COT Report – Phytoestrogens and Health (2003)

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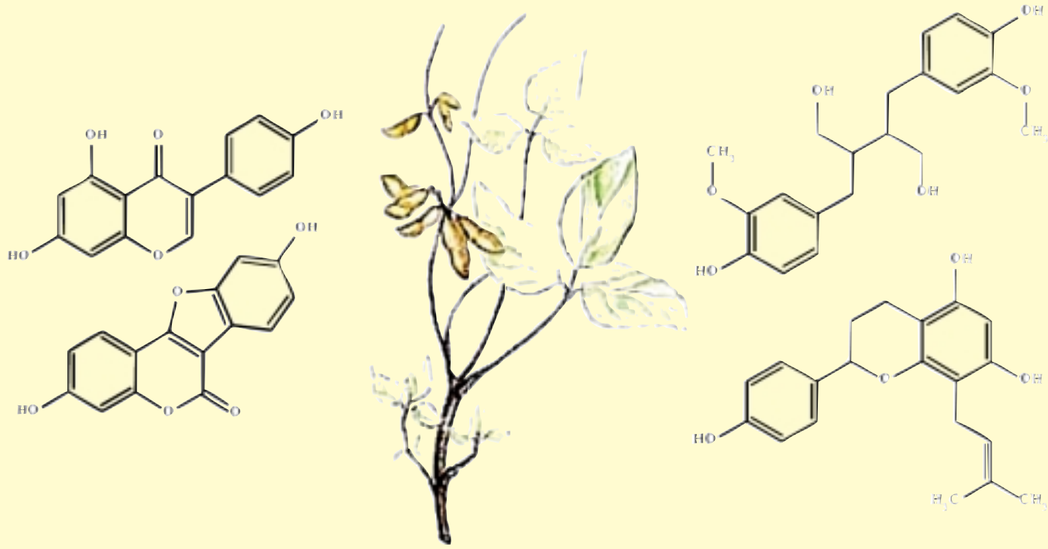
Committee on
TOXICITY

Committee on Toxicity of Chemicals in Food,
Consumer Products and the Environment

Phytoestrogens and Health



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Committee on
TOXICITY

Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment

Phytoestrogens and Health

Chairman
Professor I Hughes

Chairman of the Working Group on Phytoestrogens and Health:

Professor HF Woods

Contents

1. Executive summary

1.1 This report of the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) considers the public health implications of exposure to phytoestrogens in the diet. The report was drafted by a specially convened Working Group of the Committee with the following terms of reference:

"To advise on the health implications of dietary phytoestrogens through review of published scientific research and the Food Standards Agency's phytoestrogen research programme."

1.2 In order to address these terms of reference, the Working Group considered the following key points:

- On the basis of current evidence, does ingestion of soy-based infant formula pose any risk for human infants?
- Are there health implications for other sub-groups of the population from the ingestion of dietary phytoestrogens?
- To consider the evidence for beneficial effects of dietary phytoestrogens.
- To make recommendations for further research.

Structure of the report

1.3 The Working Group held a total of five meetings between April 2000 and March 2001. The Working Group considered peer reviewed published reports published up to 30 April 2002[1]. Preliminary research reported in the form of meeting abstracts was not included unless the work was published in full, in a peer reviewed scientific journal by 30 April 2002. The Working Group *acknowledged* that anecdotal reports and reviews had been published expressing opinions on the health implications of phytoestrogens. The Group *considered* these reports but *concluded* that such information could not be considered as objective or definitive evidence of adverse or beneficial effects of phytoestrogens. Therefore, the Group, where appropriate, *noted* such opinions but agreed it would not base any conclusions on the health implications of dietary phytoestrogens from this evidence.

1.4 A draft report was prepared by the Secretariat and Members of the Working Group and the report discussed at a series of four drafting group meetings from March 2001 to May 2002. The draft report was submitted to the COT for consideration in two parts at meetings held in February and July 2002. Some modifications were made in light of Members' comments.

1.5 The report was issued for public consultation from October to December 2002. In addition, the Scientific Advisory Committee on Nutrition considered the evidence and conclusions presented in the report relating to soy-based infant formula and provided an opinion.

[1] Unpublished data from the FSA phytoestrogen research programme as well as studies received *in press*, which were considered by the Working Group prior to April 2002 and subsequently published have been included in the report.

1.6 The Working Group considered the consultation submissions in January 2003 and further modifications were made to the report. Additional studies published after the literature cut-off date of 30 April 2002 were identified in some submissions. These were considered by the Working Group and included in the redrafted report only if they substantially altered key issues or conclusions. The draft report was submitted to the COT for consideration and endorsement at a meeting held in February 2003. Details of the background to the Working Group and its methods of working are given in **Chapter 2** of the report.

1.7 For the purposes of this report, the Working Group agreed a definition of a phytoestrogen as: *any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous oestrogens usually by binding to oestrogen receptors*. **Chapter 3** describes the chemical forms of the major phytoestrogens: the isoflavones, coumestans, lignans, and the prenylated flavonoids. The analytical methods used to identify and measure phytoestrogens in foods and biological matrices are also discussed in this chapter. In **Chapter 4**, the sources and concentrations of phytoestrogens in food are described and assessments of dietary phytoestrogen exposure in adults and infants are considered.

