3 results in the failure of EPCR to bind protein C and has been suggested as a risk factor for both arterial and venous thrombosis (145). Another point mutation has been implicated in thrombosis, the T-318G substitution is associated with late foetal loss (146). Many polymorphisms have been described in the EPCR gene which leads to varied risk of thrombosis (147-149), these functional changes in the gene may increase or decrease the risk of thrombosis, particularly in carriers of prothrombotic mutations (150).
Antithrombin pathway

Antithrombin, is a serine protease inhibitor expressed in the liver. FIXa, FXa, FXIa and thrombin are all inactivated by antithrombin. The ability of antithrombin to inhibit FIXa, FXa, FXIa, and thrombin is accelerated by heparin sulphate proteoglycans which is the basis of the anticoagulant action of heparins. Antithrombin when bound to free thrombin and FXa removes these from the circulation thereby limiting their activity to the site of clot formation. When antithrombin binds thrombin it forms the thrombin-antithrombin (TAT) complex which can be measured in a clinical setting as a marker of thrombin generation (151). Deficiency in antithrombin is a genetic disorder that is associated with venous thromboembolism, venous thrombosis in pregnancy and foetal loss. There are two types of deficiency which classify the thrombotic level of the disorder (152). Antithrombin deficiencies are uncommon with a prevalence of between 1 in 500 and 1 in 5000 in the general population (153). The regulation of the antithrombin gene involves two elements that are capable of promoting antithrombin gene transcription in HepG2 and COS1 cells. Two liver-enriched transcription factors, hepatocyte nuclear factor 4 (HNF4) and CCAAT enhancer-binding protein (C/EBPa) bound to the 5' upstream element have also been identified which modulate the transcription of the antithrombin gene (154).

Figure 1.12: The antithrombin pathway (125)
Other inhibitors of the coagulation pathway include Cl-inhibitor which inhibits FXIIa, FXIa and kallikrein, $\alpha_1$ antitrypsin which inhibits FXIa, and $\alpha_2$ macroglobulin which is a secondary inhibitor to many proteinases involved in the coagulation pathway, including kallikrien, thrombin and the fibrinolytic enzyme plasmin (155-157).

**Fibrinolysis**

Fibrinolysis is a mechanism that limits the formation of fibrin clots. The fibrinolytic system is a series of enzymes which is initiated after the formation of fibrin, and functions by breaking down this protein into fibrin degradation products (Figure 1.13). Fibrinolysis also plays a role in ovulation, embryo implantation, tissue remodelling and inflammation (158).

The active enzyme of the fibrinolytic pathway responsible for fibrin breakdown is plasmin. Plasmin is formed by the activation of the zymogen plasminogen. The formation of plasmin can occur through various different activators. These plasminogen activators along with their inhibitors play a crucial role in controlling fibrinolysis. FXIIa, FXIa and kallikrien are all capable of converting plasminogen to plasmin. The main activator is t-PA, a serine protease produced by the vascular endothelium. The tPA-mediated activation of plasminogen is slow until accelerated by the presence of fibrin. Conversion of fibrinogen into fibrin is accompanied by conformational changes that result in the exposure of multiple binding sites and modulation of various activities. The exposure of tPA- and plasminogen-binding sites provides effective activation of plasminogen on the fibrin surface. Interaction of plasminogen with fibrin is mediated through lysine (Lys)-binding sites. Interaction of tPA with fibrin is mediated primarily by its finger domain and one of its two kringle domains in a Lys-independent and Lys-dependent manner, respectively (22).

Urokinase-type plasminogen activator (uPA) also functions as a plasminogen activator. This is a naturally occurring enzyme found in human urine, blood and on the extracellular matrix via the urokinase receptor.
Figure 1.13: The fibrinolytic system. Tissue plasminogen activator (tPA), urokinase type plasminogen activator (uPA), Plasminogen activator inhibitor 1 (PAI-1) (159).

1.2.3 (iv) Proteins of the fibrinolytic system

*Plasminogen*

Plasminogen is a glycoprotein produced in the liver in its inactive form. There are two distinct forms of plasminogen and it is the native full length protein, 791 amino acids in length, which has the highest affinity for fibrin (160). Plasminogen can bind to fibrin via lysine binding sites on the heavy-chain portion of the plasminogen molecule; the subsequent binding of a plasminogen activator leads to cleavage of the plasminogen molecule at the Arg561–Val bond and the formation of the active protease plasmin, the enzyme responsible for the lysis of fibrin to fibrin degradation products (161). Within the plasminogen molecule are five kringle domains which interact with lysine-like ligands and facilitate the binding of plasminogen to fibrinogen. The gene also encodes for HNF-1 and AP-3 sites which regulate the transcription of the gene [156], and there is also an IL-6 binding site (162). Plasminogen deficiency results in venous thrombosis but there are limited clinical studies on this, a mutation in the gene, 601 Type-1, is highly frequent in the Japanese population resulting in the patients suffering with venous thrombosis (163).
*Tissue plasminogen activator (tPA)*

The major activator of plasminogen *in vivo* is tissue plasminogen activator (tPA), a serine protease produced by endothelial cells and metabolised by the liver. In the absence of fibrin, tPA is an inefficient activator of plasminogen but once bound to fibrin, activation is greatly accelerated and plasminogen is converted to plasmin. The plasma levels vary but have been shown to increase during the menopause (164) leading to an increased risk of thrombosis. As a marker of the up-regulation of endogenous fibrinolysis, tPA might be expected to be associated with a lower incidence of vascular disease but as it is mainly a marker in a complex between t-PA and its inhibitor PAI-1, rather than a measure of free t-PA, it might be expected to be associated with a higher incidence of vascular disease (165). Regulatory binding sites on this gene include an Ap-1 site, HNF-3β, NFκB, c-Jun and CREB binding sites (166).

*Urokinase plasminogen activator (uPA)*

uPA is an inactive zymogen expressed and secreted as a single-chain polypeptide. After secretion, uPA binds to its receptor, uPA receptor (uPAR) and is cleaved at position 157 by plasmin into an active two-chain molecule. The mature uPA protein remains specifically bound to its receptor and provides an inducible, transient and localized cell surface proteolytic activity. It then functions to catalyse the cleavage of plasminogen to plasmin (167).

1.2.3 (v) **Inhibitors of fibrinolysis**

The control of fibrinolysis is also under the influence of various fibrinolytic inhibitors. These include α₂-antiplasmin and plasminogen activator inhibitors and thrombin-activatable fibrinolysis inhibitor (TAFI). The function of these inhibitors is to inactivate and remove the enzymes involved in the downstream cleavage of fibrin.

*α₂-Antiplasmin*

α₂-Antiplasmin is the main plasmin inhibitor expressed in the liver. It rapidly binds to plasmin via lysine binding sites to form an irreversible complex, the plasmin-antiplasmin (PAP) complex. In a clinical setting this PAP complex can be measured as a marker of plasmin production. This inhibitor also regulates fibrinolysis by inhibiting adsorption of plasminogen to fibrin and making fibrin more resistant to plasmin through cross-linking via FXIIIa (168).
Inhibitors of the activation of plasminogen also control and regulate fibrinolysis. There are four types of plasminogen activator inhibitors, PAI-1, PAI-2, PAI-3 and protease nexin.

**PAI-1**

PAI-1 is the primary inhibitor of tPA, uPA and plasminogen, it also plays a role in angiogenesis and cell migration (169-170). PAI-1 is produced by the endothelium and is released through stimulation from thrombin and endotoxins. PAI-1 is bound to the majority of circulating tPA hence preventing unwanted fibrinolysis in the developing thrombus. Genetic disorders of PAI-1 deficiency have been reported to cause haemorrhaging and an increase in its concentration has been linked with obesity and also with some cancers which could possibly be the link between these diseases and thrombotic events (171-172). Inhibitors of PAI-1 are currently being developed that could possibly benefit several diseases such as CVD and metabolic syndrome as PAI-1 may play a role in the initial development of such diseases (173). Both tPA and PAI-1 antigen levels in plasma are linked with cardiovascular risk, however both are clustered with other risk factors including insulin resistance and triglyceride levels (174-176). Active PAI-1 has a short half life in circulation and can not be stored in cells. As a result, transcriptional control of PAI-1 plays an important role in regulating tissue and plasma PAI-1 levels. PAI-1 is regulated by a diverse range of signals including steroid and peptide hormones, hypoxia, acute phase responses as well as mechanical and physical stresses. PAI-1 expression can be modulated by the cytokine IL-1 in murine and human hepatocyte cell lines (177-178). TGFβ and glucocorticoids can also alter the expression of PAI-1 in non-parenchmal hepatocyte cells via an extracellular signal-regulated kinase (ERK) signalling pathway (179). Numerous regulatory elements in the human PAI-1 promoter have been identified including, a VLDL response element, SMAD, SP1 and TGF-β responsive sites (180). In endothelial cells, an NFκB site has been shown to mediate TNF-α induced expression of PAI-1 (181).
**PAI-2**

PAI-2 is important in pregnancy as it can only be found in the plasma of pregnant women. PAI-2 is produced mainly by trophoblasts in the placenta (182) and inhibits uPA and tPA. In comparison with PAI-1, we know little about the role of this pregnancy induced serpine except that it may play a role in inflammation and wound healing (183). PAI-3 and protease nexin are also both inhibitors of uPA and tPA.

**TAFI**

Thrombin activatable fibrinolysis inhibitor (TAFI) is a carboxypeptidase synthesized in the liver as a 55kDa protein (184). The protein becomes activated by cleavage at a single site Arg-92, from trypsin, plasmin, thrombin or meizothrombin. It circulates in plasma to inhibit the continuous cleavage of fibrin by removing c-terminal lysine residues that are essential for plasmin formation (185). Altered TAFI levels are associated with a reduced capacity to remove fibrin clots from circulation and therefore results as a risk factor for coronary heart diseases (186). As part of the study from the Leiden Thrombophilia Study, results showed that those presenting with VTE had higher levels of TAFI expression (187) indicating an increase in these protein leads to an increased risk of thrombosis. Regulatory sites on the gene include p300, ATF and C/EBPα transcription binding sites (188).

The overall mechanism of fibrinolysis leads to the enzymatic breakdown of fibrin which results in fibrin degradation products (FDPs). One unique marker of fibrin degradation is the presence of D-dimer in the blood that is used in assays on measuring thrombotic disorders, for example VTE, as it is a specific marker of fibrin clot formation as well as dissolution (189).

Decreased overall fibrinolytic potential and high plasma levels of thrombin-activatable fibrinolysis inhibitor have been consistently associated with risk of venous thrombosis, whereas little evidence exists for a role of plasminogen, alpha2-antiplasmin, tissue plasminogen activator, and plasminogen activator inhibitor 1 in venous disease (190).
1.3 Haemostasis in post menopausal women

1.3.1 Effect of the menopause

It has long been established that the plasma concentrations of some coagulation factors increase with age such as FV, FVII, FVIII, FIX and fibrinogen (191) and VWF (192). The Northwick Park Heart Study (NPHS) was the first longitudinal study to investigate the relationship between haemostatic variables and the menopause. After adjustment for age, significant increases for FVII, antithrombin and fibrinogen were observed in naturally menopausal women, those with surgical menopause had similar results but due to the small number of patients these changes were not statistically significant (193-194). Since then several other studies have reported an increase in FVII, fibrinogen and PAI-1 in menopausal women (119, 195-199).

Conflicting data from the Study of Women's Health Across the Nation (SWAN) study showed that there was no statistically significant association between haemostatic factors and menopausal status. They reported that higher circulating endogenous estradiol suppressed PAI-1 and tPA and there was no association between estradiol and FVII-c (200). These results were supported by in vitro work that showed that 17β-estradiol inhibits PAI-1 synthesis in endothelial cells (201). C-reactive protein (CRP) is reported to remain unchanged during the menopausal transition (202). CRP is an acute phase reactant involved in inflammatory reactions which has been shown to be a marker for cardiovascular disease (203-204). Most studies have not assessed endogenous estradiol in relation to haemostatic factors, but focused on the effects of oral contraceptives, post menopausal hormone therapy, or cross-sectional comparisons of pre- vs. post-menopausal women.

1.3.2 Effect of HT on haemostasis

Hormone therapy (HT) is prescribed to menopausal women in order to alleviate climacteric symptoms. It was long believed that HT also has the ability to prevent certain diseases including CVD and osteoporosis and therefore prolong life in postmenopausal women (205). Although this presumption was largely based on lipid data, the oestrogen component of HT was also shown to have a positive effect on haemostatic proteins in particular plasma fibrinogen (206-207). Oestrogen was also believed to increase the
overall potential for fibrinolysis (208). Publication of the WHI randomised trial results contradicted these studies on menopausal women using HT and showed that HT was not beneficial in preventing cardiovascular disease and also increased the risk of venous thrombosis in women taking HT (14-15). The pathogenesis of this increased risk was not understood but changes in the haemostatic system are believed to be involved. Table 1.2 provides a summary of the most common changes in plasma.

<table>
<thead>
<tr>
<th>Haemostatic Marker</th>
<th>Effect of HT</th>
<th>Levels following HT administration</th>
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</thead>
<tbody>
<tr>
<td>Fibrinogen, Factor VII</td>
<td>↓</td>
<td>3g/ltr, 616ng/ml²⁰⁹³</td>
</tr>
<tr>
<td>Antithrombin, Protein C, Protein S</td>
<td>↓</td>
<td>0.96IU/ml, 1.02IU/ml, 92.9% activity²¹⁰³</td>
</tr>
<tr>
<td>tPA and PAI-1</td>
<td>↓</td>
<td>6.3ng/ml, 3IU/ml²⁰⁹³</td>
</tr>
<tr>
<td>PF 1.2, FDPs, PAP, APC resistance, Factor VIIa</td>
<td>↑</td>
<td>1.52nmol/L²⁰⁹³, 309.2ng/ml, 523.8ug/l, 3.05, 86mU/ml²¹⁰³</td>
</tr>
<tr>
<td>TFPI</td>
<td>↓</td>
<td>72.4ng/ml²⁰⁹³</td>
</tr>
</tbody>
</table>

Table 1.2: The plasma changes in haemostasis after hormone therapy.

Although HT reduces the concentration of plasma coagulation factors such as fibrinogen and factor VII, it also reduces the levels of key coagulation inhibitors such as protein C, protein S and TFPI. In addition the development of assays which can measure prothrombin to thrombin conversion e.g. Prothrombin Fragment 1.2, Thrombin-Antithrombin complex and fibrin turnover via FDPs, has allowed investigators to measure the impact of oral oestrogen use in vivo. Studies have shown that HT can lead to increased prothrombin fragment 1.2, D-dimer and also decreased protein S and antithrombin activity (210). APC resistance has also been shown to be increased with HT use (210-211). Increased levels of markers of thrombin activation and decreased levels of inhibitors of coagulation lead to an unstable haemostatic state that can culmination into a prothrombotic one. Post-menopausal women have been reported to have increased levels of antithrombin, protein C and protein S therefore the changes observed during HT may simply be reversing the menopause induced changes. However in individuals with a thrombophilia or other risk factors for thrombosis this HT induced activation may lead to a clinical event. Although numerous studies have been carried out investigating the
effects of HT on haemostasis, the preparations used have varied in terms of composition and route of administration. These variations are known to be significant with respect to thrombosis risk and are also reflected in differences in haemostatic changes.

1.3.2 (i) Role of HT composition

Combined oral HT consists of a synthetic progestin combined with oestrogen (see section 1). Progestogens include progesterone, natural micronized progesterone and synthetic progestins derived from progesterone (pregnanes and 19-norpregnanes) such as medroxyprogesterone acetate (MPA) and norethisterone acetate (NETA) or from testosterone (19-nortestosterones). Progestins have varying pharmacological properties depending on the molecules from which they are derived, either progesterone or testosterone. Very small structural changes in the parent molecule may induce considerable differences in its activity (212). The effects of progestins are related to interactions not only with progesterone receptors but also with other steroid hormone receptors. Norpregnane derivatives, including nomegestrol acetate and promegestone, appear to have a very high activity, and unlike nortestosterone derivatives, they do not possess androgenic, estrogenic, or glucocorticoid activity (213). Norpregnane derivatives bind almost exclusively to the progesterone receptor and do not interfere with the other steroid receptors. Their affinity for the progesterone receptor is higher than the progesterone one. In addition, these progestins have both antiestrogenic and antigonadotropic actions (5).

In healthy postmenopausal women, estradiol combined with norethisterone acetate (NETA) increased D dimer levels, showed no change on fibrinogen and decreased protein C (214). In a different study D-dimer was also increased on HT treatment but this time with a different progestin component, medroxyprogesterone acetate (MPA) (215). Norris et al showed that there was no significant difference between HT with two different progestins, trimegestone (a norpregnane progestin) and dydrogesterone, on some of the haemostatic markers but there was a difference in others, namely protein C and PAP complex indicating that for these markers, the type of progestin may be important (210). A difference in tPA was shown in one study which reported that 17\(\beta\)-estradiol plus NETA decreased this antigen more than estradiol plus drospirenone (216). In HT formulations that used unopposed oestrogens, levels of FVII were higher than those with combined HT and the markers of fibrinolysis tPA and PAI-1 were lower.
Changing the progestin components of HT can have a marked effect on fibrinolytic activity as shown in a comparative study of norpregnane and pregnane progestins combined with 17β-estradiol (217). Since PAI-1 is a risk factor for cardiovascular disease the choice of progestin to include in HT may be of importance particularly with women predisposed to CVD due to lifestyle factors or a previous cardiovascular event.

Data from epidemiological studies suggests that norpregnane derivatives may be thrombogenic, whereas micronized progesterone and pregnane derivatives appear safe with respect to thrombotic risk however the numbers of women in each subgroup was small and further data is required to confirm these findings (218). Studies have reported an increase in VTE risk in users of combined HT compared to those treated with unopposed HT (219-220), emphasising the role of progestin in mediating thrombotic risk. In oral contraceptive users, progestin type has played a significant role in the risk of thromboembolic disease in pre-menopausal women. Haemostatic changes in oral contraceptive users are also affected by progestin type and dose and are thought be implicated in the increased risk of VT in this group.

The dose and type of oestrogen can also influence haemostatic changes in post menopausal women using HT. Recently preparations have come on the market containing low doses of 17β-estradiol (1mg). These preparations are associated with fewer changes or no changes in haemostatic factors. A recent study of low dose oral HT did not show any adverse coagulation changes (221). A study of low-dose hormone therapy showed that a transdermal preparation of combined 17β-estradiol/NETA resulted in a more favourable outcome on coagulation than an oral preparation of an equivalent dose (222). This same oral low-dose was given to a cohort of patients and compared with a conventional dose of HT in a study by Eilertsen et al. The effects between the two groups, a reduction in coagulation factors and inhibitors were similar although more marked in the conventional dose group (223). This study also showed that HT therapy induces an APC resistant phenotype which may be related to dose (224).

Eilertson et al also found an increase of the acute phase reactant; C-reactive protein (CRP), after both low dose and conventional dose HT, changes were significantly more pronounced in the conventional dose group (225). A study comparing the effects of different routes of administration showed that oral HT significantly increased CRP compared to placebo whereas transdermal treatment did not (225). Oral conjugated equine oestrogen (CEE) treatment has also been shown to exert a greater effect on CRP
production compared with transdermal E2 (226). Combined HT and oestrogen-only HT have shown a difference in CRP production. In contrast to these results, the phytoestrogen genistein had no effect on CRP production after treatment over six months compared with HT and placebo (227). A positive role for the inclusion of a progestogen in HT on inflammation is signified by a study which reported that the progestin NETA modifies CRP production to a lesser effect than HT with no progestin, in comparison to transdermal HT which showed no influence of CRP (214). CRP has been shown in various studies to predict cardiovascular events. Specific markers of coagulation; fibrinogen, fibrin and FDPs, have been shown to induce CRP production, specifically in vascular smooth muscle cells (228) and CRP has induced tissue factor expression in vitro (229). CRP is a member of the pentraxin family. It comprises 5 noncovalently associated protomers arranged symmetrically around a central pore and has a molecular weight of 118 000 Da. It is a non-glycosylated protein in humans and the gene has been mapped to chromosome 1. The production of CRP is predominantly under the control of IL-6. However, the cytokines IL-1 and tumour necrosis factor may also contribute to hepatic synthesis and secretion of CRP (230).

There are two different types of oestrogens used in HT, CEE’s and 17β-estradiol. Both preparations elicit pharmacologic oestrogen-like activity but the response varies. CEE and estradiol can both be regarded as natural oestrogens but only estradiol as a natural human steroid. Estradiol is a simple compound and is the major biologically active oestrogen in humans whereas CEE is a complex compound with many different components (231). Their different metabolism is shown by the response of treatment with transdermal 17β-estradiol which provides higher estradiol levels than corresponding doses of CEE that provide higher levels of estrone and estrone sulfate. This difference reflects the hormonal compositions of the different drugs as well as the consequences of the hepatic first-pass metabolism effect with oral use (232).

Although many studies have investigated the effects of combined HT on haemostasis, oestrogen-only preparations which are taken by women without an intact uterus also have effects. In a study comparing both estradiol-alone and estradiol with a progestin versus placebo both preparations caused a shift towards a procoagulant state (233). Also APC resistance was increased in estradiol-alone in both a transdermal and oral group with a more pronounced effect in the oral group which may give rise to venous thrombosis in users of this type of HT (234). The dose of oestrogen is probably a crucial factor, reduced fibrinogen, FVII and antithrombin have been shown in oral and transdermal preparations,
but oral oestrogen changes in haemostasis are dose dependant (235). The net effect of oestrogen depends on the type oestrogen used, the dose and the route of administration

1.3.2 (ii) Route of administration

Biological evidence lends support to the elevated thrombotic risk among users of oral HT and to the difference between HT combinations by route of administration. Lowe et al showed that oral but not transdermal use of HT is associated with increased levels of FIX, APC resistance and a decrease in tPA antigen and PAI-1 activity. This group showed that transdermal HT was favourable in terms of thrombotic risk with lower levels of FVII, FIX and FVIII compared to oral HT (211). A randomised control trial has shown that, compared to oral HT, transdermal HT had little or no impact on coagulation and fibrinolysis particularly with respect to prothrombin fragments (F1+2) and antithrombin (236). Acquired APC resistance, a risk factor for venous thrombosis, has been found in oral HT users, but not in transdermal HT users (237). Scarabin at al reported that transdermal oestrogen combined with micronized progesterone had little or no effect on blood coagulation activation as shown by the absence of significant variation in plasma concentration of prothrombin fragment 1+2 (236).

These results emphasise the importance of the route of administration in prescribing hormone therapy. Transdermal oestrogen therapies do not invoke the same hepatic response as seen in oral HT (238). Oral HT can result in wide fluctuations in plasma hormone levels between patients and even within patients on a day to day basis. By avoiding the hepatic portal vein the transdermal mode of HT delivery avoids the high hormone levels of the liver (239). Upon oral ingestion of HT, the majority of the oestrogen is carried to the liver via the hepatic portal vein. Here oestrogens undergo hepatic modification to various oestrogenic conjugates, therefore reducing the bioavailability of oral oestrogen. As a results of this 'first pass' effect, large doses of oestrogen are needed to ensure therapeutic levels of active hormone are available. For example, 1-2mg oral E2/day provides the equivalent therapeutic dose as 50-100μg transdermal E2/day (238). High levels of oestrogens in the hepatic portal vein lead to alterations in the expression and clearance of proteins involved in haemostasis and therefore an increased thrombotic risk. In addition, oestrogen metabolism is different following oral therapy where the predominant metabolites are estrone and 2-hydroxyestradiol, interaction of these metabolites with their receptors could lead to
differences in oestrogen receptor mediated transcription of many genes involved in
haemostasis and thrombosis both in the liver and in the vascular endothelium. Transdermal therapies avoid this first past effect and conversion of 17β-estradiol to estrone is slower producing a different metabolic profile and downstream effects of on
gene expression. The result of these effects is that the transdermal route of administration
appears to have a less marked effect overall on the haemostatic system compared to the
oral route (222, 240).

1.3.3 Effect of selective oestrogen receptor modulators (SERMs) on haemostasis

RCTs have examined the relationship between haemostatic parameters and the SERMs
tamoxifen, raloxifene, bazedoxifene and lasofoxifene. Tamoxifen therapy is the most
widely investigated and used SERM and is used in patients with breast cancer (241).
Another SERM used in studies on oestrogen-related cancers is ICI 182,780, also known
as Fulvestrant. This compound has shown to be an oestrogen receptor antagonist with no
agonist effects, which works both by down-regulating and by degrading the oestrogen
receptor (242-243). Raloxifene therapy is used for its beneficial effects in patients with
osteoporosis by increasing bone density (244) but has been shown to increase
thromboembolic disease (245). HT with a combination of E2 or raloxifene showed a
slight increase in APC resistance compared to placebo (224), has shown reduced
fibrinogen levels compared with a low dose HT group (223) and a small reduction in
CRP levels compared with a conventional dose HT. Bazedoxifene and lasofoxifene are
two others used in studies of SERMs which have so far shown adverse thrombotic effects
(246-247).
1.3.4 Effect of phytoestrogens on haemostasis

Due to the structural similarity of phytoestrogens to oestrogen and the prothrombotic effects of oestrogen, the effect of phytoestrogens on haemostasis is clearly of interest. Phytoestrogens may act as a natural SERM and studies have analysed whether these compounds may elicit distinct effects on coagulation and fibrinolytic markers. In 2008 Rios et al reported that there was no significant oestrogenic effect of soy isoflavones on haemostasis in postmenopausal women. This study provided postmenopausal women with 40mg isoflavones or a casein placebo capsule. They found that in the isoflavone group there was a decrease in prothrombin fragments 1.2, antithrombin, protein S and protein C. They also found an increase in D-dimer and PAI-1. The results were not statistically significant between the two groups and the placebo group showed a decrease in the inhibitors antithrombin and protein C (249). As postmenopausal women have increased levels of these two inhibitors it may be that isoflavones are counteracting this as the control group are giving the same results and therefore a definitive conclusion cannot be made.

Another study of soy protein consumption by postmenopausal women showed decreased levels of FVIIc in both the treatment and control groups. The soy treatment group showed a decrease in prothrombin fragments 1.2 but was not statistically significant compared to the placebo. No effect of fibrinogen, PAI-1, D-dimer or VWF was detected (250). These results suggest that soy protein supplementation does not affect biological
markers of coagulation or fibrinolysis. This conclusion is supported by Colacuri who in the same year showed no change between an isoflavone treated group versus placebo on thrombomodulin, VWF, tPA, PAI-1, D-dimer, prothrombin and fibrinogen serum levels (251). Another study reported that there was no change in fibrinogen, FVII or PAI-1 levels in perimenopausal women using either a low or high dose soy supplementation versus placebo (252).

In light of these findings soy isoflavones were thought to be neutral with respect to haemostasis and hence may be a safer alternative with respect to cardiovascular risk compared to HT. A particular phytoestrogen, genistein, showed no change in some haemostatic markers while there was a decrease in D-dimer suggesting that genistein could possibly have a cardioprotective role (253). Genistein may also have a neutral if not beneficial effect on cardiovascular risk as it has been shown that treatment with this phytoestrogen does not increase CRP levels whereas HT does (227) and it may also inhibit PAI-1 production in an inflammatory setting via TNFα signal transduction (254).

Although the data from the studies performed to date is encouraging these studies had several weaknesses. It is difficult to assess the intake of soy in the diet and maintain a rigid phytoestrogen-free diet. The metabolism and absorption of isoflavones between patients may vary significantly just as oestrogen concentrations do in women taking HT and the formation of different metabolites from ingestion of these isoflavones may lead to substantial differences with respect to biological effects. For example; daidzein, the major isoflavone in soy is metabolised to equol and O-desmethyl angolensin (O-DMA) in the gut. Approximately 30-50% of the population produce equol and 80-90% produce O-DMA. Dietary studies show that maximal clinical response to soy is found in equol producers (27).

There was also a difference in treatment regimens used in these studies. Rios et al used a capsule of 40mg isoflavones which included a mixture of genistein, daidzein and glycine (249). Teede et al supplied women with 40g soy protein containing 118mg isoflavones twice per day in powder form (250) and Dent et al provided their subjects with 80.4mg/day versus 4.4mg/day for the high and low group, respectively, in the form of a powder and food (252). These differences will all result in different absorption levels between subjects and therefore give variable results.
Another problem evident in these studies is the subject sample number. Small groups of patients such as were used in these studies are inadequate to provide the sufficient statistical power to reach a valid conclusion. From the limited and variable data a definitive conclusion on the safety of phytoestrogen use for thrombotic risk in menopausal women is not available.

1.4 Molecular effects of oestrogens and phytoestrogens

The molecular effects of oestrogens and oestrogen-like substances are mediated via specific nuclear receptors which can bind hormones such as oestrogens and can initiate downstream effects via genomic and non genomic pathways. One of the first discovered nuclear receptors was the oestrogen receptor (ER) (255). ER is a member of the nuclear hormone receptor superfamily and is responsible for mediating the physiological effects of endogenous oestrogen hormones, synthetic oestrogens and anti-oestrogens. This superfamily also includes receptors for thyroid and retinoid hormones and other lipophilic ligands (256). The ER binds steroid hormones and so is termed a steroid receptor which, once ligand-bound, modulates transcription of selected genes under specific conditions (Figure 1.15). There are two subtypes of the oestrogen receptor ERα and ERβ. Genes encoding these receptors are found on two different chromosomes, 6q and 14q respectively (257-259). There are also several splice variants of each ER, the specific function of these variants in normal developmental physiology and in the pathogenesis of disease is relatively unknown (260-261). ERα was initially cloned from rat uterus (255) and it was not until a decade later that ERβ was identified by Kuiper et al from cloning in rat prostate (262). The ER is a part of the class of classical receptors and it has a high affinity for its ligands. Along with the functional domains identified, it was concluded that the ER could regulate gene expression at the mRNA level in a cell type, promoter and ligand-specific manner (259, 262). ERα and ERβ have different transcriptional effects in certain ligand, cell-type and promoter contexts. These two subtypes of ER can also be co-expressed to form functional heterodimers. When co-expressed ERβ has been shown to have an inhibitory effect of ERα-mediated gene expression and in many instances opposes the actions of ERα (261).
Figure 1.15: The classical model of the ER activation process. Estradiol (E2), oestrogen receptor (ER), oestrogen response element (ERE), basal promoter sequence (TATA) and polymerase II (pol II) (263).

1.4.1 Tissue distribution of oestrogen receptors

Oestrogen receptors are present in many different tissue types including tissues not considered classic targets for oestrogens. Both the ERα and ERβ receptors are widely distributed. ERα is mainly expressed in the ovaries, uterus, breast, liver, kidneys and heart while ERβ is mainly in the prostate, colon, ovaries and the lungs. They are also coexpressed in a number of tissues including the brain and bone (261) (Figure 1.16). These receptors interact with ER ligands and cause them to be responsible for growth, differentiation, and the functioning of these various target tissues in the human body.

Figure 1.16: Distribution of ERα and ERβ in the human body (264)
1.4.2 Structure and functional domains of oestrogen receptors

ERα and ERβ have high sequence homology, 97%, in their DNA binding domains and a relatively high sequence homology, 54%, in their ligand binding domains (265). The general structure of the ER consists of five domains each with distinct modes of action. There is a variable amino-terminal A/B, a highly conserved DNA binding domain (DBD), followed by the D domain and a moderately conserved carboxy-terminal the ligand-binding domain (LBD), and also a c-terminal F domain (Figure 1.17). The DBD and LBD are functionally the most important and are highly conserved among the nuclear receptors.

![Oestrogen receptor domain structure](image)

Figure 1.17: Oestrogen receptor domain structure.

Transcriptional activation is facilitated by two distinct activation functions (AF); AF-1 at the N-terminus and AF-2 in the c-terminal LBD. These activator functions recruit coregulatory complexes to a DNA-bound receptor. AF-1 is a constitutive activation function which contributes to the transcriptional activity of the receptor. AF-1 does not appear to require ligand for its function which is in contrast to AF-2 which has a ligand-dependant activation function which is induced upon hormone binding to the receptor. AF-1 function is also barely evident in ERβ compared with ERα (266). Upon ligand binding the receptor becomes dimerized and produces one of three different forms of ER; ERαα, ERββ or ERαβ, which have different ways of signalling and therefore different effects on ER-regulated genes (267).

1.4.2 (i) The DNA binding domain (DBD)

Nuclear receptors bind DNA by their DBD. The classical DBD consists of two zinc fingers, each of which has a zinc molecule surrounded by four cysteine residues. The first of the two zinc fingers is called the P-box and it is this region which directly binds to the
hormone response elements (HREs) in the promoters of target genes. The second zinc finger is called the D-box and this is involved in the dimerization of the nuclear receptors. The DBD recognizes specific sites on DNA HREs or in this case oestrogen response elements (EREs).

1.4.2 (ii) The ligand binding domain (LBD)

The LBD is a multifunctional domain containing regions that mediate ligand binding and regions for dimerization of receptors. In addition to these, the LBD also functions in ligand-dependent activation, heat-shock protein association, nuclear localization and repression and the binding of nuclear receptor coregulators via AF-2 (268). The LBDs are conserved in both the ERα and ERβ and both exhibit similar affinities for 17β-estradiol (269-270). In the absence of ligand, the ER is in an inactive state within the cells' nuclei. Stabilized in this manner the ER is in association with heat shock protein-90 (Hsp90) which masks the DNA binding domain of the receptor. Binding of a ligand to ER triggers conformational changes to occur and these changes culminate to a change in the rate of transcription either through activation or repression of ER-regulated genes. The binding of ligand to ERs transforms the receptor into its active state by mediating the dissociation from HSPs and allowing dimerization of the receptor leading to transcription. The phytoestrogens bind to ER in a manner that exerts similar effects to E2 (41) but has preferential binding to the beta isoform (271).