1.8 **Chapter 5** reviews the absorption, distribution, metabolism and excretion (ADME) of phytoestrogens. The role of the gut microflora in the metabolism and bioavailability of phytoestrogens is discussed. Consideration is given to the transfer of phytoestrogens across the placenta from the mother to the fetus. Transfer of phytoestrogens to infants via breast milk is also discussed.

1.9 Phytoestrogens may cause oestrogenic effects by either interacting directly with oestrogen receptors or indirectly by modulation of endogenous oestrogen concentrations. Phytoestrogens may also induce non-oestrogenic effects. The structure of oestrogen receptors and the way in which oestrogens or phytoestrogens interact with these cellular components to induce biological effects are reviewed in **Chapter 6**. The non-receptor mediated effects of phytoestrogens are reviewed in **Chapter 7**. The experimental methods used to assess the oestrogenic activity of phytoestrogens and how these results may be interpreted are described in **Chapter 8**.

1.10 It has been shown that exposure to potent oestrogens during development can cause long-term adverse effects on human development and fertility, raising concerns that phytoestrogens may cause similar effects. The role of hormones in human sexual development and reproductive function is reviewed in **Chapter 9**. The effects of phytoestrogens on sexual development and reproductive function are also considered.

1.11 Concerns have been raised about potential effects of phytoestrogens on thyroid function. The current evidence and other potential interactions with the thyroid gland are discussed in **Chapter 10**. The effects of phytoestrogens on the central nervous and immune systems are reviewed in **Chapter 11**.

1.12 There is growing interest in the use of phytoestrogens as alternatives to oestrogen replacement therapy for treatment of conditions such as osteoporosis and the menopause. There have also been many reports on the cardioprotective effects of soy. The current evidence for the influence of phytoestrogens on these conditions is discussed in **Chapters 12**, **13** and **14**. Consideration is given also to hormonal effects of phytoestrogens in men and premenopausal women.

1.13 Epidemiological data have indicated that a diet containing large amounts of soy, a rich source of isoflavones, may lower the risk of several types of malignant disease including hormone-dependent cancers. The influence of phytoestrogens on cancer is reviewed in **Chapter 15**.

1.14 The advice and policy in the UK and other countries regarding soy-based infant formula is reviewed in **Chapter 16**.

1.15 The information considered in Chapters 2 to 16 is summarised in **Chapter 17**. **Chapter 18** comprises the Working Group's conclusions on public health issues and recommendations for future research.

Conclusions

1.16 The remit of the Working Group was to review phytoestrogens generally, rather than soy, specifically. However, the Working Group did consider the literature on soy to ensure inclusion of all relevant information.

1.17 Phytoestrogens are biologically active when administered to animals and humans and have been shown to elicit their effects *via* a number of mechanisms:

- Interaction with oestrogen receptors (ER) to modulate the expression of oestrogen-responsive genes[2].
- Inhibition of enzymes involved in oestrogen biosynthesis and metabolism.
- Modulation of thyroid hormone biosynthesis.
- Inhibition of protein kinases and interaction with components of the cell cycle as well as proliferation, differentiation and apoptosis pathways.
- Inhibition of topoisomerase.
- Antioxidant reactions.

[2] Some of the mechanisms outlined hereafter may also be dependent on ER activity.

Evaluation of risks and benefits of dietary phytoestrogens

1.18 Evaluation of the public health implications of phytoestrogens is complex as these compounds can elicit agonist and antagonist actions *via* the oestrogen receptor and non-oestrogenic effects, which are age, tissue and gender dependent. There are also significant inter-species differences in ADME and timing of sexual development making extrapolation of the effects seen in animals to humans complex.

1.19 Many of the reports on the benefits of consuming phytoestrogens are based upon observations in Eastern populations such as the Japanese and Chinese that have traditionally consumed soy. In addition, suggestions that dietary phytoestrogens do not pose significant health risks have been attributed to the lack of reports of adverse effects in these populations. However, it is uncertain whether data from Eastern populations can be extrapolated to Western populations, as there may be differences in how phytoestrogens are handled between such populations.

1.20 Given the level of complexity, the Working Group *considered* it inappropriate to evaluate the public health implications of phytoestrogens to the population as a whole or communicate the implications in a single statement.

1.21 An evaluation of the risks and benefits of dietary phytoestrogens is critically dependent on the nature, timing, conditions and extent of exposure. However, currently detailed intake data for the UK population as a whole, or for specific subgroups of consumers, is very limited.

1.22 *In vitro* studies suggest that at physiological concentrations, interactions with oestrogen receptors are the primary cause of biological effects. *In vivo* studies support this view, as the principal biological effects observed on administration of phytoestrogens are similar to those of oestrogen and can be blocked by ER antagonists. Many experimental studies have used subcutaneous administration, which can significantly influence the biological activity of phytoestrogens. In addition, these studies, for the most part, use high concentrations of phytoestrogens. This makes interpretation difficult, as these experimental conditions are not equivalent to the level of dietary exposure in humans.