1.4.3 Modulation of the oestrogen receptor with ligands

The ability of nuclear receptors to alternate between activation and repression in response to specific molecular signals is now known to be attributable in large part to a diverse group of cellular factors, the coregulators. Coregulators are either in the form of activators or repressors of gene transcription. The p160/src family, namely SRC1, SRC2 and SRC3, the CBP/P300, and the TAP/DRIp complex act as coactivators of transcription while negative coregulators include Rip40, DAX-1 and SHP, and corepressors include NCoR and SMRT (4). The first direct evidence of ligand-dependent recruitment by nuclear receptors of ancillary molecules was determined by Shang et al (272). A recurring structural feature of many coactivators is an alpha-helical LXXLL motif, or nuclear receptor box, present from a single copy to several copies in many
coactivators, which is implicated in their ligand-dependent recruitment by the receptor AF-2. The SRC/p160 coactivator family, for example, has a conserved cluster of NR boxes located in the central region of each member of the family (273). Transcriptional repression by corepressors is comparable to the mediation of receptor transcriptional activation by coactivators. The recruitment of corepressors, generally occurring in the absence of ligand, depends on a critical conformation of the receptor AF-2 domain, as well as upon nuclear receptor box-like helical motifs in the corepressor known as CoRNR boxes. In knock-out mice, studies showed that coactivators are required for physiological and developmental functions of steroid and thyroid hormones in living animals, and that corepressors too have crucial roles in the development of certain organs (274).

A spectrum of post-translational modifications such as phosphorylation and ubiquitination are known to regulate the functional relationships between nuclear receptors, their coregulator complexes and their target gene networks.

1.4.4 Molecular basis of agonism and antagonism

Oestrogens and anti-oestrogens both bind in the LBD of ER. This binding must differ as oestrogens activate transcription whereas anti-oestrogens do the opposite. These anti-oestrogens act by competing for this LBD binding site on ER, altering conformation so that the receptor fails to activate transcription of the target gene. The LBD consists of twelve helices termed H1-H12. The key structural difference upon ligand binding to ER lies in the orientation of helix 12. This part of the LBD determines the transcription from the target gene and which type of coregulator is recruited. Helix 12 is at the entry site to the LBD and forms a moveable lid over the pocket and contains residues which are vital for the function of AF-2. The binding of a ligand, causes an allosteric effect which changes the orientation of H12 (275).

The helices are arranged in a three-layer anti-parallel sandwich. Upon agonist binding the conformation of the LBD changes form apo- to holo-structure causing H12 to be repositioned across the ligand binding pocket and also the repositioning of AF-2. In an agonist bound configuration such as E2 binding, H12 is repositioned to form a hydrophobic cleft with H3 and H5 which allows for binding of LxxLL motifs, where L is leucine and x is any amino acid, found in coactivators and therefore activation of gene transcription (276-277).
The position of helix 12 in the LBD of ER is vital in the ability of the ER to carry out its function on its target genes. When unbound to ligand, the LBD of many nuclear receptors is bound to a set of corepressors such as NCoR1 and SMRT which recruit histone deactylases (HDACs) (244). It is these HDACs which generate a condensed chromatin structure and cause gene repression. The corepressor nuclear-receptor box (CoRNR box) docks into the hydrophobic groove of the LBD and prevents transcription.

In the presence of antagonists, H12 is shifted and mimics coactivators binding and therefore blocks coactivator recruitment. When antagonists bind, they form over the ligand binding cavity also but due to their bulky side chains they cause H12 to be displaced. This formation prevents the agonist-induced conformation and promotes binding of corepressors. This is the basis of action of the SERMs tamoxifen and raloxifen and pure anti-oestrogens. They each contain a bulky side chain which means they cannot fill the LBD and therefore they cause the H12 to be either repositioned (278), dissociated from the LBD (279), or bind outside the AF-2 region (280), and therefore sterically hinder agonist conformation and transcription. The allosteric effect of coactivators on the LBD can also cause changes in the AF-1 site through molecular crosstalk (281).

### 1.4.5 G-protein coupled receptor (GPR30)

GPR30 is a member of the G-protein coupled receptor 1 family and encodes a multi-pass membrane protein that localizes to the endoplasmic reticulum. This receptor binds oestrogen, resulting in intracellular calcium mobilization and synthesis of phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) in the nucleus. This protein plays a role in the rapid nongenomic signalling events widely observed following stimulation of cells and tissues with oestrogen (282-283). In 2000, Filardo et al. demonstrated MAP kinase activation by oestrogen in breast cancer cell lines expressing GPR30 but not in cell lines lacking this receptor (284). Subsequent studies have reported that GPR30 may mediate the regulation of cellular functions particularly by playing a role in oestrogen signalling. GPR30 was shown to directly bind oestrogen indicating that this receptor may modulate the functions of this ligand (285). GPR30 has also been shown to bind to ERα and induce a response in MAPK pathway (286). GPR30 is localized predominantly in the endoplasmic reticulum but some studies have shown it present on the plasma membrane (287). This receptor has been shown to activate the protein kinase (PKA) pathway which through phosphorylation of CREB induces Bcl-2 expression and apoptosis resistance and
therefore cell survival (288-289). GPR30 has been detected in many tissues including the liver, endothelium, aorta, smooth muscle, ovaries and uterus (290).

1.4.6 Genomic effects

Oestrogen plays an important role in the expression of genes involved in a variety of biological processes such as reproduction, development and breast tumour progression. These effects are mediated in the cell primarily through interaction with ERα and ERβ. This type of classical interaction leads to modulation of transcription or interaction with other transcription factors to mediate their activity. The classical mechanism of steroid hormone action or genomic effects involves the binding of oestrogen to the receptor (Figure 1.18). This releases heat shock proteins (HSPs) from the receptor and following conformational change and translocating to the nucleus the ER-ligand complex can bind to EREs in target gene promoters and stimulate transcription of the gene (Figure 1.19). The classical oestrogen response element is composed of two inverted hexanucleotide repeats; nucleotide sequences in the promoter region that are specifically recognised by ERs. The consensus ERE was initially described based on an oestrogen responsive sequence in the species *xenopus laevis* vitellogen A2 promoter: 5'-GGTCANNNT-GACC-3' (291). An example of a gene regulated by ER containing a palindromic ERE sequence is the coagulation factor FXII (292).

Transcriptional activity is regulated by AF-1 and AF-2 domains and also chromatin-remodelling complexes, coactivators and corepressors. Chromatin structures must be remodelled in order for transcription factors to bind DNA. Coactivators do not bind DNA but instead are recruited to the promoter through protein-protein interactions via the LXXLL motif e.g. SRC1, CREB-binding protein (CBP), p300 and AIB1. Corepressors include NCoR and SMRT which can silence transcription (294). These coactivators and corepressors may contain histone acetyltransferase (HAT) and HDAC activity leading to the activation and repression of transcription, respectively (295).
Protein-protein interactions involve the ER-ligand complex interacting with transcription factors such as nuclear factor kappaB (NF-κB), activator protein-1 (AP-1) and Specific protein-1 (SP-1) (295) (Figure 1.19).

Figure 1.19: The various pathways that oestrogen receptors can modulate gene transcription. The first panel depicts the classical interaction of the activated receptor with oestrogen response elements (EREs) on DNA. The other three panels are representations of the indirect effects of oestrogen receptors on transcription interactions. This occurs through protein-protein interactions with the Sp1, AP1, and NFκB proteins (4).

AP-1 can bind oestrogen receptors by interacting with the transcription factors Fos and Jun (296) (Figure 1.20). This binding may confer a difference in translation of genes as shown by Paech et al who reported luciferase transcription from ligand bound ERα activation at an AP-1 site but not when regulated through an ERE, findings that were reversed with ERβ activation using the same ligands (293).

Figure 1.20: ER interaction with Fos/Jun at an ER-dependant AP-1 response element (293).
NFkB consists of a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival. ER has the ability to inhibit NFkB activity which therefore suppresses the induction of inflammatory genes by NFkB. In vivo hepatic NFkB activation in ovariectomised mice was blocked by oestrogen replacement which was ER dependent (297). An E2-ERα complex has also been shown to displace CBP from NFkB thereby regulating its function in MCF-7 breast cancer cells (298). Also, breast cancer cells devoid of functional ERs result in overexpression of NFkB and therefore NFkB-regulated genes (299). NFkB acts as a regulator of transcription of cell adhesion molecules. It has been shown to decrease vascular cell adhesion molecule-1 (VCAM-1) gene expression upon 17β-estradiol binding (300).

SP-1 is a transcription factor which is known to interact with oestrogen receptors. In response to oestrogenic stimulation, SP-1 binds to the oestrogen responsive DNA regulatory region, with ER enhancing the binding of SP-1 to the DNA and recruiting coactivators. ERα interacts with EREs on SP-1 protein to activate the transcription. This activation requires interactions of both proteins with their cognate DNA elements as well as additional nuclear factors to form a functional ERα/SP-1-DNA complex. ERα/SP-1 can activate transcription from a consensus GC-rich SP-1 binding site in transient transfection studies in MCF-7 human breast cancer cells, and this response is also observed with ERα variants that do not contain the DNA-binding domain. Several genes that are induced by estrogens in MCF-7 cells are activated through one or more GC-rich sites in their regulatory regions and these include the cyclin D1, Bcl-2 and c-fos (301). ERα/SP-1 and ERβ/SP-1 action is dependent on ligand structure and cell context and ERβ/SP-1 is primarily associated with decreased ligand-dependent gene expression. The ERα/SP-1 complex, like ERα/AP-1, represents a pathway for hormone activation of genes in which the receptor does not bind DNA, and results of ongoing studies suggest that ERα/SP-1 plays an important role in transcriptional activation of multiple growth regulatory genes in breast cancer cells (105).

Phytoestrogens regulate transcription through binding to ERs in a similar manner to 17β-estradiol. In a study comparing the effects of 17β-estradiol versus phytoestrogens on ER binding to an ERE, it was shown that 17β-estradiol, genistein, daidzein and equol all induced an increase in ER binding to the ERE in a concentration-dependent manner. The efficacy of binding to ERα was greater with equol and daidzein than genistein, while the
latter compound preferentially bound to ERβ (302), indicating a downstream effect on the promoter of ER target genes and their regulation.

The results showed that the relative binding affinity of these compounds to ERα and ERβ differed, with a greater binding affinity of genistein to ERβ, whereas equol favoured binding of ERα.

1.4.7 Non-genomic effects

In contrast to the classical pathway of oestrogen receptors the non-classical pathways work more rapidly, taking only seconds and use existing proteins for effect. Non-genomic effects of oestrogens through ERs on the cell membrane or in the cytoplasm or other non-classical type receptors such as GPR30 recruit second messengers such as nitric oxide (NO), receptor tyrosine kinases (RTKs), and the protein kinases phosphatidylinositol-3-kinase (PI3K), serine-threonine kinase Akt, mitogen activate protein kinase (MAPK), and protein kinases (PKA and PKC) (303) (Figure 1.21).

Activation of MAPK leads to downstream cytoplasmic events or transcriptional events involving potentiation of AF-1 activity. Once ligand-bound the oestrogen receptors rapidly phosphorylate Src and SHC protein kinases, which results in the growth factor receptor binding protein-2 (SHC-GRB2)-SOS complex formation. Following the formation of this complex the proteins Ras, Raf, p38, JNK and ERk-1/2 become activated, translocate to the nucleus and regulate transcription. MAP kinases can also phosphorylate the oestrogen receptor (303). Studies have shown that the 17β-estradiol-ERα complex can activate MAPK which results in the activation of endothelial nitric oxide synthase (eNOS) which mediates vasodilation in the vascular endothelium (304). This shows that the rapid activation of eNOS is mediated by ERα functioning in a non-genomic manner. A second signal transduction pathway, PI3K, has been identified to stimulate eNOS and Akt through ligand activated ERα binding to its p85α subunit (305). Due to the ability of ERs to bind indirectly to promoters through these nongenomic pathways, the variability of target genes capable of responding to induction by oestrogens or phytoestrogens extends beyond those genes which respond specifically to direct ER activation.

Rapid non-genomic effects caused by 17β-estradiol are now well documented in various tissues however reports of the nongenomic effects of phytoestrogens are scarce. Despite this, studies in pituitary cells have shown that daidzein and genistein can activate the
JNK1/2/3 pathway via membrane bound ER\(\alpha\) and that daidzein also activates the ERK1/2 pathway in this manner (306). Phytoestrogens have also resulted in rapid stimulation of signalling pathways in other tissues such as brain (307). Genistein has shown to mediate the production of NO via the cAMP/PKA cascade, to directly stimulate plasma membrane-associated adenylate cyclases (308), and also to be a particular inhibitor of tyrosine kinases (309), nongenomic process which may result in the activation of various other complex signalling pathways.

Figure 1.21: Genomic and nongenomic effects of oestrogen (310).
1.5 Summary of evidence which led to this research

Menopause is associated with reduced secretion of the ovarian hormones oestrogen and progesterone. An increased risk of CVD has been demonstrated in post menopausal women which has been largely attributed to the decline in the hormone oestrogen. CVD is responsible for 54% of all deaths in women across Europe and 43% of the deaths in men, killing more people than all cancers combined. The mechanism by which the loss of oestrogen at menopause is associated with increased cardiovascular risk is not fully understood. Although this is the case, oestrogen can modulate the production of several factors known to be involved in the development of atherosclerosis including activators and inhibitors of the haemostatic system. It was long believed that hormone therapy has the ability to prevent certain diseases including CVD but as a result of the unfavourable results of the randomised controlled trials, many clinicians and patients have sought alternative therapies, including the use of dietary supplements, to provide relief from menopausal symptoms without the unfavourable side effects. Phytoestrogens are widely used as a ‘natural’ alternative to hormone therapy for the relief of menopausal symptoms. So far, the safety of phytoestrogen use is not available due to the limited and variable data previously reported. Conflicting results in studies on phytoestrogens effects on haemostasis have led to this research, in this study pure phytoestrogen compounds are used to examine the molecular effects on haemostasis in cell and animal models and report also on the effects of these compounds via oestrogen receptors.
Chapter 2

Material and Methods
2.1 Animals

All animal tissues were a kind gift from Prof W. Wuttke, Dept of Clinical and Experimental Endocrinology, University of Gottingen, Germany. 87 virgin female Sprague-Dawley rats raised in the animal facility of Gottingen’s University Clinic were used. Animals were kept in groups of six Makrolon® cages (type IV) under a 12-hour light, 12-hour dark cycle at ambient temperature (22-24°C) with a relative humidity of 50-55% and free access to water. In order to eliminate exposure to soy derived oestrogenic compounds found in regular rodent chow, they were fed with soy-free food (Ssniff Spezialdiaten GmbH, Soest, Germany) for 3 months. The animals (mean body weight 244g), were then bilaterally ovariectomized (ovx) under ketamin (18.75mg/animal, Ketavet, Pharmacia & Upjohn, Erlangen, Germany) anaesthesia. Treatment was then administered on the day of ovariectomy for a further 3 months, see Table 2.1.

2.2 Treatment

After ovariectomy, animals were randomised, placed in groups of six per cage and divided into 7 treatment groups (n=10-12/experimental group). The control group received soy-free food only (n=20) (Ssniff SM R/M, 10mm pellets). The supplemented chow was prepared by mixing the test substances with this soy-free formulation to homogeneity before the process of pelleting. Concentrations per kg body weight per day are shown in Table 2.2.

2.2.1 Allocation and Dosage

Careful choice of dosage is required for this study design because the exposure level of the animal should provide a proper relation to human exposure levels. The doses were chosen according to standards established in the laboratory of Prof Wuttke (311-312). For 17β-Estradiol and genistein, two or three doses respectively were selected to detect a possible switch from organ-selective to non-selective oestrogenic effects. The dose for 17β-estradiol was chosen to reflect the shift from the physiological into the supra-physiological range of treatment by a factor of 4. 17β-estradiol was administered as its ester 17β-Estradiol-3-benzoate (E2B) to improve the low oral bioavailability of 17β-estradiol and reduce the liver load. For genistein, the low dose was chosen to represent the exposure to genistein in the Asian diet or by “regular” supplement intake,
while the high dose correlates to a strong soy supplement intake. Dose dependant selectivity of effects could be expected by the 10-fold difference between the doses. The dose of equol was set equimolar to genistein to enable direct comparison of final effects among the three compounds. The subcutaneous dose was chosen to reflect the dose given in oral hormone therapy drugs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Label</th>
<th># animals</th>
<th>Dose/kg food/wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy free</td>
<td>SF</td>
<td>20</td>
<td>n/a</td>
</tr>
<tr>
<td>Oral Estradiolbenzoate</td>
<td>E2 low</td>
<td>11</td>
<td>4.3mg</td>
</tr>
<tr>
<td></td>
<td>E2 med</td>
<td>10</td>
<td>10mg</td>
</tr>
<tr>
<td></td>
<td>E2 high</td>
<td>12</td>
<td>17.3mg</td>
</tr>
<tr>
<td>Equol</td>
<td>Equol</td>
<td>10</td>
<td>400mg</td>
</tr>
<tr>
<td>Genistein</td>
<td>Gen low</td>
<td>12</td>
<td>100mg</td>
</tr>
<tr>
<td></td>
<td>Gen high</td>
<td>12</td>
<td>1000mg</td>
</tr>
</tbody>
</table>

Table 2.1: The number of animals allocated to each treatment group and dose.

The intake of food for the animals was twice weekly. Based on this and the number of animals per cage the amount of supplemented food each animal received was calculated as follows:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Label</th>
<th># animals</th>
<th>Dose per kg bw/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy free</td>
<td>SF</td>
<td>20</td>
<td>n/a</td>
</tr>
<tr>
<td>Oral Estradiolbenzoate</td>
<td>E2 low</td>
<td>11</td>
<td>0.19mg</td>
</tr>
<tr>
<td></td>
<td>E2 med</td>
<td>10</td>
<td>0.43mg</td>
</tr>
<tr>
<td></td>
<td>E2 high</td>
<td>12</td>
<td>0.75mg</td>
</tr>
<tr>
<td>Equol</td>
<td>Equol</td>
<td>10</td>
<td>17.4mg</td>
</tr>
<tr>
<td>Genistein</td>
<td>Gen low</td>
<td>12</td>
<td>6mg</td>
</tr>
<tr>
<td></td>
<td>Gen high</td>
<td>12</td>
<td>60mg</td>
</tr>
</tbody>
</table>

Table 2.2: The daily food intake calculated according to the number of animals per group, which were feeding daily and measured twice per wk.
2.3 Organ Collection

After 3 months of treatment, animals were decapitated under CO₂ anaesthesia between 8:00am and 12:00am. The abdominal cavity was opened with a longitudinal incision, and then organs were removed and weighed and transferred to 2ml polypropylene tubes, frozen in liquid nitrogen to minimize degradation of RNA, and stored at -70°C for future experiments. Liver and aorta tissues were collected from the laboratory of Prof Wuttke.

2.4 Ethics

The Local Ethics Committee approved all animal experiments for Animal Care and Use at the Georg August University in Gottingen, Germany according to the German animal welfare regulations under permission given by the district authorities of Braunschweig, Germany (number 509. 42502/01-36.03) and conformed to the FRAME’s guidelines.

2.5 Test Compounds

Estradiol

Chemical name 17β-Estradiol-3-benzoate (E2B)

Chemical family Steroids

![Figure 2.1: Molecular Structure of E2B](image)

Molecular weight 376.5g/mol
Molecular formula C₂₅H₂₈O₃
CAS# 50-50-0
Genistein
Chemical name 4', 5, 7-trihydroxyisoflavone
Chemical family flavonoids, isoflavones

![Molecular structure of Genistein](image)

Molecular weight 270.2 g/mol
Molecular formula C₁₅H₁₀O₅
CAS# 446-72-0

Equol
Chemical name 3,4-Dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol, 4',7-
Dihydroxyisoflavane
Chemical family Flavonoids

![Molecular structure of Equol](image)

Molecular weight 242.27 g/mol
Molecular formula C₁₅H₁₄O₃
CAS# 94105-90-5

Soy Free Chow
The composition of the soy-free food is shown in Appendix I.
2.6 Real Time PCR

Gene expression was determined by two-step real-time reverse transcription polymerase chain reaction (RT-PCR). Firstly, RNA extracted from the tissue samples was reverse transcribed to cDNA, then Real-Time PCR was performed. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter as an indicator of amplicon production during each PCR cycle (i.e. in “real-time”) as opposed to the endpoint detection.

2.6.1 RNA extraction

2.6.1 (i) The RNeasy® mini kit (Qiagen, West Sussex, UK)

This kit was used to purify total RNA from tissue samples and also from hepatocyte cell cultures, with the RNase-Free DNase set to prevent DNA contamination. The RNA produced is completely free of both DNA and protein.

Reagents required

RNeasy Mini Spin Column
Collection tubes (1.5ml)
Collection tubes (2ml)
Buffer RLT
A working solution was prepared with β-mercaptoethanol in a 50ml falcon tube. This was covered in tinfoil, and kept at room temperature for 4 weeks. RLT buffer was prepared as follows; 10μl of 14.3M β-mercaptoethanol per 1ml of Buffer RLT.
Buffer RW1
Buffer RPE
Buffer RPE was supplied as a concentrate. Before using for the first time, 4 volumes of 100% EtOH were added, as indicated on the bottle to obtain the working solution
RNase-free water

RNase-Free DNase set (Qiagen, W. Sussex, UK)
RDD buffer
RNase-Free water
DNase I

This set provides on-column digestion of DNA during the purification steps of the mini kit protocol.

DNase I stock solution was prepared before using the RNase-Free DNase set for the first time. Lyophilized DNase I (1500 Kunitz units) was dissolved in 550μl of the RNase-free water provided, securing that no DNase I was lost by opening the vial. The vial was then gently inverted to mix as it is extremely sensitive to physical denaturation.

For long term storage of the DNase I, the stock solution was removed and divided into single-use aliquots and stored at -20°C for a maximum of 9 months. Thawed aliquots were kept at 4°C for up to 6 weeks.

DNase was prepared in RDD buffer provided in the set by adding 10μl of DNase to 70μl of RDD buffer (80μl/sample).

Ethanol (Lennox, Dublin, Ireland)

70% EtOH was prepared with DEPC water and filtered through a 20μm membrane with syringe into a sterile universal tube.

DEPC (Sigma, Wicklow, Ireland)

Diethylene Pyrocarbonate (DEPC) water was prepared as follows; 500mls of double distilled H2O was autoclaved. Once autoclaved, 500μl of DEPC was added, incubated at room temperature for 1hr and autoclaved again.

The liquid nitrogen (LN2) container was cleaned out with DEPC water, dried and kept in the fridge to cool before use.

Sample preparation

Tissue: A mortar and pestle was used for disrupting 20-30mg of the tissue. This equipment was treated with an RNase inhibitor (RNase Zap®), followed by 70% EtOH and washed with DEPC water prior to homogenisation. It was then placed on tin foil in an ice box and pre-cooled with LN2 before addition of the tissue section.

Frozen tissue was cut with a sterile blade to 20mg. The section was then immersed in LN2-filled mortar and disrupted with the pestle under continuous liquid nitrogen until a
fine tissue powder appeared. The LN2 was allowed to evaporate but the tissue was not allowed to thaw.

Cell cultures: Cells were grown in a monolayer and lysed directly in the plate. The culture medium was completely aspirated and 600μl of RLT buffer was added to the well. The lysate was then pipetted into a microcentrifuge tube. This was pipetted to mix the sample to ensure no cell clumps were visible before proceeding to the homogenisation step. Following the procedure below the lysate was pipetted directly onto the purple shredder column in a 2ml collection tube and centrifuged at max speed for 2mins to homogenise. The supernatant was transferred into a new 2ml collection tube and the pellet was discarded and the procedure from “preparation of the lysate” below was followed.

**Qiagen Shredder system**

The Qiagen Shredder System QIA shredders® are a commercially available spin column-based shearing system, used for homogenization of tissue initially disrupted by a mortar and pestle. It allows for up to 100μg of RNA to bind to the membrane.

**Protocol**

**Homogenisation**

One shredder column (purple) and one mini column (pink) were placed in 2ml collection tubes.

The mortar containing the ground tissue powder was taken into the hood and using a new pestle, 600μl of RLT buffer was added to the powder, and ground into liquid form again. The lysate was pipetted onto the purple shredder column in a 2ml collection tube and centrifuged at max speed for 5 minutes to homogenise.

The working space was cleaned for RNA extraction at this step, using alcohol, RNase Zap and DEPC water.

The supernatant was transferred into a new 2ml collection tube and the pellet was discarded.
Preparation of the lysate

70% ethanol, at a ratio of 1:1, was added to the cleared lysate and mixed well by pipetting.

Up to 700μl of the mixed lysate was then transferred to a pink mini column in a 2ml collection tube.

This was centrifuged for 15 seconds at 10,000rpm.

The sample was removed from the centrifuge and the flow through was discarded, the collection tube was changed and the remaining lysate was centrifuged.

Wash 1

350μl RW1 buffer was pipetted onto the column and centrifuged for 15 seconds at 10,000rpm to wash.

The flow through and collection tube were discarded and the mini column was transferred to a new 2ml collection tube.

Wash 2

80μl of the DNase incubation mix was pipetted directly onto the membrane of the spin column and left on the column for 15 minutes at room temperature.

After this incubation, 350μl RW1 buffer was pipetted onto the column followed by centrifuging for 15 seconds at 10,000rpm to wash.

The flow through and collection tube were discarded and the mini column was transferred to a new 2ml collection tube.

Wash 3

500μl RPE buffer was pipetted onto the column and centrifuged for 15 seconds at 10,000rpm.

The flow-through and collection tube were discarded and the mini column was transferred to a new 2ml collection tube.

Wash 4

500μl RPE buffer was pipetted onto the column and centrifuged for 2 minutes max speed to dry the membrane.
The flow through and tube were discarded and the column was placed in a new 2ml collection tube and centrifuged again for 1 minute at full speed to eliminate any possible carryover of Buffer RPE or any residual flow-through.

**Elution**

The spin column was transferred to a 1.5ml collection tube and 30μl RNase-free water was pipetted onto the membrane.

This was centrifuged for 1 minute at 10,000rpm to elute.

The column was discarded and the RNA yield was determined by measuring the absorbance at 260nm (A260) in a spectrophotometer. This purified RNA was then stored at -80°C.

2.6.1 (ii) RNAqueous® micro kit (Ambion, Texas, USA)

The RNAqueous® micro kit was used to purify total RNA from HUAEC.

**Reagents required**

- Micro Filter cartridge assembly
- Micro elution tubes (1.5ml)
- Wash solution 1* concentrate
  - Wash solution one was prepared for use by adding 10.5ml 100% ethanol
- Wash solution 2/3 concentrate
  - Wash solution 2/3 was prepared for use by adding 22.4ml 100% ethanol
- Lysis solution
- Elution solution

**Ethanol** (Lennox, Dublin, Ireland)

**RNase-free microfuge tubes (2ml)** (Ambion, Texas, USA)

**Sample preparation**

Cells were grown in a monolayer and lysed when they reached ~80% confluency. The cells were lysed directly in the wells by aspirating the cell culture medium and disrupting the cells with 100μl of lysis buffer. The buffer was pipetted up and down on the wells.
and the cells suspension was removed and pipetted into an RNase-free microfuge tube and vortexed to mix.

50µl of 100% ethanol was added to the sample and vortexed briefly

The cell suspension was pipetted onto a micro filter cartridge assembly and centrifuged for 10 seconds at max speed

180µl of wash solution 1 was added to the filter cartridge

This was centrifuged for 10 seconds at max speed

180µl of wash solution 2/3 was added to the filter cartridge

This was centrifuged for 10 seconds at max speed

This was step was repeated.

The flow-through was discarded and with the filter cartridge assembled on the same collection tube, the tube was centrifuged for 1 min at max speed.

A micro elution tube was labelled and the micro filter cartridge was transferred onto it

10µl of elution solution (heated to 75°C) was added to the filter and incubated at room temp for 1 minute.

The tube was then centrifuged for 30 seconds at max speed

This was repeated with a second volume of elution solution to give a final volume of 20µl.

The column was discarded and the RNA yield was determined by measuring the absorbance at 260nm (A260) in a spectrophotometer. This purified RNA was then stored at -80°C.
2.7 Nano-Drop 1000 Spectrophotometer (ND-1000)

RNA concentration and quality was measured using the Nanodrop® (Figure 2.4).

2.7.1 Loading and measuring samples

The measurement pedestal surfaces were cleaned. 1μl RNase-free water was loaded onto the lower measurement pedestal and read as the “blank” measurement. The surfaces were cleaned using a soft lint-free wipe to prevent sample carryover and the test samples were measured by loading 1μl of samples onto the pedestal and reading the RNA concentration and quality. Quantification is made based on the tightly controlled path length of 1mm. Example of results from measured rat liver RNA is shown in Table 2.3.

2.7.2 Interpretation of results

A260/280: ratio of sample absorbance at 260 and 280nm. The ratio of absorbance at 260 and 280nm provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum. A ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm. The A260/280 ratio is considerably influenced by pH; lower pH results in a lower ratio and reduced sensitivity to protein contamination.

A260/230: Ratio of sample absorbance at 260 and 230nm. This is a secondary measure of nucleic acid purity, being usually higher than the respective 260/280 values for “pure” nucleic acids. It is usually in a range of 1.8-2.2; if lower, it may indicate co-purified contamination.
Concentration (ng/μl): Sample concentration in ng/μl based on absorbance at 260nm minus the absorbance at 340nm (i.e. normalized at 340nm) and the selected analysis constant.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA yield (ng/μl)</th>
<th>260:280</th>
<th>260:230</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1612.0</td>
<td>2.16</td>
<td>1.75</td>
</tr>
<tr>
<td>2</td>
<td>1095.5</td>
<td>2.15</td>
<td>1.87</td>
</tr>
<tr>
<td>3</td>
<td>1531.0</td>
<td>2.15</td>
<td>1.64</td>
</tr>
<tr>
<td>4</td>
<td>1757.2</td>
<td>2.15</td>
<td>1.72</td>
</tr>
<tr>
<td>5</td>
<td>1306.7</td>
<td>2.14</td>
<td>2.08</td>
</tr>
</tbody>
</table>

Table 2.3: The typical results from the Nanodrop found in the rat liver tissue.
2.8 cDNA synthesis

The High Capacity cDNA Reverse Transcription Kit was used to enable the quantitative conversion of total RNA into single stranded cDNA (Applied Biosystems, CA, USA). Random primers ensure that first strand synthesis occurs efficiently with all species of RNA molecules present. An essential requirement for the relative quantification of cDNA is that the reverse transcriptase reaction generated products in a manner directly dependant on the amount of input RNA template.

Reagents required

High capacity cDNA kit
10X RT buffer
10X RT Random primers
25X dNTP Mix (100mM)
Multiscribe ™ Reverse Transcriptase 50U/μl

Protocol

2μg of RNA was reverse transcribed into a final volume of 50μl

The appropriate amount of RNA to give 2μg was calculated and added to RNase-free water to produce a final volume of 50μls as follows;

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA conc (μg/μl)</th>
<th>2μg RNA= (μl)</th>
<th>H2O (μl)</th>
<th>Total volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.600</td>
<td>3.3</td>
<td>46.7</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>0.220</td>
<td>9.0</td>
<td>41.0</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2.4: Example of RNA calculation for cDNA synthesis

A No Template Control (NTC) was also included.
The solution was mixed by pipetting.
The samples were placed on ice to cool.
All kit components were thawed on ice.
A 2X RT mastermix was prepared by calculating the volume of each of the components needed for the number of reactions, by referring to Table 2.5.
Table 2.5: The volumes required of each component to prepare the 2X RT mastermix for one sample

<table>
<thead>
<tr>
<th>Component</th>
<th>μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>25X dNTPs</td>
<td>4.0</td>
</tr>
<tr>
<td>10X RT Random Primers</td>
<td>10.0</td>
</tr>
<tr>
<td>Multiscibe Reverse transcriptase</td>
<td>5.0</td>
</tr>
<tr>
<td>Rnase-free water</td>
<td>21.0</td>
</tr>
<tr>
<td><strong>Total per reaction(μl)</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

50μl of 2X master mix was added to each sample and centrifuged briefly to mix then placed on ice before loading on the thermal cycler.

The samples were placed on the thermal cycler using the following programme conditions:

- 10 minutes at 25°C
- 120 minutes at 37°C
- 5 seconds at 85°C, then hold at 4°C

When complete the samples were snap-frozen and placed at -80°C for storage.

**Concentration of the resulting cDNA**

The concentration of each sample was 2μg/100μls = 20ng/μl.
2.9 Taqman Real-Time PCR®

Principle

Real time PCR is used to quantify gene expression; amplified DNA is quantified during the exponential phase of the PCR, allowing the concentration of a particular target DNA or RNA relative to a standard to be quantified. The greater the initial concentration of target sequences in the reaction mixture the fewer the number of cycles required to achieve a particular yield of amplified product. The PCR reaction exploits the 5’ nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan® probe during PCR. The TaqMan® probe contains a reporter dye at the 5’ end of the probe and a quencher dye at the 3’ end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter as shown in Figure 2.5. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

![Figure 2.5: 5’to 3’nuclease activity of AmpliTaq Gold® DNA Polymerase during PCR](image-url)
**Reagents required**

Taqman® Universal PCR mastermix (Applied Biosystems, CA, USA)

RNase free water (Qiagen, W.Sussex, UK)

Microamp® Optical 96 well reaction plates (Applied Biosystems, CA, USA)

Microamp® Optical Adhesive Covers (Applied Biosystems, CA, USA)

Taqman gene expression assay (Applied Biosystems, CA, USA) (Table 2.7a, 2.7b)

**Controls for Taqman PCR**

It is necessary to include at least three No Amplification Controls (NAC, a minus-reverse transcriptase control) as well as three No Template Controls (NTC, a minus sample control) in each reaction plate. To achieve a 99.7% confidence level in the definition of +/- thresholds for the target amplification, six replicates of NTCs must be run. NAC is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase; NTC includes all of the RT-PCR reagents except the RNA template. It is necessary to rule out the presence of fluorescence contaminants in the samples or in the heat block of the thermal cycler so as to avoid false positive results.

If a product is amplified, it indicates that one or more of the RT-PCR reagents is contaminated with DNA which may be the amplicon. If the absolute fluorescence of the NAC is greater than that of the NTC after PCR, fluorescent contaminants may be present in the sample or in the heating block of the thermal cycler.