1.23 Many studies have used phytoestrogen-containing foods such as soy or flaxseed as a test material and assumed that phytoestrogens are responsible for the biological effects seen. However, it is impossible to exclude the possibility that there are other active components in these foods that could also contribute to the effects observed. Most research in the phytoestrogen field has focused on the isoflavones and thus, comparatively little is known about the prenylated flavonoids, coumestrol and lignans. Current analyses suggest that there are very few sources of prenylated flavonoids or coumestrol in the diet. However, the lignans are relatively common.

Does ingestion of soy-based infant formula pose any risk for human infants?

1.24 In the UK, soy-based infant formulae have been used since the 1960s and are currently fed to approximately 1% of non-breast fed infants aged 4-10 weeks rising to approximately 2% of infants aged

10-14 weeks. However, detailed information on the prevalence of, and reasons for, soy-based infant formula feeding is unavailable.

1.25 The concentration of phytoestrogens found in soy-based infant formulae is several orders of magnitude higher than that found in human breast milk. It has been estimated that intake by infants of isoflavones from soy-based formulae is approximately 4 mg/kg body weight/day. The Working Group *concluded* that infants fed soy-based formulae are the population subgroup exposed to the highest concentrations of isoflavones and that exposure via breast milk is low by comparison. No data on the transfer of lignans from the maternal diet to breast milk have been published.

1.26 There is little published information to suggest that isoflavones affect thyroid function in infants fed soy-based formulae. However, the Working Group *considered* that isoflavones may lower free thyroxine concentrations. Although a normally functioning thyroid may compensate for this, by stimulating thyroxine production, it is possible that infants with congenital hypothyroidism may be unable to increase thyroxine production. These individuals may represent a small susceptible sub-group of the population, therefore the Working Group *recommends* that physicians and other health care workers are made aware of the potential interactions between isoflavones in soy-based infant formulae and thyroid function. The Working Group *advise* that it is appropriate to monitor thyroxine levels in infants with congenital hypothyroidism who are fed soy-based infant formulae in order to establish the susceptibility of this sub-group.

1.27 Few studies have examined the effect of isoflavones on the immune system. Studies in rodents have suggested that isoflavones may alter some parameters of immune function but the effects were inconsistent. However, the Working Group *considered* that investigations of human infants fed soy-based formulae provide reassurance that phytoestrogens in soy do not have a significant impact on the integrity of immune function in such children.

1.28 A recent study conducted in male neonatal marmosets suggests that feeding with soy-based infant formulae can alter some parameters of reproductive health during the neonatal stage. The Working Group *acknowledged* that this work is still in progress, and therefore, no definitive conclusions can be made about likely human health implications. The Working Group *advise* that future findings from this work be evaluated fully once it has been completed.

1.29 Only a single study specifically examining the long-term health effects of soy-based formula feeding on sexual development and fertility in humans has been published. The Working Group *considered* that these data do not provide definite evidence for adverse clinical effects on sexual development or reproductive health, but *noted* the association between soy-based formula feeding and small increases in the duration and discomfort of menstruation. However, the study was based on recall and did not include any direct measurements of hormone levels or other parameters in the subjects. The Working Group *acknowledged* that it was difficult to draw general conclusions from the results of a single study.

1.30 The Working Group *considered* that the findings from these studies do not provide definitive evidence that phytoestrogens present in soy-based infant formulae can adversely affect the health of infants. However, the findings, together with those from studies on the mechanism of action and biological activity of phytoestrogens reviewed in this Report, provide evidence of potential risks. For this reason, the Working Group *expressed* concern about the use of soy-based infant formulae. The Working Group *noted* that the Scientific Advisory Committee on Nutrition (SACN) *expressed* similar concern when considering evidence presented in this Report. SACN also *considered* there to be no substantive medical need for, nor health benefit arising from, the use of soy-based infant formulae. However, it was *noted* that soy-based infant formulae were the only vegan infant formula option available if babies were not exclusively breast fed[3]. In light of the concerns expressed, the Working Group *recommends* that the Department of Health review current advice on the use of soy-based infant formulae.

Are there health implications for other sub-groups of the population?

1.31 The Working Group *considered* that it was of more value to identify and characterise health implications in specific population groups rather than provide an overall evaluation for the general population. At present, there are only limited data on the intake of phytoestrogens by specific population groups in the UK. However, those consuming a vegetarian or vegan diet may ingest larger amounts of soy, an assumption supported by what intake data are available. The Working Group has identified a number of population subgroups that may be expected to have a higher than average intake of phytoestrogens:

- Vegetarians and vegans (isoflavones and lignans).
- Particular ethnic groups e.g. Japanese and Chinese (isoflavones).
- Consumers of soy-based foods (isoflavones).
- Consumers of phytoestrogen-containing dietary supplements (mostly isoflavones).