A positive control, known to express the gene of interest, was also included in triplicate. An endogenous control is also included so that any skewed results can be corrected. This control must be expressed at a similar level in all study samples, it must also give similar PCR efficiencies when using the comparative $C_T$ method and finally it must be more abundantly expressed than the target gene. 18S was the internal control gene used in all experiments, it was abundant in the tissues and its expression is not affected by treatment with oestrogens.
Procedure

The preparation of the samples and mastermix was carried out on ice in the laminar flow cabinet. Each cDNA sample was diluted with RNase-free water to give a concentration of 8ng/μl per well.

For each gene to be measured, 3μls of sample at 8ng/μl is required. Each sample of 20ng/μl was diluted 1:2.5 with RNase-free water to give a concentration of 8ng/μl.

The appropriate amount of master mix for the number of samples to be assayed was prepared according to Table 2.6;

<table>
<thead>
<tr>
<th>Component</th>
<th>1x (μl)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal PCR buffer mix</td>
<td>5.0</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>TaqMan® gene expression assay</td>
<td>0.5</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>RNase free water</td>
<td>3.5</td>
<td>Qiagen</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9.0</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6: Volumes of each component required for a Taqman assay in one well of a 96-well plate.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Name</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proc</td>
<td>Protein C</td>
<td>NM_012803.1</td>
</tr>
<tr>
<td>Serpinc1</td>
<td>Antithrombin</td>
<td>NM_001012027.1</td>
</tr>
<tr>
<td>Fgg</td>
<td>Fibrinogen gamma</td>
<td>NM_012559.2</td>
</tr>
<tr>
<td>Plat</td>
<td>Tissue plasminogen activator</td>
<td>NM_013151.2</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
<td>NM_017200.1</td>
</tr>
<tr>
<td>Thbd</td>
<td>Thrombomodulin</td>
<td>NM_031771.2</td>
</tr>
<tr>
<td>F2r</td>
<td>Thrombin receptor</td>
<td>NM_012950.2</td>
</tr>
<tr>
<td>Procr</td>
<td>Endothelial protein C receptor (EPCR)</td>
<td>NM_001025733.2</td>
</tr>
<tr>
<td>ESR1</td>
<td>Oestrogen receptor alpha (ERα)</td>
<td>NM_012689.1</td>
</tr>
<tr>
<td>ESR2</td>
<td>Oestrogen receptor beta (ERβ)</td>
<td>NM_012754.1</td>
</tr>
<tr>
<td>Gper</td>
<td>G-protein coupled receptor 1</td>
<td>NM_133573.1</td>
</tr>
<tr>
<td>18s</td>
<td>Eukaryotic 18S rRNA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7a: List of gene targets selected for Taqman analysis of rat liver and aorta tissue
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Name</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F7</td>
<td>Coagulation factor VII</td>
<td>NM_000131.3</td>
</tr>
<tr>
<td>Fgg</td>
<td>Fibrinogen gamma</td>
<td>NM_000509.4</td>
</tr>
<tr>
<td>F2</td>
<td>Coagulation factor II (Prothrombin)</td>
<td>NM_000506.3</td>
</tr>
<tr>
<td>F3</td>
<td>Tissue factor</td>
<td>NM_001993.3</td>
</tr>
<tr>
<td>F2r</td>
<td>Thrombin receptor</td>
<td>NM_001992.3</td>
</tr>
<tr>
<td>Plat</td>
<td>Tissue plasminogen activator</td>
<td>NM_033011.2</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
<td>NM_001032281.2</td>
</tr>
<tr>
<td>Thbd</td>
<td>Thrombomodulin</td>
<td>NM_000361.2</td>
</tr>
<tr>
<td>Procr</td>
<td>Endothelial protein c receptor</td>
<td>NM_006404.3</td>
</tr>
<tr>
<td>Serpine1</td>
<td>Plasminogen activator inhibitor 1</td>
<td>NM_000602.2</td>
</tr>
<tr>
<td>Prosl</td>
<td>Protein S</td>
<td>NM_000313.2</td>
</tr>
<tr>
<td>Proc</td>
<td>Protein c</td>
<td>NM_000312.2</td>
</tr>
<tr>
<td>SerpinC</td>
<td>Antithrombin</td>
<td>NM_000488.2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
<td>NM_000567.2</td>
</tr>
<tr>
<td>ESR1</td>
<td>Oestrogen receptor alpha (ERα)</td>
<td>NM_000125.3</td>
</tr>
<tr>
<td>ESR2</td>
<td>Oestrogen receptor beta (ERβ)</td>
<td>NM_001040275.1</td>
</tr>
<tr>
<td>Gper</td>
<td>G-protein coupled receptor 1</td>
<td>NM_001505.2</td>
</tr>
<tr>
<td>18s</td>
<td>Eukaryotic 18S rRNA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7b: List of gene targets selected for Taqman analysis of human cell systems
Loading the TaqMan plate

1μl of sample or control was added in triplicate to each well of a 96 well plate by pipetting into the bottom of the well. 1μl of sample #1 was pipetted into the first three wells (A1-A3), followed by 1μl of sample #2 into the next three wells (A4-A6) and so on until each sample for the first gene had been loaded. This was then repeated for the next gene and continued until all samples had been loaded.

Next the first gene probe mastermix was added to the first set of samples, the pipette was angled down to the side of the well and the tip contents fully expelled, the pipette tip was changed for each new probe. This was continued with the next few probes until all wells had sample plus probe in them.

The plate was sealed with a plastic thermostable cover (MicroAmp® Optical Adhesive film, Applied Biosystems, US) ensuring that all sides and corners were stuck down well with no gaping areas for evaporation.

The side tabs were removed and the plate was centrifuged at 500rpm for 30secs.

Figure 2.6: ABI Prism 7000 Sequence Detection System
Figure 2.7: Typical layout of a TaqMan PCR plate including 5 samples and 3 controls in triplicate.

Plate reading

The ABI Prism 7000 was switched on and the computer logged in.
The plate was inserted into the prism in the correct orientation; well A1 was in the top left corner.
The software was opened and a new document for absolute quantification in a 96-well plate was selected.
The detector was assigned and added to the plate document and the negative control samples were highlighted and NTC was selected.
In the instrument tab, the volume was changed to 10µl (volume in each well), the document was saved, and then the run was started.
The thermal cycler conditions were preset as shown in Table 2.9.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time (mins)</th>
<th>Reps</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>0.4</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.8: ABI Prism 7000 thermal cycler protocol
Reviewing the results of a PCR run

The data was automatically saved in the hard drive and analysed by the SDS software provided with ABI Prism 7000 Sequence Detection System.

An amplification plot was received and the baseline and threshold values selected, the results were then saved and exported to an excel format. A typical amplification plot is shown in Figure 2.8.

![Typical amplification curve from the SDS software.](image)

Figure 2.8: Typical amplification curve from the SDS software.

Calculation of results

The file was retrieved in Excel and the mean Ct value of each triplicate sample was calculated.

Results are expressed as the ratio of target gene cDNA to the internal control using the $2^{-\Delta \Delta CT}$ method (313).

Those expressed relative to an untreated control are noted in the results section
2.10 Taqman low Density Arrays (TLDA)

Principle

The ABI Prism 7900HT Sequence Detection System is a second-generation sequence detection system instrument designed for automated, high-throughput detection of fluorescent PCR-related chemistries. The Micro Fluidic Card (Applied Biosystems, CA, USA) is used in conjunction with the 7900HT system for profiling gene expression using the Comparative C_T Method of relative quantification. The card evaluates from one to eight cDNA samples or controls generated from total RNA in a two-step RT-PCR experiment as described in section 2.8. The TLDA was used to measure relative gene expression in the liver tissue of the rats. 15 genes were chosen to be analysed alongside 18S as the endogenous control. The Taqman microfluidic card can be seen in Figure 2.9 and the layout of the TLDA for this study can be seen in Figure 2.10.

![Figure 2.9: Taqman microfluidic card](image)

Reagents required

- Taqman® Low Density Array Card (Applied Biosystems, CA, USA)
- Taqman® gene expression mastermix (Applied Biosystems, CA, USA)
- RNase-free water (Qiagen, W.Sussex, UK)
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Name</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F7</td>
<td>Coagulation Factor VII</td>
<td>NM_152846.1</td>
</tr>
<tr>
<td>F5</td>
<td>Coagulation Factor V</td>
<td>NM_001047878.1</td>
</tr>
<tr>
<td>F8</td>
<td>Coagulation Factor VIII</td>
<td>NM_183331.1</td>
</tr>
<tr>
<td>F9</td>
<td>Coagulation Factor IX</td>
<td>XM_346365.3</td>
</tr>
<tr>
<td>F10</td>
<td>Coagulation Factor X</td>
<td>NM_017143.2</td>
</tr>
<tr>
<td>Fga</td>
<td>Fibrinogen alpha</td>
<td>NM_001008724.1</td>
</tr>
<tr>
<td>Fgb</td>
<td>Fibrinogen beta</td>
<td>NM_020071.1</td>
</tr>
<tr>
<td>F11</td>
<td>Coagulation factor XI</td>
<td>NM_001047848.1</td>
</tr>
<tr>
<td>F2</td>
<td>Coagulation factor II (Prothrombin)</td>
<td>NM_022924.1</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
<td>NM_031756.1</td>
</tr>
<tr>
<td>Pros1</td>
<td>Protein S</td>
<td>NM_031086.2</td>
</tr>
<tr>
<td>Serpinel</td>
<td>Plasminogen activator inhibitor 1</td>
<td>NM_012620.1</td>
</tr>
<tr>
<td>Plg</td>
<td>Plasminogen</td>
<td>NM_053491.2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
<td>NM_017096.2</td>
</tr>
<tr>
<td>Plat</td>
<td>Tissue plasminogen activator</td>
<td>NM_013151.2</td>
</tr>
<tr>
<td>18s</td>
<td>Eukaryotic 18S rRNA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.9: List of gene targets selected for Taqman low density array analysis of rat liver tissue
Procedure

Each cDNA sample was removed from the freezer, new 1.5ml centrifuge tubes were labelled while the samples were thawing.

The samples were then vortexed and centrifuged briefly to mix.

For each sample, the following components were added to the labelled 1.5ml centrifuge tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl) per fill Reservoir</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA sample (500ng) + RNase-free water</td>
<td>50.0</td>
</tr>
<tr>
<td>Taqman Universal PCR Master Mix (2X)</td>
<td>50.0</td>
</tr>
<tr>
<td>Total Volume</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 2.10: A break-down of the components for each TLDA reservoir

Each sample of cDNA was at a concentration of 20ng/µl. 25µl of cDNA was added to 25µl of RNase-free water to give 500ng of cDNA in 50µl and then 50µl of mastermix was added to give a total volume of 100µl. There are 48 wells per assay on the Micro Fluidic Card which converts to 10ng of cDNA per well.

The centrifuge tubes were mixed by vortexing and centrifuged to remove any air bubbles.

The card was loaded as follows;

The Taqman Array Micro Fluidic Card was removed from its packaging and 100µl of the desired sample-specific PCR reaction mix was pipetted into the fill port of each fill reservoir.

After the fill reservoirs were loaded with the cDNA samples, the array card was centrifuged in a Sorvall/Heraeus bucket, and centrifuged for 1,200rpm for two 1 minute.

The ramp parameters were set at 9 for both the up and down rate.

After the two 1 minute spins, the card was removed, checked for uniform and consistent filling from reservoir to reservoir and the card was then sealed using the appropriate card sealer.

The reservoirs were then trimmed from the Taqman array and the plate was then brought to the 7900HT to be analysed.
7900HT Micro Fluidic Card Workflow

Step 1. Load the Sample

Step 2. Fill the Micro Fluidic Card Wells

Step 3. Seal the Micro Fluidic Card

Step 4. Prepare the Micro Fluidic Card for loading into 7900HT System

Step 5. Perform Real-time PCR

Step 6. View and Analyze Data

Figure 2.11: Schematic of loading and analysing a Micro Fluidic Card.

Expression analysis

The Taqman array is shipped with an array information CD.
This CD is used for setting up the SDS file and running the array.
A new file was selected within the SDS software and labelled accordingly.
Under file and import we selected a *txt file for this TLDA and the software automatically configures the plate settings to those saved on the disc.
The file was saved as an *sds file and the plate was run as per the manufacturers instructions.
Once all plates were run, the files were analyzed in a study format.
The final stage in the analysis is the computation of gene expression values from the $C_T$ data which is done in the same manner as for TaqMan PCR as described above.
2.11 Protein Analysis

Western Blotting and enzyme-linked immunosorbent assay (ELISA) were used to analyse protein expression. The initial preparation of the samples involved both nuclear protein extractions and total protein extractions.

2.11.1 Nuclear protein extraction
(Ne-Per® Reagents, Pierce, US)

Principle

The Ne-Per nuclear and cytoplasmic reagents used in this kit enable the step-wise separation and preparation of cytoplasmic and nuclear extracts from mammalian tissue. Non-denatured, active proteins from tissue samples are purified in under two hours using two reagents which disrupt the cell membranes releasing its cytoplasmic contents and a third reagent which lyses the intact nuclei and is recovered by centrifugation to produce a nuclear extract.

Reagents required

**Halt Protease inhibitor** (Pierce)

- Protease inhibitor at 100X
- One tube contains 100μl of inhibitor
- Used 10μl per 1ml lysis buffer
- Added inhibitor to CER1 and NER solutions

**Sodium Orthovanadate** (Sigma)

- Added to CER1 and NER solutions to a final concentration of 3mM

**Phenylmethylsulphonyl fluoride** (Sigma)

- Added to CER1 and NER solutions to a final concentration of 1mM
Reagent volumes:

<table>
<thead>
<tr>
<th>Mass of samples</th>
<th>CERI</th>
<th>CERII</th>
<th>NER</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mg</td>
<td>200μl</td>
<td>11μl</td>
<td>100μl</td>
</tr>
</tbody>
</table>

Table 2.11: Reagent volumes per sample according to the weight of tissue

Procedure

All centrifugation steps were carried out at 4°C. All cell samples and extracts were kept on ice throughout the procedure.

The tissue was weighed out and cut into small pieces, placed in 1.5ml centrifuge tube and the appropriate volume of CERI inclusive of inhibitors was added. The sample was subjected to homogenisation using a motorised pestle. A good resuspension is necessary to maximise the integrity of the extract.

The pellet was resuspended by vortexing the tube on the highest setting for 15 seconds. This was followed by a 10 minutes incubation period on ice.

Ice-cold CERII (according to chart above) was added to the tube. The tube was vortexed for 5 seconds on the highest setting followed by a 1 minute incubation period on ice.

This was then vortexed for 5 seconds on the highest setting and the tube was centrifuged for 7 minutes at 10,000rpm at 4°C.

The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube and stored at -80°C.

The insoluble pellet (nuclear fraction) was resuspended in 100μl of ice-cold NER with the motorised pestle followed by vortexing to fully resuspend pellet.

The sample was vortexed again for 15 seconds on the highest setting and returned to ice for 10 minutes, this was repeated 4 times.

The tube was centrifuged for 10 minutes at max speed. The supernatant (nuclear extract) was then transferred to a clean pre-chilled 1.5ml tube and stored at -80°C until used.
2.11.2 Total protein extraction
(T-Per® Reagent, Pierce, US)

Principle

This tissue protein extraction reagent is for the extraction of total protein from tissue samples. The reagent has a simple composition which is versatile enough to include the addition of inhibitors to assist in the lysis of cells and which may be used in downstream immunoassay analysis.

Reagents required

Total Protein tissue extraction reagent (Pierce, Illinois, USA)

Halt Protease inhibitor (Pierce, Illinois, USA)

Sodium Orthovanadate (Sigma, Wicklow, Ireland)

Phenylmethylsulphonyl fluoride (Sigma, Wicklow, Ireland)

Procedure

The complete tissue lysis buffer was prepared as follows:

For every 1ml lysis buffer, 5mM PMSF, 200mM sodium orthovanadate and 1X Halt Protease Inhibitor Cocktail was added.

20mg of tissue was weighed and placed in a labelled pre-chilled 1.5ml centrifuge tube on ice.

1ml of complete lysis buffer was added to the sample and homogenised with a motorised pestle.

The sample was centrifuged at 10,000 rpm for 5 minutes at 4°C

The supernatant was transferred to a fresh pre-chilled 1.5ml tube and stored at -80°C until used.
2.11.3 BCA
(BCA Protein Assay Kit, Pierce, US)

Principle

This protein assay is based on bicinchoninic acid (BCA) for the detection and quantitation of total protein. The method combines the well-known reduction of Cu$^{2+}$ to Cu$^{+}$ by protein in an alkaline medium with the highly sensitive and selective colourimetric detection of the cuprous cation using biocinchoninic acid. The reaction complex formed exhibits a strong absorbance at 562nm that is approximately linear with increasing protein concentration over a working range of 20-2,000μg/ml. Protein concentrations are determined and reported with reference to standards of a common protein such as bovine serum albumin. A series of dilutions of known concentrations are prepared from this protein and assayed alongside the unknown sample/s before the concentration of each unknown is determined from the standard curve. Here the microplate procedure was used which requires a small sample volume and the ease of working with a microplate.

Reagents required

Preparation of diluted albumin (BSA) standards

The concentration of the BSA ampoule was 2.0mg/ml. The diluent was dH$_2$O, dilutions are shown in Table 2.12
<table>
<thead>
<tr>
<th>Tube</th>
<th>Vol Diluent (µl)</th>
<th>Vol (µl) and Source of BSA</th>
<th>Final BSA conc (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 Stock</td>
<td>2.0</td>
</tr>
<tr>
<td>B</td>
<td>125</td>
<td>375 Stock</td>
<td>1.5</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325 Stock</td>
<td>1.0</td>
</tr>
<tr>
<td>D</td>
<td>175</td>
<td>175 Tube B</td>
<td>0.75</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 Tube C</td>
<td>0.50</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325 Tube E</td>
<td>0.25</td>
</tr>
<tr>
<td>G</td>
<td>325</td>
<td>325 Tube F</td>
<td>0.125</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100 Tube G</td>
<td>0.025</td>
</tr>
<tr>
<td>I</td>
<td>400</td>
<td>0</td>
<td>0=Blank</td>
</tr>
</tbody>
</table>

Table 2.12: Preparation and dilution scheme of BSA standards for the microplate BCA procedure

Preparation of BCA Working Reagent (WR)

\((\text{# standards} + \text{# unknowns}) \times (\text{# replicates}) \times (\text{Vol WR per sample})\) = Total Vol WR required

Prepare WR by mixing 50 parts of Reagent A with 1 part Reagent B (50:1, Reagent A:B) and vortex.

Microplate Procedure

The volume of WR was 200µl per well.

25µl of each standard or unknown replicate was pipetted into a microplate well.

200µl of WR was added to each well and mixed on a plate shaker (Titertek, AGB Scientific, Ireland) for 30 seconds at speed setting 2.

The microplate was covered with a clear lid and incubated at 37°C for 30 minutes

The plate was then allowed to cool to room temperature and measured on spectrophotometer at 562nm (MRX Microplate reader, Shaw Scientific, Ireland).

Calculations

The average absorbance of the blank was subtracted from the unknown and standard replicates.

A standard curve was plotted using the average blank-corrected measurement for each standard vs. its concentration in µg/ml. An example is shown in Figure 2.12.

The curve was then used to obtain the protein concentration of each unknown sample.
Figure 2.12: An example of a standard curve using the BCA protein assay.
2.11.4 SDS-PAGE

Principle

This technique separates proteins on the basis of molecular weight, the distance migrated by the protein in a given time is inversely related to the logarithm of its molecular weight. Firstly, the proteins are denatured so as they retain only their primary amino acid structure. SDS disrupts hydrophobic areas and binds to the protein, coating it with an overall negative charge, which overwhelms any positive charges the protein had due to positively charged R-groups. The resulting protein has been denatured by SDS and as a result has been linearized.

As all the proteins have the same net charge, there needs to be some matrix for them to separate through because if the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. Using an acrylamide solution, a gel will form which acts like a mesh for the proteins to travel through and the smaller proteins will travel faster than larger one.

The rate of movement is influenced by the gels' pore size and the strength of the electric field and the percentage of the gel determines the pore size of the gel 'mesh'. The higher the percentage gel, the smaller the pore size and therefore the smaller protein molecules will be able to travel through faster.

Reagents required

10% SDS:
50g SDS (Sigma)
500mls dH₂O
Store at room temperature

4X Running Buffer:
100mM Tris (Sigma) 84.75g
add 400mls dH₂O
pH to 8.8
add 20mls 10% SDS
Make up to a final volume of 500mls with dH₂O
Store at room temperature
2X Stacking Buffer:
124mM Tris 15g
add 400mls dH₂O
pH to 6.8
add 10mls 10% SDS
Make up to a final volume of 500mls with dH₂O
Store at room temperature

10% Ammonium Persulphate (APS):
1g APS (Sigma)
add 10ml dH₂O
Aliquot and stored at -20°C

10X Protein gel Buffer:
125mM Tris 15.1g
1.25M Glycine (Sigma) 94.0g
50ml 10% SDS
Make up to 500ml with dH₂O
Store at room temperature

10X TBS:
200mM Tris 24.2g
1.27M NaCl (Sigma) 80g
add 900ml dH₂O
pH to 7.6
Make up to 1 litre with dH₂O
Note: to make TBS-0.5% tween (TBST), add 5ml of Tween20 (Sigma) to 1 litre of 10X TBS. Dilute to 1X with dH₂O before use.
Calculation for 10% Gel;

<table>
<thead>
<tr>
<th>No of gels</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume</td>
<td>10ml</td>
<td>15ml</td>
<td>20ml</td>
<td>25ml</td>
</tr>
<tr>
<td>4X R.buffer</td>
<td>2.5ml</td>
<td>3.75ml</td>
<td>5ml</td>
<td>6.25ml</td>
</tr>
<tr>
<td>H2O</td>
<td>4.1ml</td>
<td>6.1ml</td>
<td>8.15ml</td>
<td>10.15ml</td>
</tr>
<tr>
<td>Bis:Acrylamide</td>
<td>3.3ml</td>
<td>5ml</td>
<td>6.67ml</td>
<td>8.33ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>100μl</td>
<td>150μl</td>
<td>200μl</td>
<td>250μl</td>
</tr>
<tr>
<td>Temed</td>
<td>10μl</td>
<td>15μl</td>
<td>20μl</td>
<td>25μl</td>
</tr>
</tbody>
</table>

Table 2.13a: Running gel components

<table>
<thead>
<tr>
<th>No of gels</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume</td>
<td>2.5ml</td>
<td>5ml</td>
<td>10ml</td>
<td>15ml</td>
</tr>
<tr>
<td>2X S. buffer</td>
<td>1.25ml</td>
<td>2.5ml</td>
<td>5ml</td>
<td>7.5ml</td>
</tr>
<tr>
<td>H2O</td>
<td>825μl</td>
<td>1.65ml</td>
<td>3.3ml</td>
<td>4.85ml</td>
</tr>
<tr>
<td>Bis:Acrylamide</td>
<td>400μl</td>
<td>800μl</td>
<td>1.6ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>25μl</td>
<td>50μl</td>
<td>100μl</td>
<td>150μl</td>
</tr>
<tr>
<td>Temed</td>
<td>2.5μl</td>
<td>5μl</td>
<td>10μl</td>
<td>15μl</td>
</tr>
</tbody>
</table>

Table 2.13b: Stacking gel components

Procedure

Preparing the running gel (Table 2.13a)
The running gel was poured to within an inch of the top of the plate to allow space for the stacking gel and comb.
This was then covered with ~300μl isopropanol to give an even finish at the top of the running gel.
The gel was left to set for ~15 minutes.

Preparing the stacking gel (Table 2.13b)
The isopropanol was poured from the running gel.
The gel was rinsed three times with dH2O.
The plates were emptied of H2O and any excess was absorbed with filter paper.
The space above the running gel was filled with stacking gel.
Immediately after this, the comb was inserted at an angle from the right side and gradually allowed to enter the gel, this was finished by filling with stacking gel. This was left to set for the appropriate time needed, ~20 minutes.

**Preparing the samples for loading**

**Materials**

4X Llaemulli buffer

**A:**
3.03 g Trizma base
2 ml of 10% SDS
added 30mls dH₂O
pH to 6.8
Made up to 50 ml with dH₂O

**B:**
To 5 ml of the “A” the following was added;
4ml Glycerol (Sigma)
SDS 1 g
0.05g Bromophenol blue (Sigma)
500 μl β-mercaptoethanol (Sigma)
This was made up to a final volume of 10 ml with dH₂O

An aliquot of 4X dye was diluted in dH₂O to a final concentration of 2X before addition to the samples.

After measurement of the samples with the BCA assay as described in section 2.11.3, the volume needed to load 20μg of protein in each well was calculated from the concentration obtained.

The appropriate volume of sample equivalent to 20μg protein was pipetted into a 1.5ml tube and an equal volume of 2X Llaemulli loading dye was added to a final concentration of 1X.

The sample was boiled at 85°C in the water bath for 5 minutes, allowed to cool to room temperature and set aside until the gel was ready to load.
Running the gel
400mls of 1X Protein Gel Buffer was prepared.
The electrophoresis tank was filled up to the tubing with 1X protein gel buffer
The seal from around the gel was removed.
The gel was inserted into the tank and held in place with the clamping apparatus.
The bottom of the gel was checked to ensure there were no air bubbles and the apparatus
was gently tilted to one side to remove any that were present.
The intermediate space was filled with the remaining buffer.
The comb was removed and the wells were ‘blown out’ with a syringe.
The molecular weight marker was loaded into the first well followed by the test samples
as shown in Figure 2.13.

Figure 2.13: A schematic diagram of the electrophoresis tank for P.A.G.E

It was ensured that the 1X buffer was level with the top of the glass plates.
The gel was run for 90 minutes at 200V and 25mA (This depends on the number of gels,
25mA is per gel, the current should be adjusted appropriately).
2.11.5. Western Blotting

**Principle**

In this technique a sample of proteins is first electrophoresed by SDS-PAGE to separate the proteins on the basis of their molecular weight. The wet gel is then placed against a sheet of nitrocellulose and placed in a special type of electrophoretic chamber. The gel is then subjected to an electric field which causes the proteins to migrate out of the gel and onto the nitrocellulose sheet, to which the proteins become tightly and irreversibly adsorbed. The nitrocellulose with its tightly bound proteins can then be ‘blotted’ or ‘probed’ with an antibody which will bind to the nitrocellulose sheet only in places where the protein(s) is recognized. A conjugated secondary antibody is used which allows for chemiluminescent detection of the protein(s). We can then determine where on the nitrocellulose blot the primary antibody binds and how intense the binding is, therefore determining the protein present in the sample. Table 2.14 gives a list of the antibodies and their dilutions used in western blotting.

**Reagents required**

**Bjerrum transfer buffer:**

- 48mM Tris 5.8g
- 39mM Glycine 2.9g
- 0.4w/v SDS 4mls of 10% SDS
- 20% Methanol (VWR, IRE) 200ml

Make up to 1 litre with dH2O

**1X TBST:**

Make 10X TBS initially
- 200mM Tris 24.2g
- 1.27M NaCl 80g
- add 900ml dH2O
- pH to 7.6

Make up to 1 litre with dH2O

Add 5ml of Tween20 to 1 litre of 10X TBS.

Dilute to 1X with dH2O before use.
5% milk TBST:
5% w/v milk powder (Marvel, UK) in 1X TBST

Developing solution:
Supersignal West Pico Solution (Pierce, Illinois, USA)

**Procedure**

**Transfer to nitrocellulose**
Ten pieces of blotting paper were cut to the same size as the gel (6.5" x 9.5") and one of nitrocellulose (6.5" x 8.5") membrane for each gel.
The nitrocellulose membrane was left to soak in Bjerrum transfer buffer for 30 minutes before use.
Five pieces of soaked filter paper were carefully placed, one at a time, in the centre of the semi-dry blotter.
A glass rod was rolled over the filter papers to remove any air bubbles.
The nitrocellulose membrane was numbered in the top left corner to indicate which gel it is and its orientation for developing.
This was then placed on top of the filter paper and soaked with buffer.
The glass plates of the gel were prised apart, the stacking gel was removed with a disposable scalpel, and the gel was rinsed with transfer buffer, and placed on to the nitrocellulose membrane.
This was smoothed out using a glass rod, removing any air bubbles.
The last five pieces of wet filter paper were placed over the gel as shown in Figure 2.14.
Holding the 2 white screws of the semi-dry blotter apparatus (C.B.S Scientific, CA, USA), the lid of the blotter was placed over the stack while gently applying pressure.
The power cords were plugged into the power supply unit (C.B.S Scientific, CA, USA), and the transfer took place at a current of 100mA (per gel) for 90 minutes with voltage set at 200V.
Upon completion the blot was washed once with TBST.
Figure 2.14: A schematic drawing of the semi-dry blotting method.
The arrow represents the direction of the transfer.

Blocking
30mls per blot of 5% Marvell in TBST was prepared in a universal.
This was then poured into a small plastic plate and the nitrocellulose membrane was
immersed in the solution.
The plate was left gently shaking for 1 hour to block any non-specific sites.
The blocking buffer was poured off and the membrane was then rinsed with TBST.

1° Antibody Incubation
The primary antibody was prepared in a falcon tube using the appropriate dilution in 5%
Marvell in TBST.
The membrane was inserted facing inwards into the tube and incubated overnight at
room temperature on the sample roller.

2° Antibody Incubation
The membrane was then washed with TBST on the shaker for 5 minutes X5.
The secondary antibody was prepared in a universal using the appropriate dilution with
TBST (30mls per blot).
After the five washes, the membrane was incubated in a small plate using the 2°
antibody, on the shaker for 90 minutes.
Development of film

Using the ‘Supersignal West Pico substrate’ kit, the following solution was prepared:

- Peroxide buffer \( 500 \mu l \)
- Luminol/Enhancer solution \( 500 \mu l \)

This 1ml solution was washed over the blot in a clear plate for 5 minutes.
The gel was placed on a clear acetate in the developing cassette and covered with a second acetate and taped down.

This cassette was brought to the dark room with x-ray film.

After an initial 5 minutes exposure, the film was first placed in developer solution until the bands were visible on the film, then placed in fixer for 2 minutes and rinsed with water then left hanging on a line to dry. If needed, a second film was placed over the membrane in the cassette and exposed for a longer time period.

Once dry the film was again placed over the membrane in the light and labelled with the bands visible from the molecular weight marker.

<table>
<thead>
<tr>
<th>Primary ab</th>
<th>Dilution</th>
<th>Secondary ab</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal ERα</td>
<td>1:200</td>
<td>Goat anti-rabbit IgG HRP</td>
<td>1:2000</td>
</tr>
<tr>
<td>Mouse monoclonal β actin</td>
<td>1:200</td>
<td>Goat anti-mouse IgG HRP</td>
<td>1:2000</td>
</tr>
<tr>
<td>Goat polyclonal F7</td>
<td>1:200</td>
<td>Donkey anti-goat IgG HRP</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit polyclonal Prothrombin</td>
<td>1:200</td>
<td>Goat anti-rabbit IgG HRP</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

Table 2.14: List of antibodies and their dilutions used in western blotting.
2.11.5 (i) Ponceau S stain (Ponceau S, Sigma, US)

This is a rapid and reversible staining method for detecting protein bands on Western blots after electrophoretic transfer.

Procedure

After electrophoresis, the blotted membrane was immersed in a sufficient volume of Ponceau S staining solution to cover the blot and left on the Clifton plate shaker for 2 minutes. After this time period the protein bands would show up stained red on the membrane and the transfer process was deemed successful.

After staining, the membrane was destained using dH2O continuously until all the red dye was removed, ~5 minutes. The membrane was removed, rinsed in 1X TBST and blocked as normal.

2.11.5 (ii) Stripping blots

In order to probe for a loading control on each blot it was necessary to strip and reprobe each blot. Stripping the blot removes the primary antibody and its reagents leaving the proteins on the membrane intact and ready to be probed with a second primary antibody.

Reagent Required

Stripping buffer

1.5g Glycine
1ml 10% SDS
0.1ml Tween
added 70mls dH2O

pH to 2.2

Made up to 100mls with dH2O

Procedure

After developing, the blot was placed in stripping buffer and washed on the Clifton plate shaker for 30 minutes. This was followed by a second wash with fresh stripping buffer for another 30 minutes.

The blot was then washed in 1X TBS for 5 minutes on the shaker x3.

The blot was then placed in blocking buffer, 5% milk/TBST, as described in 2.9.5 and the western blot protocol was followed using the loading control primary antibody.
2.11.6 Enzyme-linked immunosorbent assay (ELISA)

_Rat C-Reactive Protein (CRP) Antigen Assay_
(Patricell, UK)

**Principle**

This enzyme immunoassay is used for the quantitative determination of rat CRP (C-Reactive Protein) in rat sera. The method was adapted to detect CRP in rat tissue homogenates. Samples were added to micro-titre wells and the CRP present in the sample reacts with the anti-CRP antibodies which have been adsorbed to the surface of the wells. After the removal of unbound proteins by washing, a second anti-CRP antibody conjugated with horseradish peroxidase (HRP) was added to the wells. The conjugated antibody bound to the free antigenic determinants on the immobilized CRP forming a coat antibody-CRP antigen-conjugated antibody "sandwich". Unbound conjugated antibody and other unbound materials were washed away and the enzyme bound to the immunosorbsent was assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme was directly proportional to the amount of CRP in the sample and interpolated from the standard curve constructed from the standards.

**Reagents Required**

_Anti-rat CRP ELISA micro plate:_ 96 wells in twelve removable eight-well micro well strips in a well holder frame, each well coated with affinity purified anti-rat CRP.

_Diluent Concentrate:_ 50ml of a 5X concentrated phosphate buffered saline (PBS) solution containing bovine serum albumin, 0.25% Tween, and 0.25% Proclin 300 as a preservative.

_Wash Solution Concentrate:_ 50ml of a 20X concentrated PBS solution containing 1% Tween.

_Enzyme-antibody conjugate:_ 200μl of affinity purified anti-rat CRP antibody conjugated with HRP.
Chromogen substrate solution: 12ml of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide (H$_2$O$_2$) in citric acid buffer at pH 3.3.