1.32 The Working Group *noted* the possibility that exposure among these sub-groups will vary due to the large inter-individual differences in metabolism and bioavailability of phytoestrogens and in particular, differences in gut microflora. Specific gut microflora are responsible for the conversion of daidzein to the more potent oestrogen, equol. Thus, equol-producing individuals would be expected to be exposed to a greater oestrogenic potential than non-equol producers.

1.33 Dietary supplements containing phytoestrogens and soy-enriched foods are commercially available and are promoted as having beneficial health effects on human health. Phytoestrogen supplements are marketed as 'natural' alternative treatments for a range of conditions including the menopause, osteoporosis, cardiovascular disease and a number of cancers. Specific marketing for these conditions may lead to increased consumption within certain population sub-groups adding significantly to consumer exposure. However, at present, it is not possible to estimate the impact on consumer

[3] Scientific Advisory Committee on Nutrition response to the COT Working Group on Phytoestrogens draft report on phytoestrogens & health (2003).

exposure, as few data on the phytoestrogen concentrations in or consumption patterns of these products are available.

1.34 Isoflavones and lignans can cross the placenta after metabolism in the mother. However, it is not known how the fetus metabolises these compounds and there are no published human studies examining the potential effects of *in utero* exposure to phytoestrogens therefore, the implications of this exposure are unclear. The Working Group *advise* that further research be conducted to examine the implications of consuming a phytoestrogen-rich diet during pregnancy.

1.35 The Working Group *identified* individuals with hypothyroidism as a subgroup of potential concern. Consumption of phytoestrogen supplements, or a soy-rich diet, may provide sufficient concentrations of phytoestrogens to interfere with thyroxine replacement therapy. Although no adverse effects in hypothyroid children or adults have been reported in the published literature, the Working Group *recognised* that research had not addressed this issue specifically. In view of the increasing availability of phytoestrogen-rich food and supplements in the UK, the Working Group *recommend* that research is conducted to monitor the plasma thyroxine levels of children and adults with hypothyroidism who consume large quantities of dietary phytoestrogens.

1.36 The Working Group also *acknowledged* the theoretical possibility that under circumstances in which the thyroid status of the mother is compromised, maternal exposure to high levels of phytoestrogens may impair normal development of the fetus. The Working Group *recommend* research is carried out to address this issue.

1.37 Despite the suggested benefits of phytoestrogens in lowering the risks of developing breast cancer, studies have shown that soy supplementation of the diet can induce oestrogen-responsive gene products in nipple aspirates in premenopausal women with breast disease. Although breast cell proliferation was not evident in this study, the Working Group *suggested* that until further research is carried out, women with oestrogen-dependent breast disease should be cautious in supplementing their diet with phytoestrogen-rich foods or dietary supplements. However, the Working Group *considered* that the data are insufficient to allow a quantitative recommendation so far as the phytoestrogen intake of this population sub-group is concerned.

1.38 The Working Group *considered* an epidemiological study that suggested an association between high levels of consumption of soy-based foods and decreased cognitive function in a group of Japanese-American men and women. The Working Group *concluded* that this report did not provide sufficient evidence to confirm this association as the report lacked sufficient detail and the associations may have resulted from inaccuracies in the methods employed.

Evidence for beneficial effects of dietary phytoestrogens

1.39 Epidemiological data suggests a soy-rich diet is associated with a reduction in the risk of a number of conditions, including certain hormone-dependent diseases. However, the Working Group *considered*

many of these studies to be of limited value because they do not address specifically the roles of phytoestrogens. Any reported effects from such research therefore cannot be attributed with certainty to phytoestrogens, as other biologically active components may be causally responsible for the effects observed.

1.40 In addition, many of the studies were short-term intervention studies in adults that did not address the possibility that exposure to phytoestrogens at an earlier age may influence the risk of disease later in life. Furthermore, a significant proportion of the research has been conducted in populations such as the Japanese and Chinese and thus, extrapolation of these results to the UK population may be confounded by differences in lifestyle, diet, gut microflora, genetic make-up and ADME.

Menopausal symptoms

1.41 Studies examining the effect of soy-based products or isoflavones to relieve menopausal symptoms are inconclusive. Some studies have suggested that soy may be beneficial, especially if basal intake is low, or the vasomotor symptoms severe, but the data are equivocal, as positive results are often not statistically significant and strong placebo responses are observed.

Osteoporosis

1.42 Clinical data on the effects of phytoestrogens on bone density are limited but results of short-term human studies suggest small protective effects in the lumbar spine. The data for protective effects at other sites are equivocal. However, studies using rodent models of the menopause have consistently demonstrated that soy- or isoflavone-rich diets prevent bone loss. Large, long-term intervention studies are required to evaluate these effects in humans.

Cardiovascular disease

1.43 There is a considerable body of evidence to indicate that consumption of soy can have beneficial effects on low-density lipoproteins and total cholesterol levels. There have been attempts to attribute these effects to the isoflavones in soy. However, purified isoflavones appear not to produce the same beneficial effects, and there is little evidence to suggest that this effect is associated with the isoflavone component of soy. The Working Group *noted* that the US Food and Drug Administration reached similar conclusions when examining this issue. The effects of phytoestrogens on other factors important in the risk of cardiovascular disease such as blood pressure, thrombosis or atherosclerosis has not been extensively investigated.

1.44 There is very little epidemiological data on lignans and cardiovascular disease. Such studies would be extremely difficult to design and conduct due to the prevalence of these compounds in fruit and vegetables.

Cancer

1.45 The Working Group *concluded* that there is some evidence for beneficial effects of phytoestrogens on breast and prostate cancer based upon animal experiments. The findings in humans are less convincing.

This may be due, in part, to the much higher doses used in the animal studies. The interpretation of epidemiological studies is complicated by a number of confounding factors, including differences in lifestyle and diet and is constrained by the paucity of data on dietary intakes of phytoestrogens.

1.46 It has been suggested that exposure to oestrogens or phytoestrogens during development *in utero*, in infancy or in childhood may play an important role in the programming of hormonal homeostasis and influence the risk of developing cancer later in life. This may, in part, explain why the relatively low risk of certain cancers observed among migrant populations from the East (e.g. Chinese and Japanese) increases in subsequent generations. The Working Group *recommends* that in order to establish the clinical efficacy of phytoestrogens in these conditions in humans, long-term studies should be undertaken.

Breast cancer

1.47 Studies examining the effect of isoflavones on breast cancer incidence are inconclusive. Prospective studies have failed to show significant associations between ingestion of soy or isoflavones and breast cancer incidence. Dietary intervention studies, using phytoestrogen supplements, have indicated changes in biomarkers that may be associated with a decreased risk of breast cancer.

Endometrial and ovarian cancer

1.48 A small number of studies have investigated the effects of phytoestrogens in these conditions and to date there is no evidence to support the suggestion that phytoestrogens have protective effects on the incidence of endometrial or ovarian cancer.

Prostate cancer

1.49 Studies investigating the relationship between phytoestrogens and human prostate cancer are too few to draw conclusions and are limited to studies of soy. Experimental studies in rodents show that diets supplemented with soy or isoflavones may inhibit the development of tumours of the prostate.

Colorectal cancer

1.50 Epidemiological data suggests that consumption of non-fermented soy may lower the risk of colorectal cancer. In contrast, fermented soy products are associated with an increased risk of colorectal cancer. No studies have specifically examined the effects of phytoestrogens. Data from rodent studies are conflicting and there is no firm evidence to suggest that phytoestrogens have beneficial effects on the incidence of colon cancer.

Stomach cancer

1.51 The evidence for protective effects of soy or isoflavones on stomach cancer in humans is inconclusive. Studies in Japanese and Chinese populations have shown higher rates of stomach cancer associated with fermented soy products. However, the high salt concentrations in such foods may contribute to this higher incidence in these populations.

Lung cancer

1.52 Epidemiological studies examining associations between phytoestrogen or soy intake and lung cancer in Chinese populations are inconclusive.

Recommendations for future research

1.53 The Working Group *recommends* further research to address important outstanding issues and to aid future risk assessment of dietary phytoestrogens. The Working Group *considered* that future research should be conducted in humans where possible. The following research priorities were identified.

- Detailed exposure studies of discrete populations in the UK who ingest relatively large amounts of phytoestrogens, such as infants, vegetarians/vegans and users of phytoestrogen-rich foods and supplements, would allow a more informed view of the health implications of phytoestrogens. The Working Group *recommends* that research examining the phytoestrogen content of food, as well as intakes of, and systemic exposure to, phytoestrogens is conducted.

- The extent and nature of soy-based infant formula use in the UK is uncertain. The Working Group *recommends* research to address these areas.

- The Working Group *considers* there is a need for further research on the potential effects of phytoestrogens in infants fed soy-based infant formulae. It may be possible to use established cohorts of infants fed soy-based formula to investigate the possible long-term health effects of exposure to phytoestrogens during infancy.

- The Working Group *considers* there is a need to investigate the potential interaction of phytoestrogens with the thyroid gland in subjects with compromised thyroid function.

- The Working Group *recommends* that further research be conducted to establish whether phytoestrogens act mainly by oestrogen receptor-mediated mechanisms or by alternative mechanisms.

- Large long-term prospective studies are necessary to establish the relationship between dietary phytoestrogens and the development of some diseases, specifically osteoporosis, breast cancer and prostate cancer. Shorter intervention studies are required to assess effects on menopausal symptoms and risk markers of diseases, such as osteoporosis and cancers. Such studies should consider an evaluation of the role of metabolites, especially equol, in the biological effects observed.

  The Working Group also identified supplementary areas for future research.

- The health implications of *in utero* exposure to phytoestrogens are unclear. There is a need for research to examine what effects maternal exposure to phytoestrogens may have on the fetus and on the subsequent health status of the child.

- The potential for drug-phytoestrogen interactions has not been established. This is of potential importance for individuals consuming phytoestrogen dietary supplements while taking prescribed drugs with hormonal effects.

- The potential differences in the metabolism of phytoestrogens between Western and Eastern populations has not been determined. Knowledge of the potential differences would aid assessment of epidemiological studies.