Stop solution: 12ml of 0.3M sulphuric acid (H$_2$SO$_4$)

Rat CRP calibrator: Lyophilized rat CRP calibrator for resuspension with 1ml of dH$_2$O to give 1.35μg/ml.

Reagent Preparation

Diluent concentrate: diluted to 1X with dH$_2$O.

Wash solution concentrate: diluted to 1X with dH$_2$O.

Enzyme antibody conjugate: 100μl of enzyme antibody conjugate was added to 10ml dH$_2$O and gently mixed.

Procedure

Preparation of Standards

1ml of dH$_2$O was added to the rat CRP calibrator and mixed gently by pipetting until it dissolved.

Immediately prior to their use, the standards (std) were prepared. Firstly a 200ng/ml stock was prepared and this was then diluted to give a 100ng/ml standard with diluent, the standards to be used in the assay were then prepared according to Table 2.15.

<table>
<thead>
<tr>
<th>Standards</th>
<th>ng/ml</th>
<th>Volume added to 1X diluent</th>
<th>1X diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>300μl of 100ng/ml std</td>
<td>300μl</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>300μl of std 1</td>
<td>300μl</td>
</tr>
<tr>
<td>3</td>
<td>12.5</td>
<td>300μl of std 2</td>
<td>300μl</td>
</tr>
<tr>
<td>4</td>
<td>6.25</td>
<td>300μl of std 3</td>
<td>300μl</td>
</tr>
<tr>
<td>5</td>
<td>3.125</td>
<td>300μl of std 4</td>
<td>300μl</td>
</tr>
<tr>
<td>6</td>
<td>0 (blank)</td>
<td>0</td>
<td>200μl</td>
</tr>
</tbody>
</table>

Table 2.15: Preparation of the standard samples for the CRP ELISA.
100μl of Standard 1 was added to wells 1A and 2A
100μl of Standard 2 was added to wells 1B and 2B
100μl of Standard 3 was added to wells 1C and 2C
100μl of Standard 4 was added to wells 1D and 2D
100μl of Standard 5 was added to wells 1E and 2E
100μl of Standard 6 (blank) was added to wells 1F and 2F

Tissue preparation
Rat tissue samples were homogenised and the total protein extraction method was used, as described in section 2.9.2. Samples were diluted 1:100, in the same diluent used for the standards, before use. 100μl of sample 1 was added to wells 1G and 2G. The next sample was added to wells 1H and 2H and so on until all samples were added.

The plate was incubated on the bench at room temperature for 10 minutes. Following incubation, the contents were removed from the wells by inverting the plate over a waste container.
Each well was completely filled with 1X wash solution and removed manually by inverting the plate to shake out the contents into the sink and the wells were sharply struck onto absorbent paper to remove any residual buffer. This was repeated three times, for a total of four washes. 100μl of diluted enzyme conjugated antibody was added to each well. The plate was incubated in the dark at room temperature for 10 minutes. As above, the wells were washed out with 1X wash solution four times. 100μl of TMB substrate solution was added to each well. The plate was incubated in the dark at room temperature for precisely 5 minutes. After 5 minutes, 100μl of stop solution was added to each well with a multichannel pipette for uniformity.
The absorbance of each well was determined by reading the contents of the wells at 450nm on a microtitre plate spectrophotometer (MRX Microplate reader).

Calculations
The average background value was subtracted from the mean of the test values for each sample.
A standard curve was constructed using the mean of the results from the standards, as depicted in Figure 2.15. Sample values were interpolated from the curve and corrected for the dilution factor to determine the sample CRP concentrations.

Figure 2.15: An example of a standard curve for Rat CRP
**Rat Fibrinogen Total Antigen Assay**  
(Patricell, UK)

**Principle**

This enzyme immunoassay is used for the quantitative determination of rat fibrinogen in rat plasma and sera. The method was adapted to detect fibrinogen in rat tissue homogenates. Samples were added to micro-titre wells and the fibrinogen present in the sample reacts with the affinity purified capture antibody coated to the surface of the wells. After the removal of unbound proteins by washing, a biotin labelled anti-rat fibrinogen primary antibody was added to the wells. After washing, the bound polyclonal antibody was reacted with avidin conjugated to HRP. Unbound conjugated antibody and other unbound materials were washed away and the enzyme bound to the immunosorbent was assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme was directly proportional to the amount of fibrinogen in the sample and interpolated from the standard curve constructed from the standards.

**Reagents Required**

Coated micro plate: 96 wells in twelve removable eight well micro well strips in a well holder frame, each well coated, blocked and dried with capture antibody.

Diluent Concentrate: 50ml of a 5X concentrated phosphate buffered saline (PBS) solution.

Wash buffer Concentrate: 50ml of 10X concentrated wash buffer.

Rat Fibrinogen antigen standard: 1 vial of lyophilized standard.

Anti-Rat Fibrinogen primary standard: 1 vial of lyophilized biotin labelled rabbit polyclonal antibody.

Avidin peroxidase conjugate secondary antibody: 1 vial of concentrated HRO labelled avidin.
Chromogen substrate solution: 10ml of TMB substrate solution.

Stop solution: 10ml of 1M sulphuric acid (H\textsubscript{2}SO\textsubscript{4})

Reagent Preparation
Diluent concentrate: diluted to 1X with dH\textsubscript{2}O.

Wash buffer concentrate: diluted to 1X with dH\textsubscript{2}O.

Procedure

Preparation of Standards
The standard vial was reconstituted with 5ml of 1X diluent to give a concentration of 1600ng/ml, and mixed gently by pipetting until it dissolved.

Immediately prior to use the standards (std) were prepared. Firstly a stock solution was prepared at 800ng/ml and diluted to give a 200ng/ml standard with 1X diluent.

The standards to be used in the assay were then prepared according to Table 2.15.

<table>
<thead>
<tr>
<th>Standards</th>
<th>ng/ml</th>
<th>Volume added to 1X diluent</th>
<th>1X diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>500\mu l of 200ng/ml std</td>
<td>500\mu l</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>500\mu l of std 1</td>
<td>500\mu l</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>500\mu l of std 2</td>
<td>500\mu l</td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
<td>500\mu l of std 3</td>
<td>500\mu l</td>
</tr>
<tr>
<td>5</td>
<td>6.25</td>
<td>500\mu l of std 5</td>
<td>500\mu l</td>
</tr>
<tr>
<td>6</td>
<td>0 (blank)</td>
<td>0</td>
<td>500\mu l</td>
</tr>
</tbody>
</table>

Table 2.15: Preparation of the standard samples for the Fibrinogen ELISA.
100μl of Standard 1 was added to wells 1A and 2A
100μl of Standard 2 was added to wells 1B and 2B
100μl of Standard 3 was added to wells 1C and 2C
100μl of Standard 4 was added to wells 1D and 2D
100μl of Standard 5 was added to wells 1E and 2E
100μl of Standard 6 (blank) was added to wells 1F and 2F

Tissue preparation
Rat tissue samples were homogenised and the total protein extraction method was used, as described in section 2.9.2.
Samples were diluted 1:500, in the same diluent used for the standards, before use.
100μl of sample 1 was added to wells 1G and 2G. The next sample was added to wells 1H and 2H and so on until all samples were added.
The plate was incubated at room temperature, shaking at 300rpm for 30 minutes.
Following incubation the contents were removed from the wells by inverting the plate over a waste container.
Each well was completely filled with the diluted wash solution and removed manually by inverting the plate to shake out the contents into the sink and the wells were sharply struck onto absorbent paper to remove any residual buffer. This was repeated three times, for a total of four washes.
The primary antibody vial was reconstituted with 10ml of 1X diluent and agitated gently to completely dissolve the contents.
100μl of diluted primary antibody was added to each well.
The plate was incubated at room temperature, shaking at 300rpm for 30 minutes.
As above, the wells were washed out with diluted wash solution four times.
The secondary antibody was diluted 5μl in 5ml of 1x diluent and mixed gently by pipetting. 2.5ml of diluted secondary antibody was added to 7.5ml of 1x diluent and 100μl was added to each well.
The plate was incubated at room temperature, shaking at 300rpm for 30 minutes.
As above, the wells were washed out with diluted wash solution four times.
100μl of TMB substrate solution was added to each well.
The plate was incubated at room temperature for 5-15 minutes shaking at 300rpm.
The reaction was stopped by the addition of 50μl H₂SO₄ to each well and the final absorbance was read at 450nm on a micro-titre plate spectrophotometer (MRX Microplate reader).

Calculations
The average background value was subtracted from the test values for each sample. A standard curve was constructed using the results from the standards, plotting A₄₅₀ against the amount of rat fibrinogen in the standards, as depicted in Figure 2.16. Sample values were interpolated from the curve and corrected for the dilution factor to determine the sample fibrinogen concentrations.

**Figure 2.16: An example of a standard curve for Rat Fibrinogen**
**Rat Plasminogen Activator Inhibitor -1 (PAI-1) Total Antigen**
(Patricell, UK)

**Principle**

This enzyme immunoassay is used for the quantitative determination of rat total plasminogen activator inhibitor type 1 (PAI-1) in rat biological fluids. The method was adapted to detect PAI-1 in rat tissue homogenates. Samples were added to micro-titre wells and the PAI-1 present in the sample reacts with the anti-rat PAI-1 capture antibody coated to the surface of the wells. After the removal of unbound proteins by washing, anti-PAI-1 antibody was added to the wells. Excess primary antibody was washed away and the bound antibody, which is proportional to the total PAI-1 present in the samples, was then reacted with a secondary antibody. Following an additional washing step, TMB was then added to allow for colour development at 450nm. The amount of colour development was directly proportional to the concentration of total PAI-1 in the sample.

**Reagents Required**

- **Anti-rat PAI-1 ELISA micro plate**: 96 wells in twelve removable eight well micro well strips in a well holder frame, each well coated with anti-rat PAI-1 capture antibody, blocked and dried.

- **Wash Solution Concentrate**: 50ml of a 10X was buffer.

- **Rat PAI-1 activity standard**: 1 vial of lyophilized standard.

- **Anti-rat PAI-1 primary antibody**: 1 vial of lyophilized polyclonal anti-rat antibody.

- **Anti-rat HRP conjugated secondary antibody**: 1 vial of concentrated horseradish peroxidase-labelled antibody.

- **TMB substrate**: 10ml TMB substrate solution.
Reagent Preparation

Wash solution concentrate: diluted to 1X with dH₂O.

Anti-rat PAI-1 primary antibody: Reconstituted as directed in kit instructions.

Anti-rat HRP conjugated secondary antibody: 1μl of conjugated secondary antibody was added to 10ml of 3% blocking buffer.

1N H₂SO₄:

350ml dH₂O
Added 14ml H₂SO₄
Stirred with magnetic stirrer
Made up to 500ml with dH₂O.

3% blocking buffer: 3% milk in TBS (3g milk in 100ml TBS).

TBS Buffer:

0.1M Tris (12.1g)
0.15M NaCl (8.7g)
added 900ml dH₂O
pH to 7.4
made up to 1 litre with dH₂O.
Procedure

Preparation of Standards
The standard was reconstituted as directed in the kit giving a concentration of 50 ng/ml solution. The standards to be used in the assay were then prepared according to Table 2.16.

<table>
<thead>
<tr>
<th>Standards</th>
<th>Conc ng/ml</th>
<th>Volume added to blocking buffer</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>100 µl from vial</td>
<td>900 µl</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>500 µl of std 1</td>
<td>500 µl</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>400 µl of std 2</td>
<td>600 µl</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>500 µl of std 3</td>
<td>500 µl</td>
</tr>
<tr>
<td>5</td>
<td>0.2</td>
<td>400 µl of std 4</td>
<td>600 µl</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>500 µl of std 5</td>
<td>500 µl</td>
</tr>
<tr>
<td>7</td>
<td>0.05</td>
<td>500 µl of std 6</td>
<td>500 µl</td>
</tr>
<tr>
<td>8</td>
<td>0 (blank)</td>
<td>0</td>
<td>300 µl</td>
</tr>
</tbody>
</table>

Table 2.16: Preparation of the standard samples for the PAI-1 ELISA.

100 µl of Standard 1 was added to wells 1A and 2A
100 µl of Standard 2 was added to wells 1B and 2B
100 µl of Standard 3 was added to wells 1C and 2C
100 µl of Standard 4 was added to wells 1D and 2D
100 µl of Standard 5 was added to wells 1E and 2E
100 µl of Standard 6 was added to wells 1F and 2F
100 µl of Standard 7 was added to wells 1G and 2G
100 µl of Standard 8 (blank) was added to wells 1H and 2H

Tissue preparation
Rat tissue samples were homogenised and the total protein extraction method was used, as described in 2.9.2.
Samples were used neat.
100 µl of sample 1 was added to wells 3A and 4A. The next sample was added to wells 3B and 4B and so on until all samples were added.
The plate was incubated at room temperature, shaking at 300rpm for 30 minutes. Following incubation the contents were removed from the wells by inverting the plate over a waste container.

Each well was completely filled with the diluted wash solution and removed manually by inverting the plate to shake out the contents into the sink and the wells were sharply struck onto absorbent paper to remove any residual buffer. This was repeated three times, for a total of four washes.

100μl of diluted primary antibody was added to each well.

The plate was incubated at room temperature, shaking at 300rpm for 30 minutes. As above, the wells were washed out with diluted wash solution four times.

100μl of reconstituted secondary antibody was added to each well.

The plate was incubated at room temperature, shaking at 300rpm for 30 minutes. As above, the wells were washed out with diluted wash solution four times.

100μl of TMB substrate solution was added to each well.

The plate was incubated at room temperature for 2-10 minutes shaking at 300rpm. The reaction was stopped by the addition of 50μl H₂SO₄ to each well and the final absorbance was read at 450nm on a micro-titre plate spectrophotometer (MRX Microplate reader).

Calculations

The average background value was subtracted from the test values for each sample. A standard curve was constructed using the results from the standards, as depicted in Figure 2.17. Sample values were interpolated from the curve and corrected for the dilution factor to determine the sample PAI-1 concentrations.

![Figure 2.17: An example of a standard curve for Rat PAI-1](image)
Rat Tissue Plasminogen Activator (tPA) Total Antigen Assay
(Patricell, UK)

Principle

This enzyme immunoassay is used for the quantitative determination of total tissue plasminogen activator (tPA) in rat plasma. The method was adapted to detect tPA in rat tissue homogenates. Samples were added to micro-titre wells and the tPA present in the sample reacts with the capture antibody coated to the surface of the wells. After the removal of unbound proteins by washing, polyclonal anti-murine tPA primary antibody was added to the wells. Excess primary antibody was washed away and the bound antibody was then reacted with a secondary antibody conjugated to horseradish peroxidase. Following an additional washing step, TMB was then added to allow for colour development at 450nm. The amount of colour development was directly proportional to the concentration of total tPA in the sample.

Reagents Required

ELISA micro plate: 96 wells in twelve removable eight well micro well strips in a well holder frame, each well coated with a capture antibody, blocked and dried.

Wash Solution Concentrate: 50ml of a 10X was buffer.

Rat tPA activity standard: 1 vial of lyophilized rat tPA standard.

Anti-murine tPA biotinylated primary antibody: 1 vial of lyophilized polyclonal anti-mouse antibody.

Avidin horseradish peroxidase conjugate secondary antibody: 1 vial of concentrated HRP-labelled antibody.

TMB substrate: 10ml TMB substrate solution.
Reagent Preparation

Wash solution concentrate: diluted to 1X with dH₂O.

Anti-murine tPA biotinylated primary antibody: added 10ml 3% BSA blocking buffer to the vial and gently mixed.

Avidin horseradish peroxidase conjugate secondary antibody: Diluted 3μl of conjugated secondary antibody into 10ml of 3% blocking buffer.

1N H₂SO₄: As described previously

3% blocking buffer: As described previously

TBS Buffer: As described previously

Procedure

Preparation of Standards

The standard was reconstituted as directed in the kit giving a concentration of 1,000ng/ml solution. This stock was diluted to give a working stock solution at 5ng/ml with blocking buffer. The standards to be used in the assay were then prepared according to Table 2.17.

<table>
<thead>
<tr>
<th>Standards</th>
<th>Conc. ng/ml</th>
<th>Volume added to blocking buffer</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>400μl from 5ng/ml std</td>
<td>500μl</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>500μl of std 1</td>
<td>500μl</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>500μl of std 2</td>
<td>500μl</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>500μl of std 3</td>
<td>500μl</td>
</tr>
<tr>
<td>5</td>
<td>0.125</td>
<td>500μl of std 4</td>
<td>500μl</td>
</tr>
<tr>
<td>6</td>
<td>0.0625</td>
<td>500μl of std 5</td>
<td>500μl</td>
</tr>
<tr>
<td>7</td>
<td>0 (blank)</td>
<td>0</td>
<td>300μl</td>
</tr>
</tbody>
</table>

Table 2.17: Preparation of the standard samples for the tPA ELISA.
100μl of Standard 1 was added to wells 1A and 2A
100μl of Standard 2 was added to wells 1B and 2B
100μl of Standard 3 was added to wells 1C and 2C
100μl of Standard 4 was added to wells 1D and 2D
100μl of Standard 5 was added to wells 1E and 2E
100μl of Standard 6 was added to wells 1F and 2F
100μl of Standard 7 (blank) was added to wells 1G and 2G

**Tissue preparation**

Rat tissue samples were homogenised and the total protein extraction method was used, as described in 2.9.2.

Samples were diluted 1:3, in the same diluent used for the standards, before use.

100μl of sample 1 was added to wells 1H and 2H. The next sample was added to wells 3A and 4A and so on until all samples were added.

The plate was incubated at room temperature, shaking at 300rpm for 30 minutes.

Following incubation the contents were removed from the wells by inverting the plate over a waste container.

Each well was completely filled with the diluted wash solution and removed manually by inverting the plate to shake out the contents into the sink and the wells were sharply struck onto absorbent paper to remove any residual buffer. This was repeated three times, for a total of four washes.

100μl of diluted primary antibody was added to each well.

The plate was incubated at room temperature, shaking at 300rpm for 30 minutes.

As above, the wells were washed out with diluted wash solution four times.

100μl of reconstituted secondary antibody was added to each well.

The plate was incubated at room temperature, shaking at 300rpm for 30 minutes.

As above, the wells were washed out with diluted wash solution four times.

100μl of TMB substrate solution was added to each well.

The plate was incubated at room temperature for 5-15 minutes shaking at 300rpm.

The reaction was stopped by the addition of 50μl H₂SO₄ to each well and the final absorbance was read at 450nm on a micro-titre plate spectrophotometer (MRX Microplate reader).
Calculations

The average background value was subtracted from the test values for each sample.
A standard curve was constructed using the results from the standards, as depicted in Figure 2.18. Sample values were interpolated from the curve and corrected for the dilution factor to determine the sample tPA concentrations.

Figure 2.18: An example of a standard curve for Rat tPA
Human Plasminogen activator inhibitor-1 (PAI-1) Total Antigen Assay
(Hyphen Biomed, France)

Principle

This enzyme immunoassay is used for the quantitative determination of total plasminogen activator inhibitor-1 (PAI-1) in human plasma. The method was adapted to detect PAI-1 in cell culture medium. Anti-(h)-PAI-1-HRP immunoconjugate was added to the micro-titre wells that were coated with another monoclonal antibody specific for PAI-1: Ag. This was immediately followed by the samples to be tested and the PAI-1 antigen present in the sample binds onto the monoclonal antibody coated solid phase through one epitope, and fixes the second monoclonal antibody coupled to HRP by another epitope. Following a washing step, TMB substrate was added and a blue colour develops. The reaction is then stopped with sulphuric acid. The amount of colour development was directly proportional to the concentration of total PAI-1 in the sample.

Reagents Required

ELISA micro plate: 96 wells in twelve removable eight well micro well strips in a well holder frame, each well coated with a murine monoclonal antibody specific for human PAI-1: Ag.

Wash Solution Concentrate: 50ml of a 20X wash buffer.

Human PAI-1 standard: 3 vials of lyophilized human PAI-1 standard.

Anti-human-PAI-1-HRP immunoconjugate: 3 vial of lyophilized monoclonal anti-human antibody coupled to HRP.

Conjugate Diluent: 1 vial of 25ml conjugate diluent

F-sample diluent: 2 vials of 50ml F-sample diluent

TMB substrate: 25ml TMB substrate solution.
Stop Solution: 1 vial of 6ml sulphuric acid

Plasma PAI-1 control: 1 vial of lyophilised human plasma control (high) and 1 vial of lyophilised human plasma control (low).

Reagent Preparation

Wash solution concentrate: warmed the vial at 37°C for 15 minutes until complete dissolution of solids. Diluted to 1X with dH2O.

Anti-human-PAI-1-HRP immunoconjugate: added 4ml conjugate diluent to the vial and gently mixed.

PAI-1 standard: reconstituted vial with 2ml of F-sample diluent to give a 10ng/ml solution

Plasma Control: reconstituted both controls each with 1ml dH2O.

Procedure

Preparation of Standards

The standard was reconstituted as directed in the kit giving a concentration of 10ng/ml solution. The standards to be used in the assay were then prepared according to Table 2.18.

<table>
<thead>
<tr>
<th>Standards</th>
<th>Conc. (ng/ml)</th>
<th>Volume added to F-sample diluent</th>
<th>F-sample diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>1ml of 10ng/ml stock</td>
<td>0µl</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>500µl of stock</td>
<td>500µl</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>250µl of stock</td>
<td>750µl</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>100µl of stock</td>
<td>900µl</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>50µl of stock</td>
<td>950µl</td>
</tr>
<tr>
<td>6</td>
<td>0 (blank)</td>
<td>0</td>
<td>1ml</td>
</tr>
</tbody>
</table>

Table 2.18: Preparation of the standard samples for the human PAI-1 ELISA.
Control plasma preparation
Plasma controls (low and high) were diluted 1:5 with F-sample diluent

Sample preparation
Cell culture medium samples were diluted 1:50 in F-sample diluent.
The anti-(h)-PAI-1-HRp immunoconjugate was added to the wells (100μl/well)
Immediately after, the standards, controls and test samples were added to the plate in duplicate (100μl/well)
The plate was incubated at room temperature, shaking at 300rpm for 60 minutes.
Following incubation the contents were removed from the wells by inverting the plate over a waste container.
Each well was completely filled with 300μl of 1X wash solution and removed manually by inverting the plate to shake out the contents into the sink and the wells were sharply struck onto absorbent paper to remove any residual buffer. This was repeated four times, for a total of five washes.
200μl of TMB substrate solution was added to each well.
The plate was incubated at room temperature for exactly 5 minutes.
The reaction was stopped by the addition of 50μl sulphuric acid stop solution to each well and the final absorbance was read at 450nm on a micro-titre plate spectrophotometer (MRX Microplate reader).

Calculations
The average background value was subtracted from the test values for each sample.
A standard curve was constructed using the results from the standards, as depicted in Figure 2.19. Sample values were interpolated from the curve and corrected for the dilution factor to determine the sample PAI-1 concentrations.

![Figure 2.19: An example of a standard curve for human PAI-1](image-url)
Human Tissue Plasminogen Activator (tPA) Total Antigen Assay
(Hyphen Biomed, France)

Principle

This enzyme immunoassay is used for the quantitative determination of total tissue plasminogen activator in human plasma. The method was adapted to detect tPA in cell culture medium. Anti-(h)-tPA-HRP immunoconjugate was added to the micro-titre wells that were coated with another monoclonal antibody specific for tPA: Ag. This was immediately followed by the samples to be tested and the tPA antigen present in the sample binds onto the monoclonal antibody coated solid phase through one epitope, and fixes the second monoclonal antibody coupled to HRP by another epitope. Following a washing step, TMB substrate was added and a blue colour develops. The reaction is then stopped with sulphuric acid. The amount of colour development was directly proportional to the concentration of total tPA in the sample.

Reagents Required

ELISA micro plate: 96 wells in twelve removable eight well micro well strips in a well holder frame, each well coated with a murine monoclonal antibody specific for human tPA: Ag.

Wash Solution Concentrate: 50ml of a 20X wash buffer.

Human tPA standard: 3 vials of lyophilized human tPA standard.

Anti-human-tPA-HRP immunoconjugate: 3 vial of lyophilized monoclonal anti-human antibody coupled to HRP.

Conjugate Diluent: 1 vial of 25ml conjugate diluent

F-sample diluent: 2 vials of 50ml F-sample diluent

TMB substrate: 25ml TMB substrate solution.
Stop Solution: 1 vial of 6ml sulphuric acid

Plasma tPA control: 1 vial of lyophilised human plasma control I high (UTA) and 1 vial of lyophilised human plasma control II low (UTA).

**Reagent Preparation**

**Wash solution concentrate:** warmed the vial at 37°C for 15 minutes until complete dissolution of solids. Diluted to 1X with dH2O.

**Anti-human-tPA-HRP immunoconjugate:** added 7.5ml conjugate diluent to the vial and gently mixed.

tPA standard: reconstituted vial with 1ml of dH2O and dilute 1:2 with F-sample diluent to give a 13.5ng/ml solution.

Plasma Control: reconstituted both controls each with 1ml dH2O.

**Procedure**

**Preparation of Standards**

The standard was reconstituted as directed in the kit giving a concentration of 13.5ng/ml solution. The standards to be used in the assay were then prepared according to Table 2.18.

<table>
<thead>
<tr>
<th>Standards</th>
<th>Conc. ng/ml</th>
<th>Volume added to F-sample diluent</th>
<th>F-sample diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>741μl of 13.5ng/ml stock</td>
<td>259μl</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>370μl of stock</td>
<td>630μl</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>185μl of stock</td>
<td>815μl</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>74μl of stock</td>
<td>926μl</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>37μl of stock</td>
<td>963μl</td>
</tr>
<tr>
<td>6</td>
<td>0 (blank)</td>
<td>0</td>
<td>1ml</td>
</tr>
</tbody>
</table>

Table 2.18: Preparation of the standard samples for the human tPA ELISA.
Control plasma preparation

Plasma controls (low and high) were diluted 1:2 with F-sample diluent.

Sample preparation

Cell culture medium samples were used undiluted.
The anti-(h)-tPA-HRP immunoconjugate was added to the wells (50μl/well).
Immediately after, the standards, controls and test samples were added to the plate in duplicate (200μl/well).
The plate was incubated at room temperature for 60 minutes.
Following incubation the contents were removed from the wells by inverting the plate over a waste container.
Each well was completely filled with 300μl of 1X wash solution and removed manually by inverting the plate to shake out the contents into the sink and the wells were sharply struck onto absorbent paper to remove any residual buffer. This was repeated four times, for a total of five washes.
200μl of TMB substrate solution was added to each well.
The plate was incubated at room temperature for exactly 5 minutes.
The reaction was stopped by the addition of 50μl sulphuric acid stop solution to each well and the final absorbance was read at 450nm on a micro-titre plate spectrophotometer (MRX Microplate reader).

Calculations

The average background value was subtracted from the test values for each sample.
A standard curve was constructed using the results from the standards, as depicted in Figure 2.20. Sample values were interpolated from the curve and corrected for the dilution factor to determine the sample tPA concentrations.

Figure 2.20: An example of a standard curve for human tPA
2.12 Cell Culture

In vitro effects of phytoestrogens were studied in cell culture systems to compare with those effects found in liver and aorta tissues.

Cell Lines

Human umbilical artery endothelial cells (HUAECs) were purchased from Promocell (Promocell GmBH, Heidelberg, Germany).

Human Hepatocarcinoma cells (HepG2) were purchased from the Health protection Agency (HPA, Wiltshire, UK).

Human Hepatocarcinoma cells (Hep89) were kindly provided by Dr DC Harnish (Department of Nuclear Receptors, Wyeth Ayerst Research, Radnor, PA, USA).

The Hep89 cell line—HepG2 cells stably expressing ERα were created by transfecting the pcDNA3-ERα expression vector into HepG2 cells by electroporation using the BTX Electro Cell Manipulator 600 according to the manufacturer’s recommended settings. Stably expressing cells were selected by resistance to G418 (400mg/ml). Distinct, well isolated colonies were picked using Bellco cloning cylinders (638 mm) and assessed for the presence of ERα (314).

2.12.1 Common methods for all cell systems

Cell counting with Trypan Blue

Preparing the haemocytometer

The haemocytometer (Neubauer) was cleaned using 70% ethanol. The shoulders of the haemocytometer were moistened and a coverslip was affixed using gentle pressure and small circular motions. Newton’s rings were observed when the coverslip was correctly affixed, thus the depth of the chamber was ensured.

Preparing the cell suspension

Following trypsinization of the cells and centrifugation the cells were resuspended in 1 ml of fresh medium.

Using filter sterilized trypan blue staining solution, 20μl of the cell suspension was added to 20μl of trypan blue in a clean sterile 1ml tube (Starstedt).
Counting

Using the pipette, 10μl of the cell suspension containing trypan blue was drawn up and the haemocytometer was carefully filled by gently resting the end of the Gilson tip at the edge of one of the chambers. Care was taken not to overfill the chamber. The sample was drawn out of the pipette by capillary action, with the fluid running to the edges of the grooves only. The grid lines of the haemocytometer were focused on using the 10X objective of the microscope. One set of a 16 corner square was focused on for counting initially, as indicated in Figure 2.21:

![Figure 2.21: Haemocytometer counting chamber](image)

Using a hand tally counter, the number of cells in this area of 16 squares was counted. Only live cells that look healthy (unstained by trypan blue) were counted. The cells that were within the square and any positioned on the right hand or bottom boundary line were included. The haemocytometer was moved to focus on another set of 16 corner squares and the counting was continued until all 4 sets of 16 corner squares are counted. The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10⁴/ml. The total count from 4 sets of 16 corners = (cells/ml x 10⁴) x 4 squares from one haemocytometer grid, therefore;

The count was divided by 4 and multiplied by 2 to adjust for the 1:2 dilution in trypan blue. These two steps were also equivalent to dividing the cell count by 2.
In Vitro Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight</th>
<th>Supplier</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β estradiol</td>
<td>272.38g</td>
<td>Sigma</td>
<td>E8875</td>
</tr>
<tr>
<td>Equol</td>
<td>242.27g</td>
<td>Sigma</td>
<td>45405</td>
</tr>
<tr>
<td>Genistein</td>
<td>270.24g</td>
<td>Sigma</td>
<td>G6776</td>
</tr>
<tr>
<td>Daidzein</td>
<td>254.24g</td>
<td>Sigma</td>
<td>D7802</td>
</tr>
</tbody>
</table>

Table 2.21 List of estrogenic compounds used in cell culture work.

2.12.2 HUAEC

Reagents Required

Culture media

The base medium for the growth of HUAECs was endothelial cell basal medium (Promocell). To make the complete growth medium 0.02ml/ml fetal calf serum (FCS), 0.004ml/ml endothelial cell growth supplement, 0.1ng/ml human epidermal growth factor (hEGF), 1ng/ml human basic fibroblast growth factor (hbFGF), and 22.5μg/ml heparin and 1μg/ml hydrocortisone were added (All Promocell). The antibiotic penicillin/streptomycin was also added at 100 U/ml penicillin and 100μg/ml streptomycin (Sigma). Media was stored at 4°C for up to 6 weeks.

The base medium used in the stimulation of the above cell line was endothelial cell basal medium phenol red free (Promocell). To make the complete growth medium, 0.004ml/ml endothelial cell growth supplement, 0.1ng/ml human epidermal growth factor (hEGF), 1ng/ml human basic fibroblast growth factor (hbFGF), and 22.5μg/ml heparin and 1μg/ml hydrocortisone were added (Promocell). A final concentration of 2% dextran charcoal stripped (dcc) foetal bovine serum was also added (Sigma). The antibiotic penicillin/streptomycin was added at 100 U/ml penicillin and 100μg/ml streptomycin (Sigma). Media was stored at 4°C for up to 6 weeks.
Trypsin/EDTA
The Detach kit (Promocell) was used to in the subculture of HUAECs. This kit contained HepesBSS, 0.04%Trypsin/0.03%EDTA solution and Trypsin Neutralising solution (TNS). Aliquots were made and stored at -20°C.

Freezing Medium
Cryo-SFM was purchased from Promocell (Promocell GmBH, Heidelberg, Germany) and stored at 4°C. Cryo-SFM is a serum-free freezing medium used for cell culture systems where serum is not required.

Recovery of Cryopreserved cells
All surfaces were wiped down with 70% ethanol. Aseptic technique was adhered to at all times and all work was carried out in the laminar flow cabinet (Danlaf, Shaw Scientific, Ireland). Cell culture media was added to two 25cm² flasks (Nunc) at a volume of 200µl/cm². These flasks were incubated at 37°C, 5% (V/V) CO₂ and steam saturated atmosphere for approx 30mins (Sanyo, AGB Scientific, Ireland).
The cryovial of frozen cells (500,000 cells/ml) was taken from the liquid nitrogen container and brought to the laminar flow cabinet where the screw-top was slightly opened to reduce the overpressure and then closed again.
The vial was then brought to a 37°C pre-warmed waterbath where the tube was agitated carefully and continuously until ~90% of the contents were thawed. The tube was removed from the waterbath and agitated gently until all the contents were completely thawed.
The vial was then transferred to the laminar flow cabinet for addition of the cells to the culture flasks.
The equilibrated cell culture flasks were seeded with 10,000cells/cm² by adding 500µl cell suspension to each flask and distributing evenly across the base of the flask.
Before incubation, the cells were quickly checked for any obvious defects by using an inverted microscope (Letz DM IL, Leica, Co.Meath, Ireland).
The flasks were then placed in the incubator overnight.
The cells were checked the following day; successfully cultured cells show an 80% adherence rate, cells are uniform and display the typical cell morphology and possibly
mitosis. Once the adherence was checked the medium was replaced and the flasks were placed back in the incubator.

![Figure 2.22: HUAEC culture in phase contrast @ 10X](image)

**Subculture of adherent HUAEC cells**

Aseptic technique was adhered to at all times. The culture medium was replaced every two days and the cells were deemed ready for subculturing when 70-90% confluent. The Detach Kit (Promocell), which includes Hepes BSS, Trypsin/EDTA and Trypsin Inhibitor, was removed from the -20°C and allowed to thaw to room temperature. New cell culture flasks were filled with fresh medium and left in the incubator to equilibrate for 30 minutes.