2. Introduction

2.1 This report of the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) has been prepared by the Working Group on Phytoestrogens (subsequently referred to as the Working Group). The Working Group was set up by the COT to review the available scientific literature and to determine whether dietary phytoestrogens had any implications on human health.

Historical background

2.2 Phytoestrogens are a group of chemicals produced naturally by a number of edible plants (Mazur & Adlercreutz, 1998). In 1940, adverse effects on fertility were observed in animals that had been grazing on phytoestrogen-rich plants (Shutt, 1976). In the early 1980s, it became clear that phytoestrogens could produce biological effects in humans. As phytoestrogens are present in plant-based foods consumed by humans, notably soy, this encouraged research into the chemical identity, concentrations and biological properties of these compounds (Bingham *et al*, 1998).

2.3 The concentration of phytoestrogens in soy raised concerns that the adverse effects observed in animals could also occur in children who were fed soy-based infant formulae. It has been suggested that at this early stage in life, humans may be more sensitive to the effects of oestrogenic chemicals than adults (Sheehan, 1998).

2.4 To date, however, there have been no reports of adverse effects in human populations that have traditionally consumed soy. In fact, it has been suggested phytoestrogens can have beneficial effects on human health (Cassidy & Faughnan, 2000). For instance, it has been noted that the incidence of many hormone dependent diseases, such as breast and prostate cancer, are lower in Eastern (e.g. Japanese) compared with Western populations (Adlercreutz & Mazur, 1997). It has been postulated that components of the Eastern diet are responsible for these differences and that in particular, the ingestion of phytoestrogen-rich foods is a major contributing factor to the lower incidence of hormone-dependent diseases (Adlercreutz & Mazur, 1997).

2.5 Foods and dietary supplements rich in phytoestrogens are marketed on the basis of their potential health benefits. In 2000, the value of the European market for isoflavones was estimated to be £64 million (€106 million). Dietary supplements containing isoflavones are an expanding market and represent 9% of the phytonutrient market value. Research from market analysts predicts that the combined phytonutrient market will increase to an estimated £98 million (€163 million) by 2008 (Frost & Sullivan, 2002). Exposure through dietary supplementation could increase individual exposure by orders of magnitude, however there is no information on consumption patterns or exposure estimates from this source.

2.6 Following the concerns raised about dietary exposure to phytoestrogens, several independent scientific committees have considered the public health implications of phytoestrogens over the last ten years. In 1992, the Ministry of Agriculture, Fisheries and Food (MAFF) Working Party on Naturally Occurring Toxicants in Food identified that phytoestrogens had potential to cause adverse effects in humans. The

Working Party requested advice from the COT on the health implications of dietary phytoestrogens and in 1992, the COT reviewed the available toxicity data and reached the following conclusions:

- Phytoestrogens were capable of binding, albeit weakly, to human and animal oestrogenic receptors.
- Phytoestrogens may exert anti-oestrogenic effects.
- Because of adverse effects in animals, the concentration of phytoestrogens in soy milk products destined for consumption by infants should be analysed and monitored so that exposure data could be obtained.
- More data were needed to examine further the hypothesis that phytoestrogens are protective against breast cancer.
- Further evidence was required on the effects of phytoestrogens on luteinising and follicle-stimulating hormones and the possible health implications to women.

2.7 The COT reviewed the issue of phytoestrogens again in 1996, following concerns raised by the press and consumer interest groups about the potential adverse effects of soymilk products ingested by infants and young children (COT annual report, 1996).

2.8 At this time, the COT estimated the intake of isoflavones from soy-based infant formulae to be approximately 4 mg/kg bw/day during the first 4 months of life. This concentration was higher than those found to be associated with hormonal effects in premenopausal women. The Committee expressed concern over the potential for adverse effects, whilst acknowledging the lack of reported adverse effects in populations habitually consuming large quantities of soy. The Committee recommended that appropriate research be undertaken as a matter of high priority to determine if ingestion of soy-based infant formulae carries any risk for infants. The COT also noted that future research may be necessary to consider the potential risk of soy products to other sub-groups of the population (COT annual report, 1996).

2.9 The COT endorsed the advice of the Department of Health that breast and cows' milk formulae are the preferred sources of nutrition for infants. However, women who have been advised by their doctor or other health professionals to feed their baby soy-based infant formulae should continue to do so (COT annual report, 1996).

2.10 In the same year, the Food Advisory Committee (FAC) endorsed the COT recommendations for further research and recommended that, as a precautionary measure, manufacturers should investigate ways to reduce the levels of phytoestrogens in soy-based infant formulae (MAFF Food Surveillance Paper 51, 1996). The FAC noted that foods eaten by other groups contain soy and the need for further action would be considered in the light of the outcome of the recommended research. Following the COT and FAC recommendations, MAFF commissioned a large programme of research into the health effects of

dietary phytoestrogens. The phytoestrogen research programme is now supported by the Food Standards Agency (Appendix 8).

2.11 In 1999, the Panel on Child and Maternal Nutrition (COMA annual report, 1999-2000) endorsed the FAC advice that, as a precautionary measure, infant formulae manufacturers should investigate ways to reduce the concentration of phytoestrogens in soy-based infant formulae. However, they noted that the clinical grounds for recommending a soy-based formula to parents were diminishing, as other more suitable hydrolysates based on cows' milk were available.

2.12 In 1999, at the request of the Joint Food Safety and Standards Group of MAFF and the Department of Health, the COT reviewed a pre-publication study, which reported a higher incidence of hypospadias in boys born to women who followed a "vegetarian"[4] diet during pregnancy (North & Golding, 2000). The authors suggested that the data lent support to the possibility of a deleterious effect of phytoestrogens on the developing male reproductive system. The Committee reviewed the data but concluded there was insufficient evidence in the manuscript to support this hypothesis. The COT issued a statement stressing that there was no reason to change the existing advice concerning maternal diet in pregnancy (COT annual report, 1999a).

2.13 In 1999, the COT was also informed of work from the phytoestrogen research programme that showed that soy-based infant formulae could elicit oestrogenic effects in rodents. This study was subsequently published as 'diet and the aetiology of temporal advances in human and rodent sexual development' (Ashby *et al*, 2000). At that time, the COT agreed that a Working Group be set up to conduct a comprehensive review of the health implications of dietary phytoestrogens (COT annual report, 1999b).

Terms of Reference

2.14 The following terms of reference were agreed at the first meeting of the Working Group:

"To advise on the health implications of dietary phytoestrogens through review of published scientific research and the Food Standards Agency's Phytoestrogen Research Programme."

2.15 In order to fulfil this objective, the Working Group decided to address the following key points:

- On the basis of current evidence, does ingestion of soy-based infant formula pose any risk for human infants?
- Are there health implications for other sub-groups of the population from the ingestion of dietary phytoestrogens?

[4] The definition of vegetarian used in this study did not differentiate between vegetarian diets as defined by this report and other diets that included meat.

- To consider the evidence for beneficial effects of dietary phytoestrogens.
- To make recommendations for further research.

Membership of the Working Group

2.16 The membership of the Working Group is given at Appendix 5. The issue is particularly complex and members were appointed with a broad range of expertise.

2.17 Professor Woods, a clinical pharmacologist and chairman of the COT between 1992 to 2002, chaired the Working Group. The Membership included expertise in chemistry, developmental biology, endocrinology, epidemiology, nutrition, paediatrics and toxicology as well as two public interest representatives.

Methods of Working

2.18 The first meeting of the Working Group was held on the 3 April 2000. The Working Group met on five occasions between April 2000 and March 2001. The Working Group was supported by a Secretariat comprised of officials from the Food Standards Agency.

2.19 The Toxicology Unit at the Imperial College School of Medicine in London was contracted by the Food Standards Agency to prepare a number of literature reviews on the biological effects of phytoestrogens. This information was supplemented by additional papers prepared by Working Group Members and by the Working Group Secretariat who reviewed the literature on a regular basis to update Members on significant new research. These papers were used as a basis for discussion at the Working Group meetings and representatives from the Toxicology Unit were invited to present their papers to Members. A list of search terms used to identify relevant literature is given at Appendix 3. A list of individuals who made presentations to the Working Group is given at Appendix 4.

2.20 At the first meeting of the Working Group, Members agreed that only peer-reviewed published reports should be considered for inclusion in the review. Literature searches were conducted and the Working Group agreed that literature published after April 2002 would not be considered[5]. Preliminary research reported as meeting abstracts would not be included in the report if not published in full by 30 April 2002. The Working Group acknowledged that anecdotal reports and reviews expressing opinions on the health implications of phytoestrogens had been published and submitted to the Group as evidence. The Working Group considered such information could not be considered as objective, definitive evidence of adverse or beneficial effects of phytoestrogens. Therefore, the Group agreed, where appropriate, to note such opinions but agreed it would not base any conclusions on the health implications of dietary phytoestrogens from this evidence. Many of the studies considered have used phytoestrogen-containing foods such as processed soy or flaxseed as a test material and assumed that phytoestrogens are

[5] Unpublished data from the FSA phytoestrogen research programme as well as studies received *in press*, which were considered by the Working Group prior to April 2002 and subsequently published have been included in the report.

responsible for the biological effects seen. However, it is impossible to exclude the possibility that there are other active components in such foods that could also contribute to the effects observed.

2.21 The Working Group invited additional information from other parties involved in the phytoestrogen field and placed an advertisement in the British Medical Journal on the 6 May 2000 and on the Food Standards Agency's website. The Working Group received 17 submissions. A list of submissions received is given in Appendix 2.

2.22 The Working Group agreed to a request by the Food Standards Agency that the agendas, discussion papers and the minutes of meetings once cleared by the Chairman would be placed on the Food Standards Agency's website. However, Members agreed that if evidence was submitted 'in confidence' from interested parties and groups, then the Working Group would observe the request for confidentiality.

2.23 Members of the Working Group attended the Food Standards Agency phytoestrogen research workshop held in November 2000 in Cambridge. Eighteen project leaders presented unpublished data from the phytoestrogen research programme. A list of those that made presentations at the workshop is given at Appendix 4. The Working Group included this substantial body of unpublished data from the Food Standards Agency's programme, in their considerations. A list of projects on phytoestrogens, which are funded by the Food Standards Agency, is given at appendix 8. Published research from the phytoestrogen research programme is also included in this report.