The cell culture was examined under the microscope for 70-90% confluence. The flask was then brought into the laminar flow cabinet and the medium removed. The monolayer was then washed once with HepesBSS (100µl/cm²) by gently swirling the culture flask with the buffer inside.

The HepesBSS was removed and the monolayer was then covered with Trypsin/EDTA (80µl/cm²). The flask was kept at room temperature to trypsinize, after 1 minute the cells were examined under the microscope to observe their separation. Typically after 2 minutes the cells started to detach and the side of the flask was lightly tapped to loosen any remaining cells.

Once the cells were completely detached, 80µl/cm² of TNS was added to the flask, the flask was then agitated gently and the cell suspension was transferred to a sterile 50ml centrifuge tube (Falcon). The cells were centrifuged at 1500rpm for 6 minutes at room
temperature and brought back to the laminar flow cabinet. The tube was checked for a cell pellet/cluster attached to the bottom and the clear supernatant was carefully removed without disrupting the cells.

1ml of pre-warmed fresh medium was added to the cell cluster and the cells were resuspended by drawing the cells and medium slowly and carefully up and down with a sterile pipette tip, avoiding the introduction of air bubbles.

A cell count was then performed using the trypan blue stain. The cell suspension was then distributed in the newly equilibrated flasks at the recommended seeding density of 5,000-10,000 cells/cm² and these flasks were placed in the incubator.

After 24hrs the cells were checked for adherence under the microscope after which the medium was changed. The medium was then changed every two days until the flasks were confluent.

Cryopreservation of HUAECs

When freezing down cells in liquid nitrogen, the cells were trypsinized according to the subculturing method above. Following centrifugation and a cell count and cells were resuspended at a concentration of 1 million cells/ml Cryo-SFM. Each millilitre of cell suspension was transferred to a cryovial and frozen down gradually;

1) 4°C for 45mins
2) -20°C for 1hr
3) -70°C overnight
4) Liquid nitrogen for long term storage.

Stimulating cells with phytoestrogens

Procedure
24hrs prior to plating the cells, the medium was changed to phenol-red-free medium (prf medium)

On the day of plating the cells, each 6/12-well Nunclon® plate was prepared with 2mls prf medium/well and left in the incubator to equilibrate to 37°C.

Each flask required was trypsinized as per the “subculturing” method for HUAECs. The pellet/s was resuspended in 1ml of prf medium and a cell count was performed as per the protocol described.

The cells were plated at a seeding density of 5,000-10,000/cm².
Each plate was incubated at 37°C for 24hrs. After 24hrs, the cells were treated with the required phytoestrogen concentration (as described in the results chapter). Following 24hrs incubation RNA was extracted from the wells using the RNeasy micro kit (Qiagen) or RNAqueous® micro kit (Ambion) as described in section 2.6 above.

2.12.3 High Content screening of HUAECs

High content screening (HCS) (IN Cell 1000 platform; GE Healthcare,UK) represents a major breakthrough in bringing quantitative fluorescence microscopy to bear on the automation of cell biology and computerised image analysis, providing for the first time a fast and convenient means of conducting multi-parametric characterisation of multiple biological responses through simultaneous assessment of a multiplicity of molecular and cellular targets (315). This may include monitoring sub-cellular localisation and redistribution of individual proteins within complex cellular structures such as organelles and proteins. This technology also offers clear advantages to the more traditional biochemical or genetic analysis, as HCS can monitor and characterise physiological responses within the context of the structural and functional networks of cells in both normal and diseased states (316). In this study, this technology was used to detect the level and the localisation of EPCR and tPA antigen. The effects of the oestrogen antagonist ICI 182780 were also investigated.

Procedure

Cells were plated on 3 x 96-well plates (8 x 10³ cells/well) in 200μl medium and left overnight at 37°C. The medium was removed and the cells were then stimulated with 20μM ICI 182, 780 in 100μl of medium or in 100μl medium alone and left at 37°C. After 1 hr incubation, cells were stimulated with 100μl medium containing 50nM phytoestrogen, DMSO or 100μl medium alone, as shown in Figure 2.23. The outer wells contained medium alone to allow for evaporation-related edge effects.
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
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Figure 2.23: Plate layout for high content screening. Estradiol (E2), Genistein (Gen), Daidzein (Daid), ICI 182,780 (ICI). Those wells labelled with a + indicate the wells pre-incubated with ICI182,780.

After 24hrs, the medium was aspirated and saved for further protein analysis.

100µl of 3% paraformaldehyde (PFA) was added to the wells and the plate was stored at 37°C for 15mins.

The PFA was aspirated and 100µl of sterile PBS was added to each well to wash, this was removed and a second wash with 100µl PBS was performed.

50µl of our first primary antibody was added to each well;

Antibody: tPA (FITC labelled) (Abcam ab28376)

Used at 1:300 in 1ml PBS

Hoechst 33342 DNA stain was added to this at 1:1000 dilution.

The plates were left for 2hrs at room temperature in the dark.

Following this incubation the plates were washed with 100µl PBS (X2).

The second PBS was left on the cells to prevent drying out.

The plates were then scanned for tPA protein.

The following day the plates were washed and stained with 50µl of a second primary antibody.

Antibody: EPCR (Abcam ab56689)

Dilution 1:200

After two and a half hrs at 37°C the wells were washed twice with 100µl PBS.

A secondary goat anti-mouse (TRITC labelled) antibody was added at 1:500 for 2hrs.
The plates were washed twice with 100μl PBS and scanned for EPCR protein. Quantitative analysis of the acquired images was performed by IN Cell Investigator software.

**Acquisition and analysis**

Plates were scanned (five randomly selected fields/well) using IN Cell Analyzer 1000 automated microscope (Figure 2.24a). Images were acquired at 20X magnification using three detection channels with different excitation filters (Figure 2.25). These included a DAPI filter, which detected blue fluorescence indicating nuclear intensity at a wavelength of 461 nm; FITC filter, which detected green fluorescence indicating cell permeability at a wavelength of 509 nm and a TRITC filter, which detected lysosomal mass and pH changes with red fluorescence at a wavelength of 599 nm. Quantitative analysis of the acquired images was performed by IN Cell Investigator software (GE Healthcare) (Figure 2.24b).
Figure 2.25: High content screening image of HUAEC stained for the nucleus of the cell (blue), tPA antigen (green) and EPCR antigen (red). The staining of the nuclei is outlined with a blue line, tPA with a yellow line and EPCR staining with a green line, for each cell. In this way the system can distinguish the quantity of staining in fluorescent units, the location of the staining within the cell and the cell population.
2.12.4 HepG2 and Hep89 cell culture

Reagents Required

Culture media

The base medium for the growth of both hepatocyte cultures was eagles minimum essential medium (EMEM) (Sigma). To make the complete growth medium 10% fetal bovine serum (FCS), 2mM L-glutamine, 1% non-essential amino acids and 1% Pen/Strep were added (All Sigma). The antibiotic penicillin/streptomycin was added at 100 U/ml Pen and 100μg/ml Strep (Sigma). Media was stored at 4°C for up to 6 weeks.

The base medium used in the stimulation of the above cell line was phenol red free minimum essential medium with L-glutamine (MEM) (Gibco). To make the complete growth medium 1% non-essential amino acids and 1% Pen/Strep were added. A final concentration of 10% dextran charcoal stripped (dcc) foetal bovine serum was also added (Sigma). Media was stored at 4°C for up to 6 weeks.

Trypsin/EDTA

0.25% Trypsin/EDTA (Sigma). Aliquots were made and stored at -20°C.

PBS

Dulbecco’s phosphate buffered saline (Sigma)

Freezing Medium

EMEM with 5% DMSO

Recovery of cryopreserved cells

All surfaces were wiped down with 70% ethanol. Aseptic technique was adhered to at all times and all work was carried out in the laminar flow cabinet (Danlaf, Shaw Scientific, Ireland). Cell culture media was added to two T75cm² flask (Nunc) at a volume of 200μl/cm². This flask was incubated at 37°C, 5% (V/V) CO₂ and steam saturated atmosphere for approx 30mins (Sanyo, AGB Scientific, Ireland).
The cryovial of frozen cells (500,000 cells/ml) was taken from the liquid nitrogen container and brought to the laminar flow cabinet where the screw-top was slightly opened to reduce the overpressure and then closed again. The vial was then brought to a 37°C pre-warmed waterbath where the tube was agitated carefully and continuously until ~90% of the contents were thawed. The tube was removed from the waterbath and agitated gently until all the contents were completely thawed.

The vial was then transferred to the laminar flow cabinet for addition of the cells to the culture flasks. The cell suspension was pipetted into 5mls pre-warmed medium. A cell count was performed.

The equilibrated cell culture flasks were seeded with ~18,000 cells/cm² by adding 2.5ml cell suspension to each flask and distributing evenly across the base of the flask. Before incubation, the cells were quickly checked for any obvious defects by using an inverted microscope (Letz DM IL, Leica, Co.Meath, Ireland). The flasks were then placed in the incubator overnight.

The cells were checked the following day; successfully cultured cells show an 80% adherence rate, cells are uniform and display the typical cell morphology and possibly mitosis. Once the adherence was checked the medium was replaced and the flasks were placed back in the incubator.

![Figure 2.26: HepG2 culture in phase contrast @ 10X](image-url)
Subculture of adherent cells

Aseptic technique was adhered to at all times. The culture medium was replaced twice per week and the cells were deemed ready for subculturing when 70-90% confluent. The Trypsin/EDTA was removed from the -20°C freezer and allowed to thaw at 37°C. The required number of new cell culture flasks were filled with fresh medium and left in the incubator to equilibrate at 37°C for 30 minutes.

The cell culture was examined under the microscope for 70-90% confluence. The flask was then brought into the laminar flow cabinet and the medium removed. The monolayer was then washed once with PBS (50μl/cm²) by gently swirling the culture flask with the buffer inside.

The PBS was removed and the monolayer was then covered with Trypsin/EDTA (40μl/cm²). The flask was kept at room temperature for 2 minutes followed by 5 minutes at 37°C to trypsinize, after which the cells were examined under the microscope to observe their separation.

Once the cells were completely detached, 106μl/cm² of EMEM was added to the flask, the flask was then agitated gently and the cell suspension (~11mls) was transferred to a sterile 50ml centrifuge tube (Falcon). The cells were centrifuged at 600rpm for 5 minutes and brought back to the laminar flow cabinet. The tube was checked for a cell pellet/cluster attached to the bottom and the supernatant was carefully removed without disrupting the cells.

1ml of pre-warmed fresh medium was added to the cell cluster and the cells were resuspended by drawing the cells and medium slowly and carefully up and down with a sterile pipette tip, avoiding the introduction of air bubbles.

A cell count was then preformed using the tryphan blue stain. The cell suspension was then distributed in the newly equilibrated flasks at the recommended seeding density of 20,000-30,000cells/cm² and these flasks were placed in the incubator.

After 24 hours the cells were checked for adherence under the microscope after which the medium was changed. The medium was then changed twice per week until the flasks were confluent.
Cryopreservation

When freezing down cells in liquid nitrogen, the cells were trypsinized according to the
subculturing method above. Following centrifugation and a cell count and cells were
resuspended at a concentration of 3 x 10^6 cells/ml EMEM/5% DMSO. Each millilitre of
cell suspension was transferred to a cryovial and frozen down gradually;

5) 4°C for 45 minutes
6) -20°C for 1 hour
7) -70°C overnight
8) Liquid nitrogen for long term storage.

Stimulating the cells with phytoestrogens

Procedure

24hrs prior to plating the cells, the medium was changed to phenol-red-free medium (prf
medium).

On the day of plating the cells, each 6/12-well Nunclon® plate was prepared with 2mls
prf medium/well and left incubator to equilibrate to 37°C.

Each flask required was trypsinized as per the “subculturing” method for HepG2/Hep89
The pellet/s was resuspended in 1ml of prf medium and a cell count was performed as
per the protocol described.

The cells were plated at a seeding density of 20,000-30,000/cm²

Each plate was incubated at 37°C for 24 hours
After 24 hours, the cells were treated with the required phytoestrogen concentration (as
described in the results chapter)

Following 24 hours incubation RNA was extracted from the wells using the RNeasy mini
kit (Qiagen) as described in section 2.6.
Cell proliferation MTT assay

This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. This cellular reduction involved the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals formed are solubilised and the resulting coloured solution is quantified using a spectrophotometer. This ensures a high degree of accuracy, it enables online computer processing of the data and thereby, allows the rapid and convenient handling of a high number of samples.

Procedure

The cells seeded in 3 x 96-well plates, 50µl per well, and incubated at 37°C for 24hrs. Following incubation the cells were stimulated with a range of concentrations of genistein, equol and daidzein.

A serial dilution of 10mM stock solution was carried out to give stocks of 6000nM to 2nM.

50µl of each solution was added to the allocated wells to give a final concentration range of 3000nM to 1nM.

Replicates of 4 were provided. As shown in Figure 2.27.

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Figure 2.27: Plate layout for MTT proliferation assay. Blank: medium only. Cells: cells only. Row B, C and D 3-11 contained cells plus the described stimulant; either DMSO or the designated concentration of genistein, equol or daidzein in nM.
After 24hrs incubation period at 37°C, 10µl of yellow MTT solution was added to each well and the plates were incubated at 37°C for a further 4hrs. After this incubation period, purple formazan salt crystals were formed. These salt crystals are insoluble in aqueous solution but may be solubilised by adding 100µl of solubilisation solution and incubating the plates overnight at 37°C. The plates are then read on the Dynex technologies MRX microplate reader at an absorbance between 550 and 600nm.

2.13 Statistical analysis

All data was analysed using the SPSS statistical package version 16.0. The data was assessed for normal distribution. Unpaired students t-test compares data from two different groups of subjects and calculates the probability that the observed difference in means occurs when the null hypothesis is true. The null hypothesis states that the mean of the two normal distributions is equal. The aim was to look for genes and proteins that show a distinct difference in expression between the groups. Data is presented as the mean ± the standard error. The p-value represents statistical significance when equal variances were not assumed, with P<0.05 considered significant.
Chapter 3

Effects of phytoestrogens on haemostasis gene and protein expression in ovariectomised rats
3.1 Introduction

Studies of hormone replacement therapy have indicated that haemostatic changes play an important role in the cardiovascular and thromboembolic risk associated with post menopausal oestrogen use. Metabolism and absorption play a role in defining the effects of oestrogens and phytoestrogens in the body with the first pass liver effect mediating the prothrombotic effects of oestrogens and determining the concentration available for systemic circulation (24).

Phytoestrogens can regulate transcription through oestrogen receptor dependent mechanisms using similar pathways to estradiol, however structural differences between the two families of compounds may lead to different effects on target genes.

The first part of this project investigated the effects of phytoestrogens on expression of haemostasis genes and proteins in the liver in an animal model of the menopause. As discussed in Chapter 1, an inherent problem in human dietary studies is the difficulty in accurately controlling background soy consumption. In this study, ovariectomised rats, on a controlled diet, were used as an animal model of the menopause, thereby avoiding this problem. This model allowed the study of the effects of phytoestrogens compared with animals fed on a soy free diet and also a group whose diet was supplemented with estradiol.

Two phytoestrogens were studied, genistein which is the major isoflavone contained in soya, and equol, a biologically active metabolite of daidzein produced in the gut. Animals were treated for three months following ovariectomy.

Eighty seven Sprague Dawley rats were fed soy free chow supplemented with

1. estradiol benzoate 0.19 mg/kg body weight per day (estradiol low group)
2. estradiol benzoate 0.43 mg/kg body weight per day (estradiol med group)
3. estradiol benzoate 0.75 mg/kg body weight per day (estradiol high group)
4. genistein 6 mg/kg body weight per day (genistein low group)
5. genistein 60 mg/kg body weight per day (genistein high group)
6. Equol 21.7 mg/kg body weight per day (Equol)
7. Soy free controls (soy free group)
Animals were fed each diet for three months at the end of which they were sacrificed and liver tissue removed. For gene expression studies, TaqMan low density arrays were used to determine expression of a range of haemostasis genes relative to the housekeeping gene 18s. Expression levels greater than 2 fold were considered above background, and unpaired t-tests determined statistical significance. Protein expression was determined using western blotting and ELISAs in liver extracts.

The aim of the study was to determine the effects of genistein and equol on haemostasis gene and protein expression. These effects were compared with soy free animals and animals treated with estradiol (as a positive control).
3.2 Results

Effects of estradiol, genistein and equol on coagulation and fibrinolytic gene expression in liver from ovariectomised rats, summarised in Table 3.1.

Factor V (Figure 3.1)
All estradiol and genistein treatment groups showed a significant increase in factor V expression compared to the soy-free control group. The factor V mRNA fold change for estradiol high, genistein low and genistein high groups were all greater than 2 fold relative to levels found in the soy-free group (P>0.001). The two genistein groups had the most marked effect of factor V expression. The genistein high group resulted in statistically higher factor V expression compared with the estradiol high group (P<0.05). The equol treated group of animals showed similar levels of factor V expression to that found in the soy-free group.

Factor VIII (Figure 3.2)
Factor VIII mRNA expression levels were increased in all groups except the equol treated group. In rats treated with both high and low doses of genistein, a 4 fold increase in factor VIII expression was observed relative to soy free controls. Similar expression levels were found in the estradiol high dose however the levels found in the low and medium estradiol treated rats were lower. These changes were statistically significant compared to the soy free controls (all genistein and estradiol groups; P<0.001). The increase in factor VIII expression in the genistein high dose group was significantly higher than all three estradiol dose groups. (Genistein versus estradiol low and estradiol med; P<0.001, genistein versus estradiol high; P<0.05).

Factor IX (Figure 3.3)
Estradiol and genistein increased factor IX expression levels significantly compared with the soy-free control group (estradiol low, estradiol high, genistein low, genistein high v soy free; P<0.001, estradiol med v soy free; P<0.01). These increases also seem to be dose dependant with genistein showing the highest fold change of 2.7-3 fold. The fold change for equol did not significantly differ compared to the soy-free group.
Factor X (Figure 3.4)
Factor X mRNA expression was increased in each of the estradiol and genistein treatment groups (P<0.001) compared to the soy-free control however only the genistein treated groups had increases greater than two fold. Equol had no significant effect of factor X expression levels.

Fibrinogen
Fibrinogen is a glycoprotein containing 2 copies each of 3 polypeptide chains (α, β and γ) each encoded by a separate gene, the expression of the 3 genes was measured using the low density array

Fibrinogen α (Figure 3.5)
The mRNA expression level of fibrinogen α was increased following treatment with estradiol and genistein, specifically the low and high treatment groups of genistein and estradiol (P<0.001). The levels were increased 3-5 fold compared with soy free controls and were dose dependent for genistein. Equol treatment down-regulated the expression of fibrinogen α with a fold change of -1.8 but the effect was not significantly different from the soy-free control.

Fibrinogen β (Figure 3.6)
The mRNA expression level of fibrinogen β was increased following treatment with each dose of estradiol and genistein. The levels were increased 2-3 fold compared with soy free controls (P<0.001), except in the estradiol med group (P<0.01). The fold change for treatment with equol not significantly different to the soy-free control group

Fibrinogen γ (Figure 3.7)
Fibrinogen γ mRNA expression levels showed the greatest increase in expression in comparison to the soy free control with all treatment groups. Estradiol increased the levels 4-5 fold, genistein increased fibrinogen γ levels 7-10 fold (P<0.001) while equol increased the expression by 4 fold (P<0.01).
Factor XI (Figure 3.8)
Factor XI mRNA expression levels increased 2-3 fold with genistein treatment (p>0.001) compared to the soy-free group. In the estradiol treatment groups, levels only increased to a maximum of 1.6 fold. Significantly greater increases in expression were found between both high and low dose of genistein treatment group compared with high and low dose estradiol (P< 0.001). Equol treatment did not significantly differ from the soy-free control group.

Factor VII (Figure 3.9)
Factor VII expression was significantly increased by each dose of estradiol and also by both doses of genistein (P<0.001). Highest levels of expression, an increase of 3.7 fold, were found in the genistein low group which was significant compared with estradiol low (P<0.005). Equol decreased the mRNA expression of factor VII by 1.5 fold which was not significantly different to the control group.

Prothrombin (Figure 3.10)
Prothrombin mRNA expression was significantly increased with all six treatment groups compared to the soy free control group (P<0.001 for estradiol and genistein and P<0.01 for equol compared with soy free controls). A dose dependent increase was observed with estradiol treatment and also genistein treatment. A 4-5 fold increase in expression was found with both genistein groups; levels of expression were significantly higher in the genistein high group compared with the estradiol high group (P<0.01).

Thrombin activatable fibrinolysis inhibitor (TAFI) (Figure 3.11)
Estradiol significantly increased TAFI mRNA in a dose dependant manner to levels between 2 and 5 fold compared to the soy-free control group (P<0.001). Genistein increased TAFI levels 4 fold compared to the soy-free group (P<0.001) and equol increased the levels 2 fold compared to the control group (P<0.001). There was no significant difference between in TAFI mRNA expression between the estradiol and genistein treated groups.
Protein C (Figure 3.12)
Protein C mRNA expression levels were increased with each treatment group, with genistein having the most effect showing a 3-4 fold increase (P<0.001). Equol also significantly increased protein C but only by 1.5 fold (P<0.01). For genistein the effect was dose dependent. The genistein high group expression level of protein C was significantly higher than the upregulation found in the estradiol high group (P<0.001).

Protein S (Figure 3.13)
Protein S mRNA expression was affected in a similar manner to protein C levels but here the genistein high group showed a 6 fold upregulation (P<0.001). The genistein effect on Protein S mRNA expression was also dose dependent. Equol did not significantly change the expression of protein S compared to the soy-free control group.

Antithrombin (Figure 3.14)
Antithrombin was increased 3-4 fold by each estradiol group (P<0.001) and both genistein groups (4-5 fold) (P<0.001) and also the equol group (P<0.01) compared to the soy-free control group. There was no significant difference in fold change between the estradiol and genistein treatment groups.

Plasminogen activator inhibitor-1 (PAI-1) (Figure 3.15)
Plasminogen activator inhibitor-1 (PAI-1) mRNA expression was significantly increased 6-8 fold (P<0.001) with estradiol low and high groups. While the effect seen is not as marked with genistein treatment, both doses of genistein significantly increased PAI-1 but with fold changes of 4-5 (P<0.001). The difference in fold change between the high genistein group and the high estradiol group was also significant (P<0.01) with lower levels in the genistein group. Equol decreased the expression of PAI-1 but this result was not significantly different to the soy-free control.

Plasminogen (Figure 3.16)
Plasminogen mRNA expression levels are increased in all estradiol and genistein treatment groups (P<0.001). Genistein at low and high doses have a greater impact on the mRNA levels with significant fold changes of 4 and 3.8, respectively, compared with equivalent estradiol treatment groups (P<0.001). The equol diet did not significantly change the levels of plasminogen expression compared to the soy-free controls.
**Tissue plasminogen activator tPA (Figure 3.17)**

A significant dose dependent increase in tissue plasminogen activator (tPA) mRNA expression was found between both the estradiol low and min groups and the estradiol high treatment group (P<0.001). Both doses of genistein significantly increased the levels of tPA mRNA expression 6 fold (P<0.001) relative to the soy-free group. This was similar to the levels found in the estradiol high dose group and significantly higher than found in the low estradiol (P<0.001). Treatment with equol had no effect on the expression levels of tPA.

**C-reactive Protein (CRP) (Figure 3.18)**

C-reactive protein mRNA levels were increased following the diets supplemented with estradiol and genistein. A small but significant dose dependent increase in CRP mRNA expression was found in the higher dose estradiol group. A 2.5-fold increase in CRP expression was observed with both doses of genistein (P<0.001) which was similar to the levels found with the estradiol high dose group. Equol made no significant change to the levels of CRP expression compared to the soy-free control group.
Factor V mRNA expression in liver tissue of ovariectomised rats fed with soy-free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, **P<0.01, ***P<0.001 compared to soy-free control.
Figure 3.2 Factor VIII mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.3: Factor IX mRNA expression in liver tissue of ovarectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.4: Factor X mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.5: Fibrinogen α mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.6: Fibrinogen β mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001.
Figure 3.7: Fibrinogen γ mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.8: Factor XI mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.9: Factor VII mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.10: Prothrombin mRNA expression in liver tissue of ovariectomised rats fed with soy-free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.11: Thrombin activatable fibrinolysis inhibitor (TAFI) mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, **P<0.01, ***P<0.001.
Figure 3.12: Protein C mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, **P<0.01, ***P<0.001 compared to soy-free control.
Figure 3.13: Protein S mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate medium dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.14: Antithrombin mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.15: Plasminogen activator inhibitor-1 (PAI-1) mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.16: Plasminogen mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Figure 3.17: Tissue plasminogen activator (tPA) mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate mid dose (0.43mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, **P<0.01, ***P<0.001 compared to soy-free control.
C-Reactive Protein

Figure 3.18: C-reactive protein (CRP) mRNA expression in liver tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate min dose (0.43mg/kg bw), estradiol benzoate high dose (0.75 mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01, *** P<0.001 compared to soy-free control.
Summary of the array data (Table 3.1)

A summary of the effects of the high dose estradiol, high dose genistein and the equol treated group is illustrated in Table 3.1. The greatest changes in expression with all treatments were found with fibrinogen γ, tPA and PAI-1. For these genes, we examined the protein expression in liver tissue homogenates prepared from each treatment group (Figure 3.19a-d). We also examined protein expression of CRP prothrombin and factor VII (Figure 3.21).

<table>
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Table 3.1: Summary of mRNA fold changes found expression in estradiol (high dose group), genistein (high dose group) and equol treated groups.

Effects of estradiol, genistein and equol on coagulation and fibrinolytic protein expression in liver from ovariectomised rats (Figure 3.19)

Fibrinogen, CRP and tPA were found in nanogram levels in tissue extracts and PAI-1 was virtually non-detectable at picogram levels (Figure 3.19a, b, c, and d respectively). Fibrinogen, CRP, PAI-1 and tPA were measured using ELISA. Prothrombin, factor VII were measured using western blotting. Results are shown for rats treated with the higher dose for each treatment type.
Fibrinogen antigen (Figure 3.19a)
Protein expression largely concurred with mRNA expression results. Higher levels of fibrinogen antigen were found in the genistein treated group compared to soy free controls (P<0.05), levels in the estradiol treated group were not significantly different to soy free controls. In the equol group fibrinogen a significantly increased fibrinogen antigen level was found compared the soy-free control (P<0.001) which concurred with the 5 fold increase found in fibrinogen γ mRNA expression.

C-reactive protein (CRP) antigen (Figure 3.19b)
Similarly CRP antigen levels were higher in genistein but not estradiol treated animals compared with controls (P<0.001) and equol treatment significant altered protein levels of CRP compared to the soy-free group with a concentration greater than 3μg/mg protein (P<0.001).

tPA antigen (Figure 3.19c)
Estradiol treatment decreased the amount of tPA protein expressed whereas genistein had no effect and equol slightly increased the protein levels in comparison to the soy free control however these changes were not significant. The different effects seen between estradiol and genistein and estradiol and equol treatment on tPA antigen levels were significant (P<0.05).

PAI-1 antigen (Figure 3.19d)
PAI-1 was slightly decreased with estradiol treatment and further decreased with equol and genistein. The values obtained were at the limit of assay detection and the changes did not prove significant.
Figure 3.19a: Fibrinogen concentration (ng/mg) in soy free (SF), Estradiol benzoate (E2), Genistein (Gen) and Equol liver tissue from ovariectomised rats. Statistical significance is denoted *P<0.05, *** P<0.001 compared to soy-free control.

Figure 3.19b: C-reactive protein (CRP) concentration (ng/mg) in soy free (SF), Estradiol benzoate (E2), Genistein (Gen) and Equol liver tissue from ovariectomised rats. Statistical significance is denoted ***P<0.001 compared to soy free control.
Figure 3.19c: Tissue plasminogen activator (tPA) concentration (ng/mg) in soy free (SF), Estradiol benzoate (E2), Genistein (Gen) and Equol liver tissue from ovariectomised rats. Statistical significance is denoted *P<0.05 compared to estradiol.

Figure 3.19d: Plasminogen activator inhibitor-1 (PAI-1) concentration (pg/mg) in soy free (SF), Estradiol benzoate (E2), Genistein (Gen) and Equol liver tissue from ovariectomised rats.
Oestrogen receptor expression in rat liver tissue (Figure 3.20 and Figure 3.21a)

In order to determine whether the changes observed in coagulation and fibrinolytic gene expression were mediated via oestrogen receptors, expression of two main oestrogen receptors was determined at the mRNA and protein level. The expression of GPR30, a G-protein coupled receptor known to be involved in oestrogen signalling, was also determined at the mRNA level. The control used for oestrogen receptor expression was rat ovary RNA transcribed to cDNA. Results are expressed as target Ct - 18s Ct. Lower Ct values reflect higher expression.

Oestrogen receptor alpha (ERα) was detected in rat liver tissue at both the mRNA and protein level, similar levels of expression were observed in tissue from genistein treated, estradiol treated and equol treated rats as was found with soy free controls. Oestrogen receptor beta (ERβ) was not detected in rat liver at the mRNA or protein level. GPR30 mRNA expression showed a lower level of expression in rat liver tissue compared with control rat ovary. Of note, GPR30 expression was lower than ERα expression in rat liver.

Western blot analysis of Prothrombin and Factor VII (Figure 3.21b and Figure 3.21c)

Prothrombin and factor VII western blot analysis was performed on liver tissue samples from the soy free, estradiol high and genistein high groups using rat liver protein lysate as a positive control. Strong prothrombin expression was present in each sample (Figure 3.21b). Factor VII expression was detected in each sample group and this expression was increased in genistein treated rats compared to soy free controls (Figure 3.21c). The loading control for each blot was β-actin.
Figure 3.20: mRNA expression of oestrogen receptors in rat liver tissue compared to rat ovary.

<table>
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</tr>
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<tr>
<td>β actin</td>
<td></td>
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<td>43kDa</td>
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</tbody>
</table>

Prothrombin 72kDa

β actin 43kDa

Factor VII 50kDa

β actin 43kDa

Figure 3.21 a, b and c: SDS-PAGE and Western blot analysis of ERα (a), Prothrombin (b) and Factor VII (c) in rat liver tissue. The control for (a) is human ovarian tissue and the control for (b) and (c) is rat liver lysate.
3.3 Discussion

Post-menopausal hormone therapy can modify the risk of cardiovascular disease however clinical trials have shown that the effects are not beneficial particularly in the first year of use. Data has shown prothrombotic changes in haemostasis in women using hormone therapy (210). Some of the most recent data suggests that many of these changes are due to increased coagulation and fibrinolytic protein expression in the liver (222). Phytoestrogens have been proposed as a safer alternative to hormone therapy for the relief of menopausal symptoms however their effects on cardiovascular risk are largely unknown (11). In this study the effects of commonly used phytoestrogens on haemostasis in a rat model of menopause were investigated and compared with estradiol treated and soy free controls. Recent studies of transdermal HT have shown that the liver plays an important role in mediating the adverse prothrombotic effects of hormone preparations (250, 312). The phenotype of thrombosis, both arterial and venous, is a complex interaction between genes and a multiplicity of environmental factors including age, sex and hormone use. By studying expression of coagulation and fibrinolytic genes in the liver, we can gain an important understanding of the interaction between oestrogens and oestrogen-like substances and haemostasis at a molecular level.

The results of this study show that in ovariectomised rats, genistein has a marked effect on coagulation and fibrinolytic gene and protein expression in the liver. The effects of equol were more neutral however, enhanced expression of fibrinogen and CRP were found in this group at the mRNA and protein level. Three major risk markers for vascular disease, CRP, fibrinogen and factor VII show increased mRNA and protein expression with genistein at levels similar to those found in rats treated with estradiol, several other coagulation factors were also shown to be increased following genistein administration including the inhibitors protein S, protein C and antithrombin. Similarly, genes involved in the activation and inhibition of fibrinolysis showed enhanced expression. Relating animal models to humans is difficult but the doses in this study were chosen to reflect real human exposure levels. The estradiol doses reflect a shift from the physiological into the supraphysiological range (311-312). The low dose of genistein represents exposure through the typical Asian diet while the high dose correlates to a strong soy supplement intake (317). In view of its relevance to human physiology the equol dose used may well be of importance for postmenopausal women’s health as
pharmacologists claim that doses per kilogram of body weight applied to rats must be 10-15 fold higher than in humans to exert equipotent effects (318-319). There is evidence to suggest that some women could consume doses of up to 150mg per day of the equol precursor daidzein, resulting in an average of 2.5mg per kg body weight (319). The equivalent dose in rats would therefore be >25 mg per kg body weight which is in a similar range to the dose chosen for this study (17mg/kg).

Prothrombin, the precursor to active thrombin, is increased in this study with estradiol treatment and both phytoestrogens genistein and equol. Estradiol and genistein increased prothrombin expression in a dose dependent manner compared to the soy free control. Increasing prothrombin expression will increase the availability of thrombin following activation. The prothrombin G20210A variant results in increased expression of the prothrombin gene, and is associated with a 2-3 fold increase in venous thrombosis risk in affected individuals (118). This suggests that enhanced prothrombin expression at a molecular level is linked to increased thrombosis risk; therefore the increased (4-5 fold) prothrombin mRNA expression found in genistein treated rats could lead to a similar thrombosis risk in women taking supplements that contain genistein.

Factor VII plays an important role in the initiation of thrombus formation and is a documented risk factor for CVD. In 2001 Lowe et al showed that factor VII was increased with unopposed oestrogen (211), an increase in factors VII with oestrogen was also shown by others (210, 249, 320). At a molecular level, increased factor VII mRNA expression was found in both estradiol treated and genistein treated rats, protein expression was also increased in genistein treated rats. Teede et al reported unchanged factor VIIc activity in women treated for 3 months with placebo compared with mixed phytoestrogens including genistein and daidzein. A small decrease in factor VIIc levels was found in the soy treated group following treatment however this was also found in the placebo group (250). The study differs from this design in that it includes a mixture of phytoestrogens as opposed to a pure genistein supplement. The effects of equol and genistein in this study were different and opposite, hence a mixture of phytoestrogens will produce different results to studies of the effects of each isoflavone individually. It has previously been observed that levels of factor VIIc are reduced even in the presence of increased factor VII antigen in post menopausal women taking combined HT (217). Factor VIIc activity is strongly related to triglyceride levels (321) which can influence...
the factor VIIc assay, of note, lower levels of triglyceride were found in soy treated women in the study described above compared with placebo (250).

The results of this study have also shown that the phytoestrogen, genistein increases the expression of important genes that regulate thrombin generation. Genistein increased factors IX, X, VIII, V and XI which in vivo play a pivotal role in the rate of conversion of prothrombin to thrombin and therefore the formation of a fibrin clot. These increases were all significantly different to soy-free control and had a more marked increase in expression than estradiol which leads us to speculate that genistein is more potent that estradiol in relation to thrombotic risk. Previously the Leiden Thrombophilia Study found that an increase in plasma factor X levels were associated with a 1.6 fold risk of venous thrombosis (322). An increase in plasma levels of these coagulation factors as a result of enhanced transcription may lead to a prothrombotic state, particularly when combined with other risk factors.

Antithrombin is reduced in menopausal women and this reduction has been shown to be resolved upon hormone therapy use (323). In a study of post-menopausal women soy isoflavones have shown to decrease the level of plasma antithrombin (249), results which were also produced in the placebo group. In the present study genistein increased expression of this coagulation inhibitor in this menopause model. Antithrombin is responsible for inactivating many of the coagulation factors responsible for thrombin generation including thrombin itself but as expression of the genes involved in tenase and prothrombinase complexes were also increased, this effect may serve to balance the production of prothrombotic serine proteases should the molecular effects observed be translated into plasma levels.

Factor V has an important role in the APC pathway and in this study this coagulation factor and its inhibitors, protein C and protein S are all increased with both estradiol and genistein, particularly with the high dose genistein group. Activated protein C and its cofactor protein S can inactivate factors V and VIII. Aging and the menopause are associated with an increase in protein C and protein S (78), it remains unclear whether the effects shown with this animal model relate to a physiologically important effect in women during the menopause. So far, studies on hormone therapy have reported a
decrease in the activity of these inhibitors but this effect may simply be reversing the natural effects of menopause (210).

All three fibrinogen genes showed enhanced expression, in particular fibrinogen γ, in response to both equol and genistein, these effects were similar and in the case of genistein greater than observed with estradiol. Fibrinogen is a documented risk factor for cardiovascular disease (324). Fibrinogen β is an important regulator of synthesis of the fibrinogen molecule and polymorphisms in this gene which lead to elevated fibrinogen levels have been linked to arterial thrombosis (118-119). However debate persists whether this is a direct effect or whether it is secondary to other changes related to the atherosclerotic process. The β-chain fibrinogen gene contains several regulatory sites including IL-6 responsive elements and a hepatic nuclear factor 1 site (325). The IL-6 sites are considered to be important in mediating raised levels as a result of an acute phase response. If the increased fibrinogen β expression which was observed in the liver of the treated rats, and also the increase in the alpha and gamma fibrinogen chains were translated into increased levels in plasma, this would indicate that genistein treatment at both doses would be associated with an increased risk of cardiovascular disease. In a large study, Atteritano et al (326) showed that genistein combined with Vitamin D and calcium decreased plasma fibrinogen by 7-8% in post menopausal women which would conflict with the results of this study, however decreases in plasma levels of clottable fibrinogen have also been found in post menopausal women (40, 253) despite evidence of increased fibrin breakdown (210, 236) and an enhanced cardiovascular risk. Further work is required to determine the relationship between enhanced fibrinogen expression in the liver and the levels of fibrinogen activity in plasma.

Ovariectomised rats treated with genistein in this study showed an increase in the expression of activators and inhibitors of fibrinolysis. For tPA this increase was similar to that found with high and low dose estradiol, PAI-1 expression, although increased compared to that found with soy free animals, was less than was found in estradiol treated rats. All groups showed an increase in plasminogen expression compared with soy free controls. At the protein level, estradiol and genistein treatment did not significantly change tPA or PAI-1 but tPA was significantly higher with genistein and equol compared to estradiol. PAI-1 levels were very low, and it is possible that the ELISA used was not sensitive enough to detect differences in expression in liver tissue.
and therefore this would not be a conclusive result. As the protein results don’t fully agree with the mRNA data it is recognised that these results can provide only limited information regarding the biosynthesis of these haemostasis proteins. Both tPA and PAI-1 antigen levels in plasma are linked with cardiovascular risk, however both are clustered with other risk factors including triglyceride levels and insulin resistance (320). Active PAI-1 has a short half life in circulation and can not be stored in cells. As a result, transcriptional control of PAI-1 plays an important role in regulating tissue and plasma PAI-1 levels. The results of this study are in conflict with studies of human plasma levels that showed no effect of dietary isoflavones on plasminogen and PAI-1 levels (249). In addition, Triffletti et al showed that genistein (54mg/day) was associated with unchanged PAI-1 plasma levels, despite a decrease in fibrin degradation products (253). Discrepancies between the findings of the present study may reflect post translational modifications, and additional regulatory effects on the release and clearance of PAI-1. In addition changes in expression of PAI-1 in the vascular endothelium may have masked the effects of the increased liver derived PAI-1. Thrombin activatable fibrinolysis inhibitor (TAFI) was also increased with estradiol and also both phytoestrogens, with genistein increasing TAFI further than equol.

C-reactive protein (CRP) is a risk marker for cardiovascular disease and recent studies have identified it as a possible target for use in primary prevention (327). CRP is predominantly synthesised in the liver as an acute-phase reactant and is transcriptionally driven by IL-6 (203). In this study estradiol and genistein both increase CRP gene expression in the liver of ovariectomised rats. Protein levels of CRP were increased with both genistein and equol compared to soy free controls. In 2001, Lowe et al reported that oral oestrogen impacts hepatic synthesis of CRP directly and not as a generalised inflammatory response (211). In addition comparative studies of transdermal versus oral oestrogen use in post menopausal women have shown CRP to be increased only following oral use (226, 328) without an increase in pro-inflammatory cytokines (226, 329). The results of this study agree with this and show that the phytoestrogen genistein is even more potent in its effect on CRP expression in the rat liver than estradiol. Teede et al investigated the effects of soy versus a casein placebo on plasma CRP and found that although levels of CRP were increased in both groups, there was no difference between them (250). In this rat study, the effects of pure genistein rather than the mixture
of isoflavones used in the human study was investigated, this may explain the contradictory results found between them.

The results suggest that genistein particularly at the higher dose which is equivalent to that used in human dietary supplements activates expression of coagulation and fibrinolytic genes in the liver. Equol also has the ability to increase the expression of several haemostatic genes but to a lesser extent than genistein. Quantitation of mRNA expression has shown here the potent effects of the phytoestrogen genistein, to gain a deeper understanding of the physiological effects of this compound, protein analysis showing similar results would have been beneficial as translational regulation, post-translational modifications and also intracellular trafficking pathways are involved in the biosynthesis of many coagulation factors and must be taken into consideration for a definitive result. Analysis of plasma levels would also provide a more definitive answer on the regulation of haemostasis proteins by these phytoestrogens.

Previous studies on the tissue distribution of oestrogen receptors α and β have shown that hepatocytes only express the ERα subtype and no ERβ has been found (34, 330). Phytoestrogens bind to both oestrogen receptors but with greater affinity to ERβ (331). The reported beneficial effects of phytoestrogens with respect to cardiovascular risk are generally thought to be mediated through ERβ (35, 40). As this beta isoform is not expressed in the liver, it was suggested that phytoestrogens such as genistein do not provoke the prothrombotic effects associated with increased risk for thromboembolic disorders (250).

In the present study, expression of ER-β was found to be absent in rat liver tissue, hence the phytoestrogens used, particularly genistein, in this model are likely to enhance transcription by other mechanisms which may or may not include ER-α. One such study by Hillish et al has shown that oestrogen effects in the liver are mediated by ERα (332). From the results shown in this study, it is possible that the phytoestrogen genistein acts via ERα which has been detected at the mRNA level and at the protein level via western blot. As rat ERβ is highly homologous to ERα, approx 90% in the DNA binding domain and 55% in the ligand binding domain, this theory is plausible, although the binding affinity of genistein for ERβ is of an order of magnitude less than ERα (34). The role of ERα in mediating these effects will be investigated in the next chapter.
It can be speculated that as physiological concentrations of genistein for example, are expected to have only a modest impact on ERα activity that there may be other mechanisms by which these phytoestrogens are working and changing the expression of the coagulation factors studied. Genistein is known to influence a wide variety of other regulatory pathways. These include the ERK/MAP kinase pathway, PPAR mediated transcription and NFkB pathway (38). Genistein can also directly stimulate plasma membrane associated adenylate cyclases leading to increased cAMP levels and activation of cAMP dependent pathways (308). This indicates that phytoestrogens may exert effects on cells other than via oestrogen receptor mediated transcription possibly via non-genomic effects. GPR30, the recently described oestrogen receptor was expressed in the liver from treated rats in this study. GPR30 is known to be involved in many of the non-genomic effects of estrogens including MAP-kinase activation and downstream signalling (303). Phytoestrogens including genistein can bind to GPR30 in a similar manner to oestradiol, providing an alternative pathway for the effects of these compounds on haemostasis (333).

In conclusion this study shows that the phytoestrogen genistein, at concentrations found in normal Asian diets and in added dietary supplements, can increase expression of coagulation and fibrinolytic genes expressed in rat liver to a level that is similar and in some cases higher than is found with estradiol. These changes do not appear to depend on ER-β. In the next chapter these effects will be examined in more detail in a human hepatocyte cell model.
Chapter 4

*In vitro* effects of phytoestrogens on the regulation of haemostasis gene and protein expression in human hepatocyte cell lines
4.1 Introduction

The data presented in chapter 3 of this thesis has shown that the phytoestrogens, genistein and equol can regulate the expression of many coagulation and fibrinolytic activators and inhibitors in the rat liver. In order to identify if these results translate to a human model the ability of these phytoestrogens to regulate haemostasis genes in human hepatocyte cell lines was analysed.

The second part of this project investigated the effects of the phytoestrogens genistein, equol and its precursor daidzein, on the expression of genes and proteins from the haemostatic system in human hepatocyte cell lines (HepG2). As described previously, one of the aims of this thesis is to investigate whether oestrogen receptors regulate any haemostatic changes which have been identified. Phytoestrogens bind with greater affinity to ERβ and are thought to mediate many of their reported cardioprotective effects through this receptor (35, 334). However ERα but not ERβ was detected in the rat liver samples (see chapter 3) indicating that, if the effects on haemostatic gene expression which were observed from the rat study, were oestrogen receptor mediated, they could not be mediated by ERβ. To investigate the role of ERα, a human hepatoma cell line that had been transfected to stably express ERα (Hep89) was used (335). Any effects seen with this cell line were compared with the original ERα-negative cell line (HepG2).

Both hepatocyte cell lines were grown until they were ~80% confluent. The medium was then changed 24hrs prior to plating to phenol red free MEM with DCC stripped serum. FCS was replaced with DCC stripped serum before stimulating the cells as this type of serum has been treated with charcoal and dextran to deplete any hormones present that may interfere with oestrogen receptor binding. Phenol red was also omitted before treating the cells as it also has been known to interfere with steroid-receptors in in vitro studies (336) and could therefore also influence the results. Cells were stimulated for 24 hours with genistein, daidzein and its metabolite equol at a final concentration of 50nM (chosen to reflect the expected serum levels of phytoestrogens following supplementation) (337). Cells were also treated with 17β-estradiol and DMSO (as vehicle). Cells were harvested and RNA extracted. TaqMan real time PCR was used to determine the effects of each phytoestrogen or estradiol treatment on haemostatic gene expression. The genes chosen for study were those whose expression was altered
significantly by genistein and/or equol in the rat study. Protein expression of genes significantly affected was determined in the media from treated cells using ELISA. The effect of each treatment on the expression of oestrogen receptors, including GPR30, in Hep89 cells was also determined. Cytotoxicity assays were performed to ensure that each treatment did not compromise cell viability.

The aims of this part of the study were

1. To investigate if the results found in the animal model could be translated to a human hepatocyte cell model.
2. To determine whether any effects seen were dependent on ERα expression.
4.2 Results

Effects of the phytoestrogens, genistein, equol and daidzein on haemostasis gene expression in Hep89 cells

Factor VII (FVII) (Figure 4.1)
FVII mRNA expression was increased by estradiol and also by each phytoestrogen. Highest levels of expression were found in the equol and daidzein groups with fold changes of 4 and 3.2 respectively.

Fibrinogen γ (Figure 4.2)
Estradiol increased the expression of fibrinogen γ mRNA 3.4 fold versus the vehicle control. Both genistein and daidzein resulted in the downregulation of fibrinogen γ levels although the fold change only reached 1.3 and -1.4 respectively, compared to the vehicle control. Stimulation with equol resulted in a small change in the expression of fibrinogen γ with a fold change value of 1.7.

Prothrombin (Figure 4.3)
Prothrombin mRNA expression remained unchanged after treatment with estradiol and equol. Genistein significantly increased the level of prothrombin 3.6 fold (P<0.01) compared to the cells treated with vehicle. Daidzein also increased the expression compared to the control cells but the level was within the background physiological range (-2 to 2 fold).

Antithrombin (Figure 4.4)
Antithrombin mRNA expression levels are increased in Hep89 cells after genistein and daidzein treatment, daidzein gave the highest fold change difference of 4.4 compared to the DMSO treated cells (P<0.01). Estradiol and equol did not change the expression of antithrombin relative to the control.
Protein C (Figure 4.5)
The mRNA expression of protein C was unchanged in estradiol treated cells compared to the vehicle controls. All three of the phytoestrogens downregulated the expression of protein C by -1 to -2 fold. The effect of daidzein was significant (P<0.05).

Protein S (Figure 4.6)
Unlike the effect on protein C, estradiol increased the expression of protein S 3.2 fold. Genistein, equol and daidzein treatment only slightly regulated changes in protein S. Genistein and daidzein showed fold changes of 1.4 and 1.7 respectively, whereas equol slightly decreased the expression of protein S with a fold change less than -1.

Plasminogen activator inhibitor-1 (PAI-1) (Figure 4.7)
PAI-1 mRNA was upregulated 2.2 fold following stimulation with estradiol compared to the vehicle treated cells. Daidzein also increased PAI-1 expression in a similar manner to estradiol. Genistein and equol had the most marked effect on regulating PAI-1 expression. These phytoestrogens increased the expression of PAI-1 4.2 and 2.9 fold, respectively, each fold change was statistically significant compared to the vehicle treated cells (P<0.05).

Tissue plasminogen activator (tPA) (Figure 4.8)
All four treatments increased the levels of tPA expression relative to the vehicle control. Fold changes of 2.6 were recorded for estradiol and daidzein treatment; genistein and equol increased mRNA expression further. Tissue plasminogen activator fold change for genistein was 4.3 and stimulation with equol resulted in a fold change of 4.8 (P<0.01).

C- reactive protein (CRP)
CRP was not expressed in the Hep89 cells.
Figure 4.1: Factor VII mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls.

Figure 4.2: Fibrinogen γ mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted *$P<0.05$ compared to DMSO.
Figure 4.3: Prothrombin mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted **P<0.01 compared to DMSO.

Figure 4.4: Antithrombin mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted **P<0.01 compared to DMSO.
Figure 4.5: Protein C mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted *P<0.05 compared to DMSO.

Figure 4.6: Protein S mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls.
Figure 4.7: Plasminogen activator inhibitor-1 (PAI-1) mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted *P<0.05 compared to DMSO.

Figure 4.8: Tissue plasminogen activator (tPA) mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted **P<0.01 compared to DMSO.
Table 4.1: Summary of the effects of phytoestrogens (>2 fold) on haemostasis gene expression in Hep89 cells * P<0.05, ** P<0.01 compared with vehicle treated cells

Summar of the effects of phytoestrogens on haemostasis gene expression in Hep89 cells

Table 4.1 summarises the effects of each phytoestrogen on mRNA expression of each of the genes studied. Upregulation or downregulation greater than 2 fold was considered physiologically relevant and thus only effects greater than 2 fold are listed.

Genistein

Genistein had the greatest effects on the genes studied. Incubation at 50nM resulted in up-regulation of prothrombin and antithrombin gene expression. Upregulation of the fibrinolytic activator tPA and its inhibitor PAI-1 was also observed. All effects observed were greater than those observed for estradiol for the same genes.

Daidzein

Daidzein upregulated FVII and antithrombin III expression however the coagulation inhibitor protein C was down regulated. tPA but not PAI-1 was upregulated but to a lesser extent than was observed for genistein and equol.

Equol

The metabolite of daidzein equol also upregulated factor VII expression but to a slightly higher level than its precursor, daidzein. Equol had no effect on coagulation inhibitor
expression. In a similar manner to genistein, both tPA and its inhibitor (PAI-1) were upregulated.

**Expression of oestrogen receptors in Hep89 and HepG2 cells (Figure 4.9 and Figure 4.10)**

As expected, oestrogen receptor alpha (ERα) was detected at the mRNA level in these transfected HepG2 cells (Hep89). The beta isoform of the oestrogen receptor was not detected. No expression of ERα or ERβ was detected in the HepG2 cell line. Both cell lines expressed the oestrogen receptor GPR30. The Hep89 cell line displayed a very similar level of GPR30 present as in the wild type HepG2 cells. Human breast tissue was used as a control sample. Results are expressed as target Ct - 18s Ct. Lower Ct values reflect higher expression.

**Effect of phytoestrogens on oestrogen receptor expression in Hep89 cells (Figure 4.11 and 4.12)**

Hep89 cells incubated with equol and daidzein downregulated ERα at the mRNA level. Following genistein incubation a slight downregulation was noted but this was less than 2 fold. A similar effect was observed with estradiol. GPR30 mRNA expression was upregulated by all three phytoestrogens, the effect with equol was significant (P<0.05). The effect of estradiol on GPR30 was less with a small upregulation of 1.4 fold.
Figure 4.9: mRNA expression of oestrogen receptors in Hep89 cells compared with human breast tissue control.

Figure 4.10: mRNA expression of oestrogen receptors in HepG2 cells compared with human breast tissue control.
Oestrogen receptor alpha

Figure 4.11: Oestrogen receptor alpha (ERα) mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted *P<0.05 compared to DMSO.

G-protein coupled receptor

Figure 4.12: G-protein coupled receptor (GPR30) mRNA expression in Hep89 cells stimulated with 50nM of 17β-estradiol, genistein, equol or daidzein for 24hrs, compared to DMSO treated controls. Statistical significance is denoted *P<0.05 compared to DMSO.
The effect of varying concentrations of the phytoestrogens genistein and equol on haemostasis gene expression in Hep89 cells

To determine if the effects of genistein and equol on haemostasis gene expression were concentration dependent, gene expression was studied at three concentrations for each phytoestrogen, 10, 100 and 1000nM using TaqMan PCR. Levels of upregulation or downregulation greater than 2 fold were considered physiologically relevant.

Genistein (Figure 4.13-4.17)
Following incubation with 10, 100, and 1000nM of genistein, Hep89 cells displayed downregulation of the coagulation inhibitor protein C, following incubation with genistein at 100nM a 2.7 fold down regulation was observed, this was not observed with previous 50nM incubations or with the lower 10nM concentration used in this experiment, increasing the concentration to 1000nM neutralised this effect. PAI-1 mRNA expression was down regulated in a dose dependent manner from -3.1 for 10nM treatment on PAI-1 to a neutral effect at 1000nM. Fibrinogen α, tPA and FVII expression was largely unchanged.

Equol (Figure 4.18-4.22)
Equol treatment using the same concentration ranges showed more neutral effects compared to genistein. None of the changes reached the cut-off of 2 fold down or upregulation. The greatest effect was with protein C which was down regulated to 1.9 after 1000nM treatment.
Figure 4.13: Protein C mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM genistein versus DMSO control.

Figure 4.14: Fibrinogen γ mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM genistein versus DMSO control.

Figure 4.15: Plasminogen activator inhibitor-1 (PAI-1) mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM genistein versus DMSO control.
Figure 4.16: Tissue plasminogen activator (tPA) mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM genistein versus DMSO control.

Figure 4.17: Factor VII mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM genistein versus DMSO control.

Figure 4.18: Protein C mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM equol versus DMSO control.
Figure 4.19: Fibrinogen γ mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM equol versus DMSO control.

Figure 4.20: Plasminogen activator inhibitor-1 (PAI-1) mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM equol versus DMSO control.

Figure 4.21: Tissue plasminogen activator (tPA) mRNA expression in Hep89 cells stimulated with 10nM, 100nM or 1000nM equol versus DMSO control.
Effect of ERα expression on the phytoestrogen induced changes in haemostasis in the HepG2 cell line (Hep89 v HepG2) (Figure 4.23)

In order to determine the role of ERα in the effects which were observed, the effects of phytoestrogens on gene expression in the HepG2 (ERα negative) cell line was determined and compared with Hep89 (ERα positive) cell line. Graphs show the mean of 3 independent experiments. The genes studied were FVII, tPA, PAI-1 and prothrombin all of which showed effects in the Hep89 cells in the earlier experiments.

*Factor VII mRNA (FVII) (Figure 4.23a)*

FVII mRNA expression was increased particularly following equol treatment in Hep89 cells, this effect was not observed in HepG2 cells where ERα is absent (P<0.05). In these cells a small downregulation of FVII expression was observed suggesting a role for ERα in the increased expression observed following phytoestrogen treatment. In comparing HepG2 to Hep89 cells, the phytoestrogen genistein and daidzein increased FVII expression in ERα positive cells, lower levels were found in the HepG2 cells. In the estradiol treated cells, a small increase in FVII expression was observed in the Hep89 cells however this was not observed in the absence of the ERα receptor (P<0.01).
Prothrombin (Figure 4.23b)

Prothrombin expression is markedly different between ERα expressing Hep89 cells and ER-negative HepG2 cells. Genistein treatment upregulated prothrombin mRNA expression and this effect was amplified in the presence of the oestrogen receptor (P<0.05). In contrast, the phytoestrogens equol and daidzein upregulated prothrombin expression in the absence ERα. Hep89 cells treated with equol and daidzein did not show this upregulation. Estradiol treatment also upregulated prothrombin expression in the HepG2 cells compared to Hep89s. Equol showed the greatest difference in prothrombin expression between the two cell lines (P<0.05).

Plasminogen activator inhibitor-1 (PAI-1) (Figure 4.23c)

There are distinct changes in the regulation of PAI-1 mRNA levels in Hep89 and HepG2 cells. In Hep89 cells the phytoestrogens genistein (P<0.01) and equol (P<0.05) upregulated the expression of this inhibitor as did the synthetic oestrogen estradiol. Without the presence of ERα in the HepG2 cell line these treatments have a modest effect on the expression of PAI-1 with a 2 fold or less increase in expression compared with control treated cells. Daidzein had a similar effect on PAI-1 expression in both cell types showing an approximate 2 fold increase compared with vehicle treated cells.

Tissue plasminogen activator (tPA)(Figure 4.23d)

The presence of ERα on the expression of tPA caused significant changes in its expression following phytoestrogen treatment. Genistein treatment resulted in an up-regulation of tPA expression in Hep89 cells, an effect not observed in cells devoid of ERα. In contrast, following equol treatment, tPA was up-regulated in Hep89 cells and down-regulated in HepG2 cells, suggesting an important role for ERα in the expression of tPA (P<0.001). Daidzein increased tPA expression in Hep89 cells 3 fold compared to no change in the HepG2 cells. Estradiol positively impacted the expression of tPA, resulting in a 2 fold change compared with vehicle treated Hep89 cells, a similar result was observed in ER negative HepG2 cells.
Figure 4.23a and b: mRNA expression of (a) Factor VII and (B) Prothrombin in Hep89 versus oestrogen receptor-negative HepG2 cells stimulated with 50nM 17β-estradiol (E2), genistein, equol or daidzein compared to DMSO control. Statistical significance is denoted *P<0.05, **P<0.01 for Hep89 vs. HepG2.
Figure 4.23c and d: mRNA expression of (C) Plasminogen activator inhibitor-1 (PAI-1) and (D) Tissue plasminogen activator (tPA) in Hep89 versus oestrogen receptor-negative HepG2 cells stimulated with 50nM 17β-estradiol (E2), genistein, equol or daidzein compared to DMSO control. Statistical significance is denoted *P<0.05, **P<0.01, ***P<0.001 for Hep89 vs. HepG2.
Oestrogen receptor expression in hepatocyte cell lines (Figure 4.24)

The difference in oestrogen receptor expression can clearly be seen in Figure 4.24, showing the expression of ERα in Hep89 cells only. GPR30 can be seen at a similar expression level in both hepatocyte cell lines. ERβ is not present in either cell line. Results are expressed as target Ct - 18s Ct. Lower Ct values reflect higher expression.

Cytotoxicity of phytoestrogens (Figure 4.25)

The possible cytotoxicity of phytoestrogen incubation was assessed using the MTT assay. This assay assessed viability by the ability of the cells to cleave yellow coloured MTT reagent to purple formazan crystals. The number of metabolically active cells directly correlates to the amount of crystals formed and is measured by absorbance. Results are expressed as percentage proliferation with the absorbance of untreated cells as 100% and the treated cells a percentage of this value. As shown in Figure 4.25 the phytoestrogens, daidzein and genistein each showed no toxicity on the cells, whereas equol had a slight negative effect on cell proliferation but which was similar to the effects seen in the DMSO control for this experiment.
Figure 4.25: Cell proliferation in response to (A) genistein, (B) equol and (C) daidzein in Hep89 cells. Medium: cells with medium only (no treatment), DMSO: cells with DMSO treatment only.
4.3 Discussion

Studies have shown that HT use by postmenopausal women causes an increase in many of the coagulation and fibrinolytic proteins synthesised in the liver (210-211, 233). These changes are thought to be implicated in the increased risk of thrombosis observed in hormone users (11, 14). In this study the relationship between 17β-estradiol and the phytoestrogens genistein, equol and daidzein in haemostatic gene expression and the possible mediating influence of the oestrogen receptor was examined. Human hepatocyte cell lines were used in order to study the regulation of the production of these haemostatic markers. HepG2 is a human hepatoma cell line which provides an in vitro model for this study as this cell line expresses many of the genes involved in coagulation activation and fibrinolysis. HepG2 cells are frequently used in studies on the regulation of liver-specific gene expression and they display the majority of haemostatic factors, excluding CRP, found in the liver. They have been the subject of numerous haemostasis studies including those focusing on the relationship between coagulation FXII and estrogen (338-339). We examined the differences between these oestrogen receptor negative hepatocytes and those stably transfected with the alpha isoform (ERα). Limitations of transfection have been reported including diversions from the normal growth of the cells however here we have used very low passage numbers for our experiments to compensate for possible growth problems.

The activation of the coagulation system is balanced by the activation of the anticoagulant systems namely the antithrombin and APC pathways. Synthesis of many of these of these haemostatic proteins occurs in the liver (340) and it has been shown in this study that all of the genes investigated were amplified by RT-PCR indicating that these hepatocytes are appropriate for the transcriptional regulation of these genes in vitro. There was one exception, CRP an acute phase reactant involved in the prediction of cardiovascular events was not detected at the mRNA level in Hep89 cells, this marker is induced in vivo by inflammatory cytokines (341-342), a process which does not occur in this in vitro model. This confirms previous reports on the HepG2 cell line which state that these cells can synthesise and secrete many of the proteins known to originate from the liver excluding CRP (343-344).
Kilbourne et al also showed that ER transcriptional activity was not present in HepG2 cells, as shown by the inability of 17β-estradiol to stimulate ERE controlled reporter activity (345), unless transfected with ERα. The Hep89 cell line enabled the study of ER dependent effects on expression of these genes. Hep89 cells are transfected to stably express ERα. The mRNA expression of ERα was detected in Hep89 cells but not in the HepG2 cell line when analysed via Taqman real time PCR.

In this Hep89 cell line, changes in the expression of coagulation and fibrinolytic genes were found compared with the HepG2 cells. Of note is the effect that genistein has on this ERα expressing cell line. The increase by genistein on PAI-1, tPA, prothrombin, and mRNA expression are in contrast to the results shown in ER negative hepatocytes from the HepG2 cell line suggesting a role for ERα in mediating the enhanced transcription of these genes. In contrast the expression of protein C, an important inhibitor of coagulation, is downregulated by genistein and daidzein in a concentration dependent manner. As discussed in Chapter 1 many of the vitamin K dependent proteases have similar gene structure and are regulated in a tissue specific manner however subtle differences and the expression of cofactors can lead to different effects.

Overexpression of the prothrombin gene is associated with an increased risk of thrombosis (113). Prothrombin is a vitamin K dependent protease and its gene structure shares many of the features of other members of this family including factor VII which is upregulated by daidzein and its metabolite equol. The promoter region of the prothrombin gene contains binding sites for HNF-4α and HNF-3β as well as sites for the ubiquitous SP-1 and SP-3. As discussed in the introduction (see section 1.2.3 (i)) Sp-1 is known to interact with oestrogen receptors. ERα can interact with SP-1 and bind to GC rich regions of target genes. The response is tissue specific and also ligand dependent. In agreement with the findings of this study ERα/SP-1 interactions are normally associated with enhanced transcription whereas ERβ type/SP1 is associated with a repression of gene expression. Genistein induced an increase in transcription of prothrombin which was not observed with estradiol, equol or daidzein suggesting a ligand dependent effect. This effect was mediated by ERα as the increase in prothrombin was not evident in HepG2 cells. On the other hand, estradiol, equol and daidzein increased prothrombin expression in ER negative cells suggesting that this effect is regulated by another pathway. Ligand dependent activation may also trigger a number of coregulators to form a complex which alters chromatin structure and facilitates transcription. HNF
transcription factors are a family of transcription factors which play an important role in liver specific gene expression (346). Studies using liver specific HNF-4α null mice suggest that this transcription factor plays an important role in regulating the expression of some but not all blood coagulation factors produced in the liver (347). The activity of the prothrombin gene is determined by an enhancer region distal from the transcription start site, in this way it differs from other vitamin K dependent coagulation factors. Although data on the interaction between the prothrombin enhancer region and oestrogen like compounds are scarce, a study of the ApoA1 gene has shown that both genistein and estradiol stimulated an increase in binding to a HNF-3β site and also an increase in the expression of the transcription factor itself in liver cells (348). This suggests that HNF-3β could play a role in genistein induced increase in prothrombin expression observed in this study.

An increase in FVII expression was also observed following stimulation with daidzein and its metabolite equol in Hep89 cells, this effect was reversed in HepG2 cells which did not contain ERα, suggesting that the response is oestrogen receptor mediated. Reporter studies have suggested that FVII expression can be regulated via an oestrogen response element (ERE) on the FVII promoter, although interaction with this site is linked to repression rather than stimulation of FVII, the effect is ligand specific and may depend on the expression and binding of coregulators to activate or inhibit transcription (93). FVII expression is also regulated by HNF-4α. HNF-4 and HNF-3 are known to interact with ligand bound oestrogen receptor through protein/protein interactions to enhance transcription in the ApoA1 gene promoter (335), this mechanism may also contribute to the regulation of FVII expression. The FVII promoter also contains GC rich regions. As described above, the effect of equol on FVII expression could also be mediated via SP-1 in a similar manner to that described for the prothrombin.

Both genistein and daidzein increased transcription of the antithrombin gene. The major focus of molecular studies of antithrombin is in understanding antithrombin deficiencies which are a significant risk factor for venous thrombosis. Much remains to be understood regarding the significance of increased expression of this inhibitor. In common with the other vitamin K dependent proteins described in the liver, the promoter region contains a HNF-4α binding site and hence the increased expression observed may be mediated via this site.
A significant downregulation of protein C expression was observed following stimulation with all three phytoestrogens in the Hep89 cell line. In the case of genistein, this downregulation was concentration dependent, reaching a maximum at 100nm. This effect was reduced but not abolished in the absence of ERα expression suggesting that the receptor may not be the major determinant of phytoestrogen induced repression of this gene. The promoter region of the protein C gene contains regulatory sites for HNF-1 and two overlapping binding sites for HNF-3, an Sp-1 site and a unique regulatory element (designated PCE1). Although HNF-3 and SP-1 are common regulatory sites on several of the genes described above, the effects of phytoestrogens on protein C transcription appear to be different and probably involve effects on the expression of corepressors. ERα may also downregulate expression by interacting with other transcription factors. Further studies are required to determine the precise mechanisms involved.

The most significant alterations in expression were observed with tPA and its inhibitor PAI-1. Both genistein and equol upregulated tPA expression greater than 4 fold. Regulatory binding sites on this gene include an Ap-1 site, HNF-3β, NFκB, c-Jun and CREB binding sites (166). In addition, a multihormone response region has been identified far upstream from the human tPA gene in human fibrosarcoma cells which has been shown to be important in hormone activation (349). ERα is clearly important in mediating the effects of increasing tPA transcription since in the absence of this receptor, the upregulation of tPA was abolished. It appears likely that this response is due to a combination of ERα mediated effects which may involve some or all of the transcription factors mentioned above. Of note, in the absence of ERα, a significant downregulation of tPA expression was found following equol stimulation, this suggests that this phytoestrogen can also regulate tPA in an ER independent manner.

The regulation of PAI-1 is also complex involving numerous regulatory elements including a VLDL response element, SMAD, Sp-1 and TGF-B responsive sites (180). PAI-1 is regulated by a diverse range of signals including steroid and peptide hormones, hypoxia, acute phase responses as well as mechanical and physical stresses. PAI-1 was upregulated in all treatment groups in Hep89 cells. The most significant increase was with genistein and equol treatment with 4 fold and 3 fold changes, respectively. Comparison of PAI-1 expression following phytoestrogen stimulation in ERα positive and ERα negative cells shows that this upregulation of expression is reduced in ERα.
negative cells rather than abolished, particularly for cells stimulated with estradiol where a 2 fold upregulation is seen in the absence and presence of ERα. This is in agreement with a study which has shown that oestrogen reduces PAI-1 plasma levels (350). The effects in HepG2 cells may be mediated by transcription factors such as AP-1, Sp-1 or CREB binding sites. This suggests that several regulatory pathways both direct and indirect are involved in the gene regulation observed. There was a greater increase in PAI-1 expression in Hep89 cells after genistein and equol treatment suggesting that the upregulation of PAI-1 by these phytoestrogens may be ERα dependent. This could be mediated through an AP-1 site as oestrogen has previously been shown to regulate AP-1 dependent transcription by ERα (293) and a study of PAI-1 promoter activity in endothelial cells showed sites that were similar to AP-1, named P- and D-boxes, which had the ability to modify ER activity (351).

Increased expression of PAI-1 is important as it has been linked to a variety of pathological conditions including thrombosis and myocardial infarction (352-353). Coronary artery disease is an important manifestation of atherosclerosis and is one of the most frequent causes of death in the Western world. It has been proposed that inflammation is the driving force in atherosclerosis (354-355). Strong evidence supports the central role of proinflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6) in these pathologies (356). For confirmation of the mRNA data PAI-1 antigen expression was measured using ELISA. Although a significant difference in expression at the mRNA level was shown, this does not exactly translate to the protein level. This assay has a homogenous reactivity to the various forms of PAI-1, latent, active and bound to tPA and uPA this may be the reason between the difference seen in PAI-1 mRNA and these protein levels.

The results observed in this study give weight to the hypothesis that ERα regulates the effects of genistein and to a lesser extent equol and daidzein on certain haemostasis genes. Although these phytoestrogens are similar in structure to estradiol, they differ in the number and position of their hydroxyl groups. The activity of many synthetic oestrogen-like compounds is related to the number and arrangement of their hydroxyl groups (357). Of note genistein, which appeared to have the greatest effects both in the in vitro and in vivo rat studies contains three hydroxyl groups whereas daidzein and equol have two (358). The effect of this on ligand binding may be reflected in the potency of each compound in mediating its effects on transcription.
The results for daidzein were not as significant as the changes seen in its metabolite equol. Equol is more oestrogenic than its precursor hence the results were not unexpected, provided these transcriptional effects are mediated by ERα.

The physiological effects of oestrogen are mediated through the combined actions of the classical nuclear oestrogen receptors (ERs) and GPR30, both of which can mediate rapid signalling events and modulate transcriptional activity. GPR30 is a transmembrane receptor which can bind oestrogen and can have both genomic and non genomic downstream effects. This can be achieved via the stimulation of mitogen-activated protein kinase (MAPK) pathway, and extracellular signal-regulated kinase (ERK1/2). Following ligand binding to GPR30, this receptor becomes phosphorylated and has the ability to recruit the protein arrestin (359) which has recently been recognised to activate receptor tyrosine kinases (360). Although data is scarce in the liver, in a rat model oestrogen reduced hepatic injury via a PKA pathway stimulated by oestrogen bound to GPR30.

Activation of these kinase pathways can also modulate nuclear transcription events and cell proliferation. GPR30 may represent a mechanism by which phytoestrogens can regulate transcription, independent of the classical ERs, such as was observed for PAI-1 in this study.

A small upregulation of GPR30 expression was observed in response to all three phytoestrogens in contrast to the down regulation of ERα. Expression of ERα is known to be affected by acute and chronic changes in circulating estradiol. It would appear that phytoestrogens may have similar effects on ERα in the liver. It is also possible that the effects on haemostasis that were seen in the HepG2 cell line are mediated via GPR30 which are exacerbated by the presence of ERα in the Hep89 cell line (333) as kinase activation by either steroids or other receptor types can result in steroid receptor phosphorylation, leading to transcriptional activity (361).

Trifiletti et al conducted a small placebo-controlled trial of the effects of genistein on haemostatic markers, they found that genistein made no change to the plasma levels of PAI-1 or prothrombin fragments 1 and 2 in healthy postmenopausal women (253). The results of this study show in vitro that the effects of the phytoestrogen genistein significantly increased prothrombin and PAI-1 expression and also considerably increased tPA in the Hep89 cells, conflicting with Trifiletti’s in vivo study. Furthermore a
study showed that an isoflavone preparation may even decrease the levels of prothrombin fragments 1 and 2 (249). Genistein also made slight changes in the expression of other genes, some of these changes differ to both the results shown for the control treatment and also the estradiol treated cells which may indicate that genistein is more effective in regulating these genes. Studies have shown that estradiol has the greatest binding affinity for ERα relative to these phytoestrogens (34-35) so it is plausible that the effects of the phytoestrogens on regulating gene expression shown in this study are mediated through another pathway such as GPR30. Non genomic and genomic pathways are not mutually exclusive and the convergence of the two pathways allows the fine degree of control required for oestrogen mediated transcription.

In order to analyse if the effects of genistein and equol regulate the changes in a dose dependent manner, a concentration curve was carried out in Hep89 cells. With varying concentrations of genistein, only the protein C and fibrinogen γ results are reproducible. FVII, PAI-1 and tPA showed opposite results to the initial 50nM treated study which when repeated were not altered. PAI-1 showed the most noteworthy changes in expression with the concentration curve showing a dose dependent decrease in expression levels. The lower the concentration of genistein the higher the fold change. A dose response curve using equol again showed different results to the initial 50nM study. Although these fold change values were below the background level it shows that this study was not reproducible in these cells. It may be possible that the hepatocytes undergo growth changes over time that causes them to lose some of their cell features or activity therefore explaining these differences and rendering this dose response study inconclusive.

This hepatocyte study was designed to reflect, in a human cell model, the effects of the phytoestrogens examined in the ovariectomised rat model. This study used a standard concentration of 50nM stimulant which represents a value reflecting an approximate concentration that may be found in plasma of postmenopausal women taking phytoestrogen supplements. The normal serum concentration of 17β-estradiol is 360-550pmol/L in the luteal phase of reproduction which decreases to <73pmol/L in postmenopausal women (362). This dose and others were analysed via MTT assay and no significant cytotoxic effects were found from genistein, equol or daidzein after a broad range of concentrations were tested in the Hep89 cell line. In comparing the
hepatocyte results with the previous results on the ovariectomised rat model it is noted that the prothrombin results are the same, activation of gene expression occurred after treatment with genistein and also equol in both systems. For the hepatocytes treated with genistein, similar results are found in the expression of antithrombin, PAI-1 and tPA in the animal model. Equol has similar effects on FVII expression in both animal model and HepG2 cells. These similarities could suggest a comparable thrombotic potential between the two systems. The slight discrepancies between the animal model and the hepatocytes may be due to a combination of effects. The disparate effects of genistein and equol on some markers of haemostasis may be due to the kinetics of absorption, distribution, metabolism and excretion processes which differ between the systems used. The effects of estradiol on gene expression in the cell culture model were generally less marked than those found in the rat study. In the rat model, a preparation of estradiol benzoate was used while the hepatocytes were stimulated with 17β-estradiol. The difference in structure of these compounds could cause the variation observed between these two systems on some of the genes studied. In addition estradiol in the rat model was metabolised in the liver and hence the effects observed may have been due to interaction of the oestrogen metabolites such as estrone rather than the 17β-estradiol used in the present study. There would also be a contribution of other tissues in vivo to circulating levels of these markers which may be one explanation of the differences seen here e.g. inflammatory mediators produced in rat tissues may have influenced haemostatic gene expression. In addition genistein can also increase oestrogen activity in vivo by stimulating aromatase activity (37).

There are limitations to extrapolating results from an animal to an in vitro model, one is that the in vitro model will not respond to circulating plasma concentrations of proteins as the liver would in vivo and secondly the clearance and synthesis of plasma concentrations of these markers can not be mimicked in the same manner through cell culture as in vivo. Future analysis would verify the effects of phytoestrogens on translation, cellular processing, transport and secretion.

In conclusion this study shows that the phytoestrogens, genistein, equol and daidzein can regulate the expression of coagulation and fibrinolytic genes expressed in a human hepatocyte cell line, an effect which is augmented in the presence of ERα. In the next chapter these effects will be investigated in rat endothelial tissue and human endothelial cells.
Chapter 5

*In vivo* and *in vitro* effects of phytoestrogens on the regulation of haemostasis gene and protein expression in rat aorta tissue and human umbilical arterial endothelial cells in culture.
5.1 Introduction

As has been seen in the previous two chapters, phytoestrogens cause an increase in the expression of many of the activators and inhibitors of haemostasis both at the mRNA and protein level in animal liver tissue and human liver cells in culture. However the site at which thrombin and fibrin are formed and dissolved is the endothelium, and these cells play an important role in thrombus formation. Vascular endothelium can be both an important anticoagulant and procoagulant surface, and endothelial cells are the site of production of activators and inhibitors of the coagulation and fibrinolytic cascade (57). For this reason, the effect of phytoestrogens on vascular endothelial cells needs to be investigated. Several animal studies have shown that phytoestrogens particularly genistein can increase the availability of vasodilators such as nitric oxide, a feature shared with oestrogens (363). In order to determine whether similar effects are observed with endothelial derived proteins which influence haemostasis, this chapter investigates (1) the effects of phytoestrogens on aorta from phytoestrogen treated ovariectomised rats and (2) human aortic endothelial cells in culture.

The relationship of oestrogen receptors with the effects seen was also analysed.

For the first part of the study, two phytoestrogens, genistein and equol, were studied in ovariectomised rats.

Animals were treated for three months following ovariectomy.

Seventy seven Sprague Dawley rats were fed soy free chow supplemented with

(1) Estradiol benzoate 0.19mg/kg body weight per day (estradiol low group)
(2) Estradiol benzoate 0.75 mg/kg body weight per day (estradiol high group)
(3) Genistein 6 mg/kg body weight per day (genistein low group)
(4) Genistein 60 mg/kg body weight per day (genistein high group)
(5) Equol 21.7 mg/kg body weight per day (Equol)
(6) Soy free controls (soy free group)

At the end of the treatment period, animals were sacrificed and strips of aorta were excised. RNA was extracted from the tissues and Taqman real time PCR was used to determine the effects of each phytoestrogen or estradiol treatment on haemostatic gene expression.

For the human in vitro study, primary human umbilical arterial endothelial cells were grown until they were ~80% confluent. The medium was then changed 24hrs prior to
plating to phenol red free MEM with DCC stripped serum. Cells were stimulated for 24 hours with genistein, equol and its precursor daidzein at a final concentration of 50nM. Cells were also treated with 17β-Estradiol and DMSO (as vehicle). Cells were harvested and RNA extracted. TaqMan real time PCR was used to determine the effects of each phytoestrogen or estradiol treatment on haemostatic gene expression. Fold changes higher or lower that 2 to -2 fold were considered relevant and t-tests were performed to determine statistical significance compared with controls. The genes chosen for study were those whose expression was altered significantly by phytoestrogens in the rat study and those who are important in thrombotic disease. Protein expression of the genes significantly affected was determined in the media from treated cells using ELISA. In addition high content screening, a fluorescent labelling technique, was used to determine the expression of proteins in the cells in culture following incubation with each stimulant. The role of the oestrogen receptor in HUAECs in mediating any phytoestrogen induced changes in protein expression was investigated using the ER antagonist ICI 182,780. The cells were pre-incubated with the antagonist prior to addition of each stimulant, after 1 hour 17β-estradiol, genistein, equol, and daidzein, with estradiol and DMSO (as vehicle) controls, were added to the cells for 24 hours. Cytotoxicity assays were performed to investigate the effect of these phytoestrogens on cell proliferation and viability.

The aim of this part of the study was

(1) To determine the effects of genistein and equol (compared with estradiol and soy free controls) on haemostasis genes and proteins expressed in aortic tissue from ovariectomised rats.

(2) To investigate if the changes in the animal model were similar to those found in human arterial endothelial cells in culture.

(3) To determine the role of oestrogen receptors in any effects seen.
5.2 Results

Effects of Estradiol, Genistein and Equol on coagulation and fibrinolytic gene expression in aorta from ovariectomised rats.

*Tissue plasminogen activator (tPA) (Figure 5.1)*

Tissue plasminogen activator is increased following treatment with both low and high doses of estradiol (P<0.05) compared to the soy free control. The phytoestrogen equol also significantly increased tPA expression 3 fold, similar to the effect of estradiol (P<0.01) compared to the soy free control group. A small upregulation was observed in the genistein low dose group but this was not significant. Increased tPA levels were found with the high dose genistein group (P<0.01), although the levels were within the background range.

*Tissue factor pathway inhibitor (TFPI) (Figure 5.2)*

Estradiol increases the expression level of TFPI in the low and high doses to 2.4 and 3.4 fold, respectively, the low dose estradiol is statistically significant compared with the control (P<0.01). The low dose of genistein greatly increases the expression of TFPI to 15 fold (P<0.05) compared to the control group. The genistein high dose group showed a similar upregulation as the low dose estradiol group. Expression of TFPI was also was significantly increased in the equol treatment group compared with the control group with a fold change of 3.5 (P<0.05).

*Thrombomodulin (Figure 5.3)*

Thrombomodulin levels were increased in a dose dependent manner following estradiol treatment. The low dose resulted in a 2.4 fold increase while the higher dose doubled this value to 4.8 fold compared to the soy-free control group (P<0.05; P<0.01). The phytoestrogens genistein (low dose) and equol also significantly increased thrombomodulin expression. Genistein increased the levels 7 fold (P<0.01) while equol gave a fold change of 5.5 (P<0.05) compared to the soy-free control group. The high dose of genistein had no effect on thrombomodulin expression.
Thrombin receptor (Figure 5.4)

Estradiol increased thrombin receptor expression in a dose dependent manner. The levels of thrombin receptor in these samples were increased 4 and 6 fold for the low and high dose respectively, with the high dose group proving statistically significant (P<0.01) compared to the soy-free control group. Genistein also amplified thrombin receptor mRNA levels but in the opposite manner to estradiol. After genistein treatment the level of thrombin receptor was highest in the low dose group (P<0.01) at 8.5 fold and 3.7 fold for the high dose group, compared to the control. Equol did not significantly change the expression of thrombin receptor compared to the control group.

Endothelial protein C receptor (EPCR) (Figure 5.5)

Estradiol significantly increased EPCR compared to the soy-free control group and also in a dose dependent manner. The low dose increased EPCR expression 4 fold (P<0.01) while the high dose increased this by a further 1.5 fold (P<0.05). The low dose of genistein showed a similar effect with a fold change of 5 while the high dose of genistein actually decreased the expression of EPCR, the change was within the background range of -2 to 2 and was not significantly different to the control. The level of EPCR was unchanged after equol treatment compared with the control group.
Figure 5.1: Tissue plasminogen activator (tPA) mRNA expression in aorta tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01 compared to soy-free control.

Figure 5.2: Tissue factor pathway inhibitor (TFPI) mRNA expression in aorta tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01 compared to soy-free control.
Figure 5.3: Thrombomodulin mRNA expression in aorta tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01 compared to soy-free control.

Figure 5.4: Thrombin receptor mRNA expression in aorta tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted ** P<0.01 compared to soy-free control.
Figure 5.5: Endothelial protein C receptor (EPCR) mRNA expression in aorta tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6 mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted *P<0.05, ** P<0.01 compared to soy-free control.

Oestrogen receptor expression in rat aorta tissue (Figure 5.6)

Oestrogen receptor alpha (ERα) was detected at the mRNA level in these samples. The beta isoform was not detected. G-protein coupled receptor (GPR30) was expressed in these samples at a slightly higher level that ERα. Rat ovary RNA reverse transcribed to cDNA was used a control sample. Results are expressed as target Ct - 18s Ct. Lower Ct values reflect higher expression.
Effect of phytoestrogens on oestrogen receptor expression in rat aorta tissue (Figure 5.7 and 5.8)

ERα was similar in each of the treatment groups except for genistein high dose group which increased the level of expression of this receptor 3.9 fold (P<0.05). Although small changes in the regulation of GPR30 expression were observed between the oestrogen doses the changes are still within the background range. The low dose of genistein downregulated GPR30 expression however the effect was small and not statistically significant. The phytoestrogen equol had the most marked effect of GPR30 expression, downregulating its expression -5.5 fold which proved highly statistically significant (P<0.01).
Figure 5.7: Oestrogen receptor alpha (ERα) mRNA expression in aorta tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted $^*P<0.05$ compared to soy-free control.

Figure 5.8: G-protein coupled receptor (GPR30) mRNA expression in aorta tissue of ovariectomised rats fed with soy free food supplemented with estradiol benzoate low dose (0.19mg/kg bw), estradiol benzoate high dose (0.75mg/kg bw), genistein low dose (6mg/kg bw), genistein high dose (60mg/kg bw) and equol (17mg/kg bw) for 3 months. Statistical significance is denoted $^{**}P<0.01$ compared to soy-free control.
Effects of the phytoestrogens, genistein, equol and daidzein on haemostasis gene expression in human umbilical arterial endothelial cells (HUAECs).

*Tissue factor (TF) (Figure 5.9)*
Tissue factor mRNA expression was increased 2 fold in HUAECs after treatment with estradiol compared to the DMSO control. Stimulating the cells with phytoestrogen daidzein gave a similar result. A smaller fold change was observed with genistein treated cells (1.7 fold). Treatment with equol did not change tissue factor expression.

*Tissue factor pathway inhibitor (TFPI) (Figure 5.10)*
Stimulating the cells with estradiol downregulated TFPI expression 1.5 fold. Genistein and daidzein upregulated TFPI, 2 and 2.5 fold respectively compared to vehicle treated cells. Equol caused a 1.8 fold downregulation of TFPI; this result is within the background level and did not prove to be significantly different to the vehicle treated cells.

*Thrombomodulin (Figure 5.11)*
Thrombomodulin mRNA expression was not significantly changed by any of the stimulants used compared to the DMSO control.

*Thrombin receptor (Figure 5.12)*
Estradiol upregulated the thrombin receptor mRNA levels 2 fold in comparison to the DMSO control. Genistein was slightly lower with a fold change of 1.9 compared with the control. Equol decreased the expression of thrombin receptor -1.3 fold in comparison to the control whereas its precursor daidzein increased the levels 3.5 fold. Daidzein showed the greatest change in thrombin receptor expression.

*Tissue plasminogen activator (tPA) (Figure 5.13)*
The expression of tPA mRNA in HUAECs was downregulated -2.3 fold after stimulating the cells with estradiol compared to the DMSO control. Genistein resulted in a small increase in tPA expression compared to the control as did the phytoestrogen, daidzein. Equol downregulated tPA levels -3.5 fold compared with the DMSO control.
**Endothelial protein C receptor (EPCR) (Figure 5.14)**

EPCR mRNA expression levels were increased 3 fold with estradiol treatment compared with the DMSO control. Both genistein and equol negatively regulated the expression of EPCR with a fold change of -4.6 and -4.3, respectively. Daidzein had no effect on EPCR expression.

**Plasminogen activator inhibitor (PAI-1) (Figure 5.15)**

The mRNA expression of PAI-1 was increased 3.2 fold after stimulating the cells with estradiol. The phytoestrogens genistein and daidzein induced changes in expression levels which were within the background range. Stimulating the cells with equol decreased the expression of PAI-1 -2.2 fold compared to the DMSO control.
Figure 5.9: mRNA expression of tissue factor (TF) in HUAEC stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.

Figure 5.10: mRNA expression of tissue factor pathway inhibitor (TFPI) in HUAEC stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.
Figure 5.11: mRNA expression of thrombomodulin in HUAEc stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.

Figure 5.12: mRNA expression of thrombin receptor in HUAEc stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.
Figure 5.13: mRNA expression of tissue plasminogen activator (tPA) in HUAEC stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.

Figure 5.14: mRNA expression of endothelial protein C receptor (EPCR) in HUAEC stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.
Oestrogen receptors in HUAECs (Figure 5.16)

Oestrogen receptor alpha was not detected in these cells. The beta isoform was detected at the mRNA level. We also found expression of GPR30 in these cells, at a level which was similar to the control. The control used was human breast tissue. Results are expressed as target Ct - 18s Ct. Lower Ct values reflect higher expression.
Effects of phytoestrogens on oestrogen receptor expression in HUAECs (Figure 5.17 and 5.18)

ERβ expression was increased 2.5 fold after estradiol treatment of the cells compared to the DMSO control. Equol had the opposite effect to estradiol by downregulating ERβ mRNA expression 2.3 fold. The fold change after incubation with genistein remained similar to the DMSO control while daidzein gave the biggest change in expression of ERβ with an mRNA fold change of 2.9 compared with the DMSO control (Figure 5.17).

The expression levels of GPR30 remained unchanged after stimulating the cells with estradiol or genistein compared to the DMSO control. The phytoestrogens equol and daidzein resulted in exact opposite fold changes in GPR30 expression. Daidzein upregulated the levels of GPR30 2.3 fold while equol downregulated them to -2.3 fold, versus the DMSO control (Figure 5.18).
Figure 5.17: mRNA expression of oestrogen receptor beta (ERβ) in HUAEC stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.

Figure 5.18: mRNA expression of g-protein coupled receptor (GPR30) in HUAEC stimulated with 50nM 17β-estradiol, genistein, equol or daidzein compared with DMSO control.
Effects of phytoestrogens on HUAEC proliferation and viability (Figure 5.19)

The possible cytotoxicity of phytoestrogen incubation was assessed using the MTT assay. This assay is based on the ability of the cells to cleave yellow coloured MTT reagent to purple formazan crystals. The number of metabolically active cells directly correlates to the amount of crystals formed and is measured by absorbance. Results are expressed as percentage proliferation with the absorbance of untreated cells as 100% and the treated cells a percentage of this value. Following incubation of the cells with a range of concentrations of genistein the assay showed that a concentration of 30nM and higher induces cell proliferation. With unstimulated cells ranked at 100%, genistein can activate the cells to 250%.

Upon incubation with equol, DMSO has a slight effect on cell proliferation which increases after equol is added to the cells reaching a maximum at 10nm. Daidzein gave a similar result to genistein on these cells with an increase in proliferation at 30nM. There was a decrease in the curve at 3000nM which could suggest that at higher concentrations of daidzein the growth of HUAECs is inhibited.
Figure 5.19: Cell proliferation of HUAEC in response to (A) genistein, (B) equol and (C) daidzein. Medium: cells with medium only (no treatment), DMSO: cells with DMSO treatment only.
Summary of the results of the in vitro effects of phytoestrogens on gene expression in HUAECs

Phytoestrogens showed the greatest effect on EPCR expression; both genistein and equol downregulated EPCR mRNA. In contrast EPCR was upregulated by estradiol. In general, equol was the most effective phytoestrogen studied. In addition to EPCR, equol downregulated tPA and PAI-1 expression. Estradiol upregulated PAI-1 and downregulated tPA. The effects of genistein were relatively neutral on the other haemostasis genes studied. Daidzein increased the expression of thrombin receptor and was relatively neutral on the other genes studied. Equol also downregulated ERβ and GPR30 expression. Equol did not compromise cell viability at the concentrations studied, in fact an increase in cell proliferation was observed in comparison to untreated cells.

Effects of phytoestrogens on protein expression in HUAECs

To determine if these alterations in gene expression were translated into changes in protein expression, EPCR, tPA and PAI-1 protein were measured in response to genistein, equol, daidzein and estradiol using ELISA and high content screening, which is a method for quantitative fluorescence microscopy.

To determine the role of oestrogen receptors in any effects seen, an oestrogen receptor antagonist ICI 182,780 was pre-incubated with the cells prior to the addition of each phytoestrogen/oestrogen. The results are described below.

Plasminogen activator inhibitor-1 (PAI-1) protein expression in HUAECs (Figure 5.20)

PAI-1 protein expression was measured using an ELISA technique in the media from cells treated with each preparation. PAI-1 protein expression was increased in response to estradiol, genistein, equol and daidzein compared with DMSO treated cells.
Effect of the ER antagonist ICI 182,780

The unstimulated cells show an increase in PAI-1 protein concentration after incubation with the ER antagonist, ICI 182,780. When the cells were stimulated with estradiol or genistein, PAI-1 levels were reduced but not to the levels found in vehicle treated cells. For equol and daidzein, following incubation with the ER antagonist, there was a slight decrease in protein levels of PAI-1.

Figure 5.20: Plasminogen activator inhibitor-1 (PAI-1) protein expression in HUAECS stimulated with 50nM 17β-estradiol, genistein, equol and daidzein with or without 10μM ICI 182,780 compared with untreated HUAEC and DMSO controls. Protein concentration is expressed as ng/ml of media.
tPA protein expression in HUAECs as measured using high content screening (HCS) analysis (Figure 5.21)

tPA staining intensity was observed and quantified using INcell Investigator software and measured in relative fluorescent units. Unstimulated cells showed the highest concentration of tPA detected. Upon stimulating the cells with DMSO vehicle, estradiol or phytoestrogens, tPA protein levels were decreased.

Effect of the ER antagonist ICI 182,780

These results were unaffected by the addition of the ER antagonist to the cells. The effect of the antagonist can only be seen in the unstimulated cells which show a decrease in tPA protein compared with cells not treated with the antagonist.

EPCR protein expression in HUAECs as measured using high content screening analysis (Figure 5.22)

The EPCR staining intensity was observed and quantified using INcell Investigator software and measured in relative fluorescent units and the results indicate that the phytoestrogens genistein, equol and daidzein increase EPCR protein levels in comparison to the unstimulated cells, DMSO control and estradiol.

Effect of the ER antagonist ICI 182,780

Pre-incubation of the cells with the ER antagonist results in an increase in EPCR protein expression in all groups including the DMSO control. This increase is most marked in the case of DMSO, estradiol and daidzein and less marked with equol and genistein.
Figure 5.21: Tissue plasminogen activator (tPA) protein expression measured in relative fluorescent units in HUAECs stimulated with 50nM 17β-estradiol, genistein, equol and daidzein with or without 10μM ICI 182,780 compared with untreated HUAEC and DMSO controls.

Figure 5.22: Endothelial protein C receptor (EPCR) protein expression measured in relative fluorescent units in HUAECs stimulated with 50nM 17β-estradiol, genistein, equol and daidzein with or without 10μM ICI 182,780 compared with untreated HUAEC and DMSO controls.
Figure 5.23: High content screening images. These images represent the differences between the untreated cells (Figure 5.23a) and daidzein treated cells (Figure 5.23b). The green stain is tPA, red stain is EPCR and blue stain is the nucleus. This shows that daidzein treatment increases the protein expression of EPCR.
5.3 Discussion

The vascular endothelium lines the blood vessels and has an important role to play in maintaining haemostasis and normal vessel function. The endothelium can act as a thrombogenic surface by expression of procoagulant receptors and tissue factor and by downregulating expression of key inhibitory pathways of coagulation. In the presence of thrombin it can also provide an anticoagulant surface by expressing receptors that are crucial for the activation of protein C, such as endothelial cell protein C receptor (364) and thrombomodulin, or by expressing TFPI, the fast acting inhibitor of tissue factor. The endothelium also plays a role in fibrinolysis, expressing tPA which, in the presence of fibrin, activates fibrin dissolution.

Research has provided information on the role of the endothelium in areas such as the regulation of haemostasis (365), control of vascular tone (366) and regulation of blood pressure (367). Studies on the effects of oestrogens and phytoestrogens on the endothelium mainly focus on findings regarding vascular tone and growth and lipoprotein expression. These reported benefits were not translated into improved cardiovascular benefits in hormone therapy trials (11, 14).

In this chapter, the effects of the phytoestrogens, genistein, equol and daidzein on the regulation of haemostatic genes and proteins of the vascular endothelium in rat and human tissue were studied and compared to the effects of estradiol and appropriate controls.

The activity of coagulation and fibrinolytic pathways is a complex process involving serine proteases which were seen to be modified by phytoestrogens in the liver studies described in the earlier chapters. These effects can only translate into changes in the level of coagulation and fibrinolytic activation in vivo when combined with the effects observed in the endothelium.

In the first section of this study, using aorta samples from the rat menopausal model, a diet with a low dose of genistein (equivalent to that found in an Asian diet) gave the most marked response in the expression of endothelial derived coagulation and fibrinolytic proteins. The effects of genistein were similar if not more pronounced than those of estradiol indicating a higher potency of this compound in these tissue samples. Increase in the expression of the coagulation inhibitor TFPI were found in genistein treated rats.
In addition, key proteins involved in the activation of protein C i.e. thrombomodulin and EPCR were also increased by the low dose of genistein. These results show that two key pathways for the regulation of thrombus formation are altered significantly by phytoestrogens.

Thrombomodulin is a membrane bound protein which acts as a cofactor for thrombin which when bound can activate protein C and TAFI. By doing so it can inhibit both coagulation and fibrinolysis, with increasing levels of TAFI genistein may play a role in regulating these pathways in the endothelium. In human studies, plasma levels of soluble thrombomodulin are used as a measure of its expression since measurement of cell surface thrombomodulin is not possible. In a study of post menopausal women taking an isoflavone supplement, plasma levels of soluble thrombomodulin did not significantly change from the placebo group. Oestrogen replacement therapy has been associated with reduced plasma levels of thrombomodulin in healthy menopausal women (251). Although these levels suggest a possible reduction in anticoagulant activity, the correlation between soluble thrombomodulin and cardiovascular disease is unclear since increased levels have been found in patients with atherosclerosis (368) and have also been shown to be protective against future events in healthy individuals (369). Treatment with 17β-estradiol decreased thrombomodulin dependent APC activity in another in vitro study on endothelial cells, in contrast the same study showed that the two different SERMs had the opposite effect showing an increased expression and a potential vasculoprotective role (370). This is in agreement with the results from the rat aorta since genistein and equol, acting as SERMs, may be having a similar effect on thrombomodulin mRNA expression. Thrombomodulin is also transcriptionally upregulated by thrombin and cAMP which provide an indirect pathway by which the phytoestrogens may be mediating their effects (371). In addition, increased expression of thrombomodulin may have effects beyond coagulation including a direct role in modulating inflammation and an effect on the pathology of the vessel walls attacked by atherosclerotic lesions (371).

An essential component of the assembly of the APC complex is the endothelial cell protein C receptor (EPCR) which functions as an important regulator of the protein C anticoagulant pathway by binding protein C and enhancing activation by the thrombin-thrombomodulin complex. EPCR binds to both protein C and APC with high affinity and
by increasing the levels of this receptor, APC activity can also be increased preventing further thrombin formation at the endothelial surface. EPCR can bind activated FVII with a similar affinity as protein C, leading to a decrease in the rate of thrombosis (364). The results from menopausal rat aorta tissue show that genistein has a marked effect on EPCR expression, normally a tightly regulated receptor (372). The low dose of genistein gave a similar increase in fold change to the higher estradiol dose which could lead to an increased availability of the receptor at the endothelial surface and a potential enhanced anticoagulant effect. Although data on the role of enhanced EPCR expression is scarce it is known that mutations in this gene are associated with venous thrombosis (139). Several point mutations have been described for EPCR however they are relatively rare and their significance in relation to thrombosis risk is difficult to establish (373). Polymorphisms of the EPCR gene also influence APC activation, H1 haplotype tagged by the rare allele of 4678G/C, was reported to be associated with a reduced risk of VTE. H1 haplotype also reduced the risk of thrombosis in carriers of FV Leiden (374).

TFPI is a proteinase inhibitor expressed by the endothelium that inhibits the TF:FVIIa complex. TFPI is attached to the cell surface of endothelial cells via a glycosylphosphatidylinositol (GPI) anchor (375). TF contributes to the pathogenesis of thrombosis but is also involved in vascular and inflammatory disorders. TFPI is known to inhibit the development of thrombotic occlusion in atherosclerotic vessels, unstable angina and myocardial infarction. The ratio of TF to TFPI is an important determinant in haemostasis and thrombosis (376).

By increasing TFPI expression, genistein alters this ratio in favour of TF-mediated coagulation activation. There are few distinct studies on phytoestrogens regulating TFPI levels but studies have shown that hormone therapy, both estradiol-only and combined with a progestin, can decrease plasma levels of free TFPI leading to a possible hormone mediated downregulation of the TFPI pathway which could be thrombogenic (377-378). In the present study of TFPI mRNA expression, the phytoestrogen genistein would seem to have the opposite effect on rat endothelium as does equol. Plasma TFPI represents a small proportion of overall TFPI expression since over 80% of the protein is found bound to the endothelium; hence plasma levels may not reflect the expression of TFPI at the endothelial surface.

In platelets, oestrogen treatment was found to increase TFPI expression in an ovariecotomised porcine model, a similar finding was found with the SERM raloxifene.
In contrast an *in vitro* study showed that 17\(\beta\)-estradiol reduced the concentration of TFPI antigen in the media of HUVECS incubated for 24 hours with 17\(\beta\)-estradiol. TFPI released into the media however is similar to the TFPI found in plasma and does not represent the pool of TFPI anchored at the endothelium.

This study also showed increased expression of tPA, the main activator of fibrinolysis following treatment with two different doses of estradiol. Lower levels were found in genistein treated rats similar to those found in the soy free control group however equol treated rats showed a significant upregulation. Vascular endothelial cells are considered to be the predominant source of tPA in the circulation. Higher tPA expression would promote fibrinolysis in the presence of excess fibrin but as tPA is associated with PAI-1 this would depend on the expression levels of PAI-1. Plasma levels of tPA and to a greater extent PAI-1 have been documented as markers of atherosclerotic disease.

Protease activated receptors are members of a family of G protein-coupled receptors which are activated upon proteolytic cleavage by several proteases including thrombin. The so-called thrombin receptor or PAR-1 is the major receptor of this family expressed in the endothelium and plays an important role in platelet activation and thrombosis as well as tissue remodelling and vascular reactivity. The PAR-1 receptor plays a key role in many signalling pathways within the cell and through these signalling pathways, can upregulate genes involved in inflammation, coagulation e.g. TF and PAI-1, and cell proliferation.
Figure 5.24: Summary of PAR-1 signal transduction pathways (383).

Genistein can increase thrombin receptor expression greater than 8 fold, an increase seen at the low dose which was higher than the dose dependent effect seen after treatment with estradiol. The high dose of genistein also increased the level of thrombin receptor and equol made no significant change. Once bound by thrombin this receptor causes a number of events to take place in the cell, one such event being the secretion of PAI-1 (384). Activation of these receptors may promote coagulation activation and also increase the expression of cytokines and other inflammatory mediators involved in atherosclerosis and provide an important link between enhanced thrombin production and downstream signalling events (383).

Although genistein at the lower dose had the greatest effect of the phytoestrogens studied, the daidzein metabolite equol also showed some effects. This phytoestrogen increased levels of tPA, TFPI and thrombomodulin mRNA expression. Although the effects were not as potent as were found for genistein, in a mixed diet of isoflavones these changes may augment the genistein induced increases in expression.

Overall the impact of genistein would appear to have a positive effect on the haemostatic system in these rat aorta tissue samples by promoting the expression of key anticoagulant proteins which should serve as a defence against inappropriate thrombus formation.
These samples consist of a variety of cells and not just endothelial cells, so the effects observed are those obtained from a mixture of endothelial cells, smooth muscle cells, intimal cells and fibroblast-like cells. Studies have shown that haemostasis is vascular-bed specific, for example thrombomodulin is more important in maintaining the haemostatic balance of the heart than it is in the liver whereas tPA is the same for both (385). This suggests that phytoestrogens and other oestrogen-like compounds may not affect expression of these genes in the same way in the different cell types of the aorta and that the result obtained in this study is therefore a combination of these effects. The effects shown for thrombomodulin, EPCR and tPA are functionally the most important for endothelial cells.

Normal APC generation depends on the precise assembly on the surface of vascular endothelial cells of at least four proteins: thrombin, thrombomodulin, protein C and EPCR. Variations in this assembly may modify APC generation and alter the risk of thrombosis (374). It has been suggested that a transient reduction in the expression of APC and thrombomodulin may explain variations in APC activity observed over time (386). This study has shown alterations in EPCR, thrombomodulin and the thrombin receptor mRNA expression that suggests that estradiol and genistein, and to some extent equol, have an effect on the aortic tissue in the rat and thus a potentially modulating effect on APC activity.

In this tissue type, ERα mRNA levels were detected with no expression of ERβ which may mean the alpha isoform is enhancing these anti-thrombotic effects as was previously seen with the rat liver tissue. A significant upregulation of ERα was observed with the higher dose of genistein compared with the control group which is in contrast with receptor binding studies. Estradiol binds ERα with much greater affinity than phytoestrogens and, of the phytoestrogens that do bind, equol binds ERα with higher affinity than genistein (302). Expression of GPR30 in this tissue was also observed which may or may not be involved in regulating the effects of these compounds alongside ERα. The expression of GPR30 after treatment with each compound was similar with the exception of equol. Equol downregulated the expression of GPR30, as this phytoestrogen had a more neutral effect on the haemostasis genes studied, this could be due to reduced availability of this receptor. This would indicate the effects of genistein and estradiol on these coagulation proteins may be regulated to some extent by GPR30.
To elucidate the effects of these phytoestrogens in more detail in human cells, an *in vitro* model of endothelium, primary human umbilical arterial endothelial cells (HUAEC), was used. It was not feasible to obtain endothelial cells from pre-menopausal women undergoing hysterectomy and to do this would be far more time consuming than the scope of this study. The arterial form was chosen over the venous cells as to represent closely the effects on CVD. Vascular endothelial cells are an important target of oestrogen action through both the classical genomic activities of oestrogen receptors alpha and beta and the rapid non-genomic activation of ER. Following phytoestrogen stimulation of these cells, it was found that genistein and daidzein have similar effects to 17β-estradiol on the genes studied however the effects seen differed from those found in the rat study.

Tissue factor, the primary cellular initiator of blood coagulation, is a transmembrane receptor that is expressed in a tissue-specific manner. TF plays a pivotal role in thrombin generation in atherothrombotic disease and is transcriptionally regulated by Spl (98). TF is not normally expressed by vascular endothelial cells but in this study of HUAEC, TF was detected at the mRNA level and was increased with 17β-estradiol incubation. The phytoestrogen daidzein upregulates TF at a similar level whereas its metabolite equol does not, displaying the unique structural characteristics of these compounds. The thrombin receptor is upregulated by estradiol and daidzein treatment as was found in the rat study. Genistein slightly increased the expression of this receptor and equol showed no change. In the presence of increased TF expression and downstream coagulation activation, increasing the availability of this receptor for thrombin binding could provoke the activation of several genes involved in the atherosclerotic process.

With estradiol, a decrease in the expression of tPA was found relative to the soy-free control, an effect similar to that found with equol. As the predominant source of tPA, decreasing this activator in endothelial cells will reduce fibrinolytic activity. Fibrinolytic activity can take place on the vascular cell surface as these cells have binding sites for tPA and plasminogen. Several transcription sites known to be responsive to oestrogens exist on genes involved in fibrinolysis. These binding sites include an AP-1 site and NFκB site for tPA (166) along with a AP-3 site for plasminogen transcription (387). This may be the basis for the regulation of tPA with ligand binding of equol and estradiol through an interaction between ER and cofactors in the promoter region of the gene.
An increase in PAI-1 expression in these cells was observed with estradiol, an effect which is reversed with equol treatment of the cells. Equol may negatively regulate this inhibitor via binding to transcription sites on the gene such as SP-1, TGF-β and SMAD responsive sites. There was no change in PAI-1 expression after genistein stimulation of the cells. Previous work on PAI-1 expression in human umbilical venous endothelial cells showed that genistein can decrease its expression in a dose dependent manner at concentrations between 37 and 370μM (254), concentrations much higher than the nanomolar range used in this study.

In contrast to the results found in the rat tissue, thrombomodulin expression in these cells was unchanged with each of the phytoestrogens studied. Similarly, the effects of phytoestrogens on TFPI and thrombin receptor expression were more modest for both oestrogen and genistein in this cell culture model and were largely within the background range. In agreement with the rat data, EPCR mRNA expression is upregulated following estradiol treatment. However opposite effects on its expression were observed with the phytoestrogens genistein and equol which downregulated the EPCR expression.

This indicates that should this effect be mediated by oestrogen receptors that the effect is ligand specific. Alternatively, additional regulatory pathways may be involved in the phytoestrogen downregulation. This may be of significance in carriers of certain EPCR functional polymorphisms and other prothrombotic mutations (374) leading to a prothrombotic phenotype.

Unlike the rat tissue, only ERβ was expressed in these cells, as ERα was not detected in these cells and as AIB1 is coexpressed with ER in the vascular endothelium (388), this may facilitate the oestrogen mediated effects shown. As there are differences between the animal model and HUAEC this difference in ER expression may be the explanation. Equol downregulated the expression of ERβ and any effects seen on the genes analysed were also negative. The membrane receptor GPR30 was also detected in these cells, the expression of which was unchanged with estradiol, genistein and daidzein treatment, however as with ERβ, equol also downregulated the expression of this receptor. It appears likely that both receptors contribute to the decreased expression observed in equol treated cells.
For genes that were markedly up/downregulated by phytoestrogens at the mRNA level, ELISA and a High Content Screening technique was used to determine if these effects were reflected in protein levels. High content screening enabled the visualisation of proteins and their cellular distribution in culture through fluorescent microscopy. To investigate the effects of oestrogen receptors in these cells the ER antagonist ICI 182,780 was used. The concentration of inhibitor was chosen as used in previous studies (305, 389-390). Oestradiol, the natural ligand for the ER, binds and stimulates receptor dimerization and subsequent activation of two activation domains (AF1 and AF2). The activated receptor complex then undergoes nuclear localization and subsequent binding to oestrogen response elements. Upon binding to these sequences, transcriptional coactivators are recruited to the complex, resulting in activation of gene expression. Following ICI 182,780 binding to ER, receptor dimerization is impaired, receptor degradation is accelerated and the two activation domains are rendered inactive. This compound then results in nuclear localization, disruption and a failure to recruit transcriptional coactivators. Consequently, ER-mediated transcription is completely attenuated which leads to complete suppression of oestrogen-dependent genes, and thus ICI 182,780 can be described as a ‘pure antioestrogen’ (391).

PAI-1 was upregulated with estradiol and downregulated with equol at the mRNA level, when tested at the protein level, small quantities of PAI-1 antigen were released into the media at similar levels in each of the phytoestrogen groups as the estradiol treated cells. After the addition of the ER antagonist to the cells a slight decrease in PAI-1 expression was observed in each treatment group which may suggest a role for ERβ in mediating the production of this protein in HUAECs. These results agree with the results found on genistein induced increase in PAI-1 mRNA in hepatocytes described in the earlier chapters which was also found to be reduced in the absence of ERs. The level of PAI-1 antigen released into the media was small and may not reflect the total production of PAI-1 in the cell. In addition post translational modifications and the regulation of the secretion of this protein into the media may explain the discrepancy between the mRNA and protein results.

Using the high content screening technique, tPA was detected at the highest level in untreated cells without the inhibitor. DMSO decreased tPA whereas estradiol and the phytoestrogens gave a similar value indicating the effects seen with this protein may be due to the vehicle itself and not specific oestrogen mediated effects. The ER antagonist
had no effect on the stimulated cells but did decrease tPA protein expression in the unstimulated cells.

Using the same technique, EPCR at the protein level was determined in cells pre-incubated with phytoestrogens. Prior incubation of the cells with the ER antagonist resulted in an increase in EPCR protein expression in all groups, with the highest concentration in the daidzein treated cells. This is in contrast to the down regulation of EPCR mRNA observed in genistein and equol treated cells but in agreement with the findings of the rat study. Post translational modifications and effects on mRNA stability may explain the discrepancies observed.

Although ICI 182,780 is known as an ER antagonist it has recently been shown to be an agonist for GPR30 (392), this may explain the upregulation of EPCR expression observed in all of the cell treatments following incubation with ICI 182,780 and suggests that GPR30 may mediate the effects on EPCR transcription and translation observed. Molecular, cellular and animal studies convincingly demonstrate that oestrogen has favourable effects on vascular cells, and that many of these effects are achieved through rapid, membrane-initiated ER-dependent signalling responses (393).

Cell viability tests showed that genistein, in agreement with previous studies (394), equol and daidzein, have a proliferative effect on HUAECS. The downregulation observed with many of the genes could not therefore be accounted for by a toxic effect of the added phytoestrogen. NFκB regulates genes involved in cell viability and studies have previously described how AIB1 is present in the vascular endothelium (388), this coactivator augments NFκB expression (395) and possibly together with ligand bound ER may aid the proliferation of these cells.

Overall, summarising the effects between the two experimental systems indicates that estradiol has the same effect on thrombin receptor and EPCR expression both \textit{in vitro} and \textit{in vivo} while genistein has similar effects in both systems on TFPI, thrombin receptor and tPA expression. In contrast equol appears to downregulate expression of both EPCR, tPA and PAI-1 at least at the mRNA level in HUAECS. This emphasises the individual effects of these compounds on gene expression. Studies of soy containing diets containing a mixture of daidzein and genistein represent a combination of the effects of the two compounds which may augment or neutralise each other. This may explain why
some dietary studies state that phytoestrogens have no significant oestrogenic effects on coagulation, fibrinolysis or endothelial function (250).

For genistein and equol, the effects of EPCR expression were markedly different between the two systems with the rat model showing increased expression and the cell culture model showing a marked downregulation. Of note, estradiol upregulated EPCR expression in both systems. A similar disparity was found in experiments investigating the effects of endotoxin on EPCR mRNA expression in rats where an upregulation was found, in contrast cell culture experiments showed that incubation of endothelial cells with inflammatory mediators such as TNFα caused a down regulation of expression. Subsequent experiments showed that the upregulation observed in the animal model was dependent on thrombin and could be replicated in the cell culture studies following the addition of thrombin (386). Increased levels of thrombin or another signalling molecule produced in response to the phytoestrogen diet may have caused the upregulation seen in the rat tissue. The HUAEC cell model did not account for the significant crosstalk that occurs between anticoagulant proteins and inflammatory mediators at the vessel wall which may provide an indirect mechanism by which phytoestrogens may exert their effects on coagulation gene expression.

Some experimental evidence obtained in animal models suggests that phytoestrogens may act as oestrogen agonists under oestrogen-depleted circumstances but exert antagonist activity under circumstances of oestrogen surplus (396-397). Thus, the effect of isoflavone phytoestrogens may depend on the oestrogenicity of the surrounding milieu the expression of ER subtypes, in addition to the dose of phytoestrogens used.
6.1 Discussion

There is a strong direct link between menopause and an increase in cardiovascular disease, which is the leading cause of death in women over the age of 50. A number of observational studies have provided evidence that HT reduces the risk of cardiovascular disease (8-9). However, randomised trials of women with pre-existing disease reported that HT in post menopausal women did not prevent recurrent myocardial infarction (11) and HT caused an increase in coronary events (12) and venous thromboembolism (398). In agreement with this the Women’s Health Initiative, a large primary prevention study, reported that HT afforded no protection against cardiovascular disease-associated conditions in women taking HT with the possible exception of young women who commenced therapy soon after the menopause (14, 399)

This study shows that herbal alternatives to HT known as phytoestrogens have the ability to stimulate changes in haemostatic genes and proteins at physiological concentrations and that they can do this via oestrogen receptor and/or non-genomic signalling pathways.

Menopause is the cessation of reproductive fertility resulting from the depletion of ovarian follicles and therefore reduced secretion of ovarian steroid hormone production. This results in a sharp decrease in circulating 17β-estradiol, hormonal cyclicity ceases within the ovary and it secretes primarily androgens in a hypergonadotropic environment. Because 17β-estradiol is assumed to afford protection in premenopausal women against health risks, such as cardiovascular disease, the loss of 17β-estradiol in menopause is thought to contribute to most menopause-associated disorders hence the use of HT.

The mechanism by which the loss of oestrogen at menopause is associated with increased cardiovascular risk is not fully understood but the early increase in events following the initiation of hormone therapy would suggest that thrombotic mechanisms are important.

In researching menopause, a limited amount of mechanistic information can be obtained from studies in middle-aged women. Therefore, the elucidation of underlying cellular and molecular mechanisms that accompany menopause-associated disorders requires the use of appropriate animal or cell models in controlled experimental conditions. This is
particularly true of studies of dietary supplements where obtaining accurate information regarding soy intake is difficult in a human model.

Blood coagulation takes place in an environment of blood cells and vascular wall components, under flow conditions and in a constantly changing milieu, such as local hypoxia, venous stasis and acidosis. Hence, there is no *ex vivo* model, however sophisticated, that is able to mimic this complex situation. Animal models of venous thrombosis may be used for four different reasons; firstly, animal models may be used to study the pathogenesis of venous thrombosis and to establish factors and pathways that play a pivotal role in this pathogenesis. Secondly, experimental models can be applied to be able to accurately determine the *in vivo* biological effect of an intervention. Hence, the *in vivo* antithrombotic efficacy or pro-thrombotic effect of compounds with *in vitro* anticoagulant or procoagulant properties, respectively, may be confirmed. Indeed, not every agent that is able to block coagulation *in vitro* will have antithrombotic properties *in vivo*, hence this confirmation may be essential. Thirdly, animal models of venous thrombosis are used to facilitate dose-finding of novel antithrombotic agents. Lastly, animal models are often applied for comparative pharmacology, i.e. to predict a superior clinical antithrombotic efficacy of one agent over the other.

In this research, an animal model of the menopause was used to study the modification of haemostatic genes and proteins by phytoestrogens as an alternative to HT. This was compared to the *in vitro* results from human cell lines. *In vitro* systems are, however, by definition, nonphysiological and have important limitations. Living creatures are biologically complex and this is especially true in higher order animals including man. While data from experiments carried out in *in vitro* systems can establish mechanisms and define toxicities, *in vivo* biological systems using live animals are necessary to study how such mechanisms behave under clinical or pathophysiological conditions.

Phytoestrogens are structurally and functionally similar to 17β-estradiol and are capable of producing oestrogenic effects. In order to better understand the molecular mechanism of phytoestrogens regarding thrombotic risk some of the most common constituents of soy were studied; genistein, daidzein and its metabolite equol with estradiol and soy free/vehicle controls to determine if these phytoestrogens had an effect on haemostasis. These effects would then indicate the possible thrombotic effects of these compounds compared with oestrogens.
The liver plays an important role in mediating adverse prothrombotic effects of hormone preparations. On examining the liver from the ovariectomised rat model the phytoestrogens genistein and equol had the ability to regulate transcription of haemostasis genes compared to a soy-free control group, and in some cases the effects were more pronounced than seen with estradiol treatment. In particular genistein was found to be the most potent phytoestrogen in this tissue with dose dependent increases in mRNA and protein levels in some of the main markers of coagulation and fibrinolysis, for example; factor VII, fibrinogen, prothrombin, tPA and PAI-1. Genistein also had a marked effect on the acute phase protein CRP. The doses of this phytoestrogen were chosen to represent both normal Asian diets and herbal supplements, they increase liver-derived coagulation serine proteases that are involved in thrombin generation. If these effects were translated into increased levels in plasma then this would indicate an increased thrombotic risk similar to that found with estradiol.

These results appear to conflict with the data on human plasma levels of haemostatic activation which indicate that isoflavones including genistein have no effect on haemostasis (250) or may even decrease the level of some markers of haemostatic activation (249, 253). Most of these studies are conflicting which may be due to multiple factors such as the soy compound used or individual phytoestrogen used, the dose, the time and duration of exposure, metabolism, route of administration, bioavailability and the intrinsic oestrogenic status. In addition the inherent difficulties involved in controlling soy intake in human control groups have meant that in some studies the placebo group showed changes not dissimilar to those found in the study group (251, 400). The small amount of data and the questionable statistical power of some of the placebo based studies compared to the data available for conventional HT means that the effects of phytoestrogens on coagulation and fibrinolytic activation in plasma is far from clear.

Coagulation activation is dependent not only on the production of serine proteases from the liver but also on the expression and activation of activators and inhibitors of coagulation (e.g. TFPI) from the vascular endothelium. In addition endothelial damage and inflammatory cytokines will all influence the level of haemostatic activation detected in plasma. Although no data is presented in this study on the plasma levels of haemostatic activation of this rat model, genistein treatment was compared not only to soy free controls but to the effects of rats treated with estradiol which act as a positive
control since estradiol is known to be associated with haemostatic activation and increased thrombotic risk.

The expression of oestrogen receptors in the liver tissue was also examined. The effects of phytoestrogens, particularly genistein are thought to be mediated by ER$\beta$. It was found in this study that the effects shown in the genes studied may be regulated by ER$\alpha$ and/or other regulatory pathways but which does not include ER$\beta$. In examining the role of oestrogen signalling in modulating the expression of these haemostatic markers in vitro it was shown in human hepatocytes, that ER$\alpha$ has a role in regulating genes such as prothrombin, antithrombin, PAI-1 and tPA.

Of note the expression of GPR30, a recently described oestrogen receptor was increased in response to all three phytoestrogens but not in response to estradiol. This receptor may be involved in some of the effects observed in ER$\alpha$ negative cells.

In addition to the classical ER signalling pathway, the ER can also undergo 'crosstalk' with growth factor and G-protein-coupled signalling pathways (401-402). For example, oestrogen can activate membrane-bound ER and, via G-protein activation can then activate growth factor receptors such as the EGF receptor and human epidermal growth factor receptor 2 (403-404).

The ER itself may be activated in a ligand-independent manner by other signalling molecules such as growth factors and protein kinases that control the phosphorylation state of the ER complex and play a part in regulating activity of the ER (405). Some effects that have been suggested in this thesis to be mediated by ER$\alpha$ may be due to a combination of ER/GPR30 signalling as phytoestrogens are known to have a low binding affinity for ER$\alpha$. It is possible that the effects seen in this study in HepG2 cells are mediated via GPR30 but are exacerbated by the ER$\alpha$ in the Hep89 cells.

Genistein in addition to its oestrogen-like activities can also activate numerous pathways within the cell. At higher concentrations genistein acts as a tyrosine kinase inhibitor (406). Both genistein and equol at lower concentrations can act via the MAP kinase pathway, indeed studies in MCF-7 cells have shown that in genistein and equol treated cells, ERK-2 activation is required for ER$\alpha$ transactivation (407).
The changes seen between the rat studies and cell culture studies may be due to the fact that coagulation is regulated in a tissue-specific manner and is also possibly species specific. In addition, the effects seen in the cell culture model are those due to the direct effects of each phytoestrogen. The *in vivo* effects are more likely to be a combination of direct effect superimposed with the indirect effects of phytoestrogens e.g. effects on inflammatory mediators and the expression and activation of other transcription factors and coregulators.

Some of these effects may also be due to differences in the expression of the classic oestrogen receptors. Opposing regulation of the PAI-1 promoter by ER\(\alpha\) and ER\(\beta\) has been reported in bovine aortic endothelial cells (351). Similarly the results presented in this thesis show that equol induces an upregulation of PAI-1 mRNA in Hep89 cells expressing ER\(\alpha\) but a downregulation in HUAECs expressing ER\(\beta\), this effect was not seen for estradiol. Different residues of the ER hormone-binding domain are involved in the recognition of structurally distinct oestrogens and anti-oestrogens and these results highlight the exquisite precision of the regulation of ER activities by ligands, with small changes in ligand structure resulting in major changes in receptor character (405).

An alteration in endothelial function can contribute to a loss of the tightly regulated haemostatic balance and lead to various pathological states. This study examined the effects of the phytoestrogens in the endothelium of an ovariectomised rat model and in human arterial endothelial cells. The endothelium is the most important tissue for maintaining haemostasis, whereas the liver has showed the effects at the site of synthesis of these markers, changes in the endothelial expression alters the regulation of thrombin formation which may increase the risk of arterial thrombosis. This study showed that phytoestrogens are tissue specific in their regulation of markers of haemostasis. Each marker analysed in the rat endothelium was upregulated by both genistein and equol, in the case of proteins which are involved in the inhibition of coagulation e.g. the APC pathway, this could be considered an overall beneficial effect in protecting against thrombosis. Increases in the expression of endothelium specific genes such as thrombomodulin, thrombin receptor and EPCR may modulate APC activity. Human arterial endothelial cells were chosen as a cell culture model to study the effects observed in the rat endothelium. A key finding of these studies was the downregulation of EPCR by equol and genistein which could be important in modulating APC activity particularly in factor V Leiden positive individuals. These cells only display the beta isoform of the
The pharmacology of various oestrogens is intriguing. While many compounds are able to bind to the oestrogen receptor, they can differ markedly in their stimulatory and inhibitory effects. In addition, SERMs can demonstrate remarkable differences in efficacy in the various tissues in which oestrogens act, functioning as agonists in some tissues but as antagonists in others. The action of a particular oestrogen depends on the structure of the ligand, the receptor subtype it binds to and the regulation of this binding by coregulator proteins. The most critical components include the gene-regulatory DNA site to which the receptor either directly or indirectly binds to, as well as an array of coregulator proteins that determine the magnitude of the transcriptional response and its sensitivity to hormonal regulation. The hormone-receptor complex then recruits these coregulators, thereby linking the complex physically and/or functionally to the basal transcription complex and affecting the local chromatin structure (408). As the ER isoforms have differences in their DBD and LBD regions it would be expected that ligands would have different levels of potency or efficacy which would allow differences in transcriptional regulation, particularly in different tissue types.

To relate these thrombotic changes to risk in post menopausal is difficult but the systems used in this study were the nearest models to study these markers and chosen doses of our treatments to reflect physiological ranges. Isoflavones have previously shown significant improvements in endothelium-dependent vasodilation. They were shown to lower plasma lipids and stimulate the activity of endothelial nitric oxide synthase, thus inducing vasodilatation.

Although measurement of biomarkers of thrombosis can provide information on the risk profile of an individual, thrombosis whether arterial or venous is primarily a local event. In addition to the level of coagulation activation, this event is influenced by numerous factors including vasodilatation, endothelial activation and inflammation. Phytoestrogens are known to influence all of these processes. In addition the well described cross talk between coagulation activation and immune mediators would indicate that initial stimulus by oestrogenic compounds may be greatly amplified through feedback pathways (409). A recent study in a macrophage cell line showed that genistein and equol can
regulate transcription of a wide variety of genes including of many key inflammatory mediators (410). The effects of phytoestrogen on the determinants are likely to be multifaceted and complex and not solely dependent on their oestrogen like properties.

Figure 6.1: Schematic representation of activation of coagulation and inflammation on rupture of atherosclerotic plaque. Coagulation pathways are indicated by straight arrows; inflammatory mechanisms by dashed arrows (409).

Unfortunately, no large multi-center randomized clinical trials examining clinical end points of cardiovascular health are available for phytoestrogens and it is not currently appropriate to recommend phytoestrogen supplements or diets as a primary or secondary cardiovascular disease preventive intervention in post menopausal women. Oestrogens and phytoestrogens may have different effects on different patient subgroups e.g. those with insulin resistance syndrome, those with a polymorphism or altered lipid metabolism. On their own these risk factors may have limited effect, but together can interact with each other to produce the disease phenotype e.g. the PAI-1 4G allele is transcriptionally regulated by triglycerides which are known to be elevated in HT users (123).

From these results it can be concluded that the phytoestrogens, genistein, equol and daidzein at both physiological and supraphysiological doses can exert an oestrogenic stimulus on haemostatic markers in the liver and endothelium which may be of importance among post menopausal women. This may be of particular importance in the case of women with predisposing cardiovascular risk such as diabetes, obesity, thrombophilia or a previous cardiovascular event.
6.2 Recommendations for future research

In this study, the effect of phytoestrogens on haemostatic gene and protein expression in an animal model was investigated. The detection of plasma levels of these proteins in a similar animal model would allow the correlation of gene expression changes with plasma levels and give direct information on the likely significance of these findings with respect to thrombotic risk.

Similarly animal models of thrombophilia (e.g. factor V Leiden mice) could be used to investigate the possible synergistic effects between the mutation and the phytoestrogen induced changes in haemostasis.

Previous studies have shown the competitive binding of oestrogen-like substances for oestrogen receptors. This study shows the effects that the phytoestrogens, genistein, equol and daidzein have on regulating coagulation and fibrinolysis gene expression. It may be of interest to see the effects of genistein and oestrogen in unison to investigate if genistein can compete with the natural oestrogen estradiol and what this may mean at a physiological level.

The individual contribution of the each of the receptors to the effects seen in this study could be further investigated using specific SERMs for such as G1 for GPR30, propylpyrazole triol (PPT) and diarylpropionitrile (DPN) that exhibit relative specificity for oestrogen receptor alpha and beta, respectively. siRNA techniques could also be used in order to silence individual genes involved in cell signalling pathways which could regulate coagulation gene expression.

Macrophages are the predominant cells involved in the progression of atherosclerosis by the formation of foam cells. Monocytes and macrophages constitute a major source of blood tissue factor, a key element of the coagulation cascade. Additionally, these cells link inflammation and the procoagulant state observed in various prothrombotic conditions. The development of atherosclerotic lesions in human arteries can be regarded as a modified form of chronic inflammation. Macrophages formed from differentiated blood monocytes in culture provide a system for investigating the regulation of the procoagulant activity of these cells following stimulation with estradiol and
phytoestrogens. Investigating gene regulation following oestrogen and phytoestrogen stimulation of these cells may further add to the understanding of the effects of oestrogen-like compounds on these cells and the possible link with thrombosis.

It would be of interest to also examine the difference in the route of administration of oestrogens in hormone therapy. Some studies have shown that transdermal therapy has a favourable profile on haemostatic risk factors in comparison to the oral route suggesting that the first pass liver effect is important. In addition to the data presented in this thesis, tissues from the animal model treated in a subcutaneous manner with the phytoestrogens mentioned are available. It would be of interest to investigate gene transcription in these tissues and compare them to the oral groups presented here. This would indicate whether the differences in haemostatic activation between oral and transdermal therapy occur at the level of transcription.

High content screening was used in this study for protein analysis on the cell model. This technique is a very powerful tool for screening a variety of cell features and would be useful in determining protein content in strips of aorta tissue from the animal model for comparison with the endothelial cell analysis. This tissue could be processed using immunohistochemistry and the fast high resolution imaging of the GE INcell analyser by using a dedicated slide module for imaging tissue samples.

The ultimate tool to determine the effect of any therapy on the risk of a particular disease is the randomised trial. Placebo based, adequately powered randomised trials of phytoestrogen supplements on human postmenopausal women, similar to those undertaken for hormone therapy, would provide the definitive answer regarding the effects of phytoestrogens on cardiovascular disease risk in post menopausal women.
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Appendix
Appendix I

ssniff® Complete feeds with low phytoestrogen content for rats and mice / nude rats and nude mice*

Complete diets for all development and life stages

ssniff Spezialdiäten GmbH has produced various low-phytoestrogen diets (free from soya or/and alfalfa) for rats and mice as standard feeds for years. Nevertheless, these special diets with considerably reduced phytoestrogen content should be reserved for certain scientific questions dealing with this subject directly or indirectly (i.e. endocrinological studies on the effect of phytoestrogens, estrogens, bone metabolism etc.). However, it can be not recommended using these low-phytoestrogen diets as standard feedingstuffs for breeding and maintaining rats and mice and for most „conventional“ research projects for the following reasons:

□ Since the introduction of laboratory animal diets, phytoestrogens have been contained in these feed mixtures and also occur in the natural feed of rats and mice. Consequently if there were to be a change from „conventional“ laboratory animal feed to low-phytoestrogen diets, the reproducibility of experimental data would have to be queried (see historical data/toxicology).

□ Phytoestrogens evidently develop a large number of positive effects, at least in humans, (see inter alia the review paper by Stetchell and Cassidy 1999). That is why soya products are increasingly used in human food. Although some of the effects observed ex vivo and also in vivo require verification by systematic, broad-based examinations, it can still be derived despite this that phytoestrogens might also be able to contribute to reducing chronically progressive diseases in laboratory animals too.

□ In an extensive study various laboratory animal diets were examined for their influence on the sexual development of rats (Odum et al. 2001). According to this study similar effects were ascertained for instance on the uterus with AIN76A, that contains no phytoestrogens, and Purina 5001, with a very high phytoestrogen concentration (about 180 µg genistein and 150 µg daidzein/g feed). The authors come to the conclusion that other (unknown) substances in the diets may be responsible for the findings surveyed.
This study shows that the endocrinological processes and reactions in the organism are very complex and partly not predictable.

Moreover, in a recently published study with rats it could be demonstrated, that diets free from phytoestrogens evidently induced a dramatic impairment of the breeding performance and affected the health status of the offspring (Souzeau et al. 2005).

ssniff® complete feeds low in phytoestrogens are designed for rats/mice maintenance, mice breeding, rat breeding, as well as for nude mice and rats (breeding/maintenance). They are generally intended for the ad libitum intake, however, from case to case it may be necessary to restrict the daily feed consumption, e.g. under certain study conditions or in animals susceptible to get obese.

Moreover, further diets with reduced phytoestrogen content - also with increased energy density - are available for the breeding of rats and mice.*

**Phytoestrogen reduced diets for breeding and maintenance of rats/mice and nude rats/nude mice** *

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<td>Product</td>
<td>R/M-H</td>
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| Crude protein, CP (%) | 19.3 |
| Crude fat, CL (%)    | 3.3  |
| Cude fibre, CF (%)   | 4.4  |
| ME (MJ/kg)           | 12.8 |
| CP/ME (g/MJ)         | 15.1 |

* Further special diets on request

Status: February 2006
ssniff® R/M-H Low-Phytoestrogen

Complete feed for rats and mice - maintenance, soy-free

Description

This diet was developed for the maintenance of rats and mice. The feed contains no soybean and alfalfa product and is therefore characterized by very low concentrations of the phytoestrogens genistein, daidzein and coumestrol. Accordingly, that type of diet is particularly suitable for studies, in which the effects of estrogens and similar substances or other sex hormones should be investigated.

Feed composition

*descending order of feedingstuffs (FMV)*

Grain and grain by-products, tuber products, minerals, vegetable oils, vitamins, trace elements.

**Crude Nutrients (%)**

Dry matter 88.0
Crude protein (N x 6.25) 19.3
Crude fat 3.3
Crude fibre 4.4
Crude ash 6.0
N free extracts 55.1
Starch 36.1
Sugar 3.1

**Minerals (%)**

Calcium 1.00
Phosphorus 0.70
Sodium 0.20
Magnesium 0.21
Potassium 0.68
**Fatty acids (%)**

C12:0 ——
C14:0 0.01
C16:0 0.39
C16:1 0.01
C18:0 0.06
C18:1 0.51
C18:2 1.49
C18:3 0.19
C20:0 0.01
C20:1 0.02
C20:5 ——
C22:6 ——

**Energy (MJ/kg)**

Gross Energy (GE) 16.5
Metabolizable Energy (ME) * 12.8

**Amino acids (%)**

Lysine 1.01
Methionine 0.38
Met+Cys 0.75
Threonine 0.82
Tryptophan 0.25
Arginine 1.01
Histidine 0.46
Valine 1.08
Isoleucine 0.88
Leucine 1.71
Phenylalanine 1.05
Phe+Tyr 1.88
Glycine 0.93
Glutamic acid 3.77
Aspartic acid 1.81
Proline 1.43
Alanine 1.09
Serine 1.01

_Vitamins per kg_
Vitamin A 15,000 IU
Vitamin D3 1,000 IU
Vitamin E 110 mg
Vitamin K (as menadione) 5mg
Thiamin (B1) 18 mg
Riboflavin (B2) 22 mg
Pyridoxine (B6) 19 mg
Cobalamin (B12) 100 µg
Nicotinic acid 120 mg
Pantothenic acid 39 mg
Folic acid 6 mg
Biotin 460 µg
Choline-Chloride 2,530 mg
Inositol 100 mg

_Trace elements per kg_
Iron 168 mg
Manganese 80 mg
Zinc 101 mg
Copper 14 mg
Iodine 2.1 mg
Selenium 0.4 mg
Cobalt 2.1 mg

57 % from Carbohydrates
34 % from Protein
9 % from Fat

*ME calculated according to the pig formula, Annex 4 of the German feed regulation*
Main products
V1550-0 Meal, single ground
V1554-0 10 mm pellets
V1554-3 10 mm pellets, autoclavable
*(vitamin fortified: not shown)*

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