2.24 A draft report was prepared by the Secretariat and Members of the Working Group and the report discussed at a series of four drafting group meetings from March 2001 to May 2002. The draft report was submitted to the COT for consideration in two parts at meetings held in February and July 2002. Some modifications were made in light of Members' comments. The Membership of the COT is given at appendix 7.

2.25 The report was issued for public consultation from October to December 2002. The Working Group received comments from 43 individuals, groups or organisations. A list of the submissions received is given in Appendix 2. Comments on the draft report were also sought from an independent expert, Professor Steven Barnes, Professor of Pharmacology and Toxicology, University of Alabama, USA. In addition, the Scientific Advisory Committee on Nutrition considered the evidence and conclusions presented in the report relating to soy-based infant formula and provided an opinion.

2.26 The Working Group considered the submissions in January 2003 and further modifications were made to the report. Additional studies published after the literature cut-off date of 30 April 2002 were identified in some submissions. These were considered by the Working Group and included in the redrafted report only if they substantially altered the key issues or conclusions. The draft report was submitted to the COT for consideration and endorsement at a meeting held in February 2003.

2.27 The conclusions set out in this report represent the opinion of the Working Group on Phytoestrogens and have been endorsed by the COT. The opinions expressed in the report are independent of any other body.

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3. Chemistry and analysis of phytoestrogens

Chemical structures and nomenclature

Introduction

3.1 Some naturally occurring compounds present in plants have been found to possess oestrogenic properties, these chemicals have been termed 'phytoestrogens'. The majority of phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids. Flavonoids are present in many plants and it has been estimated that they can constitute up to 7% of the dry weight of some plants (Kuhnau, 1976). Three classes of flavonoid, the coumestans, prenylated flavonoids and isoflavones, are phytoestrogens that possess the most potent oestrogenic activity. A class of non-flavonoid phytoestrogens, the lignans has also been identified. The relationship between these types of phytoestrogen and the names of the compounds most commonly found in food from these four groups are summarised in Figure 3.1.

3.2 The Working Group has defined a phytoestrogen as: **any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous oestrogens usually by binding to oestrogen receptors**.

3.3 Phytoestrogens possess oestrogenic properties due to their structural similarities to the hormone oestradiol. The structural similarities between members of the four main groups of phytoestrogen identified above and oestradiol are shown in Figure 3.2.

3.4 Much of the work on phytoestrogens has focused on examining the concentration and biological activity of the isoflavones, coumestans, prenylated flavonoids and lignans. Although other phytoestrogens have been identified there are limited data on their biological properties and their concentrations in plants and foods. The Working Group did not consider other sources of dietary oestrogens such as phytosterols or mycoestrogens[6].

[6] Phytosterols were not considered by the Working Group as data on their oestrogenic properties are equivocal (Makela *et al*, 1995; Mellanen *et al*, 1996; Baker *et al*, 1999). The Scientific Committee on Food (SCF) reviewed these studies and considered that the available evidence provided sufficient reassurance of the absence of endocrine effects of phytosterols *via* the oral route of administration (SCF, 2000). Mycoestrogens were not considered as the health implications of these compounds have been evaluated at both international and European levels by the Joint Expert Committee on Food Additives and the SCF, respectively.

Figure 3.1 The relationship between the various groups of phytoestrogens (given in bold) and members of each group.

The compounds in brackets are not inherently present in plants but are oestrogenic products resulting from metabolism of members of that class of phytoestrogen. This cannot be considered as an exclusive list as other phytoestrogens may be identified as constituents of food in the future.

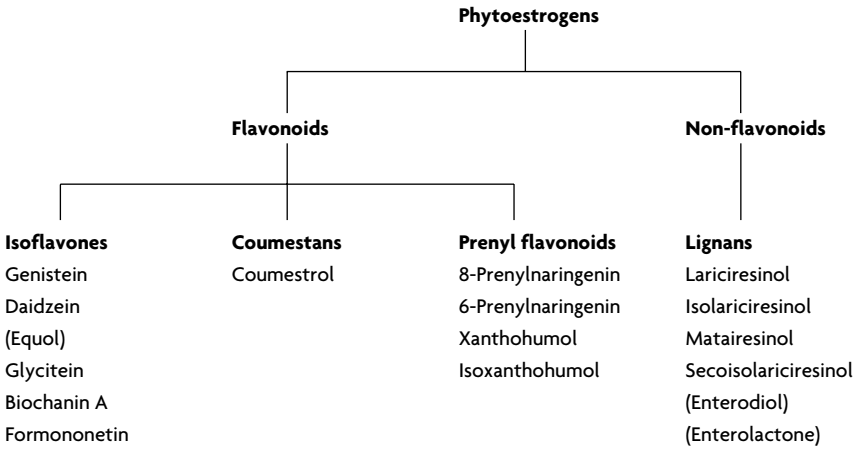
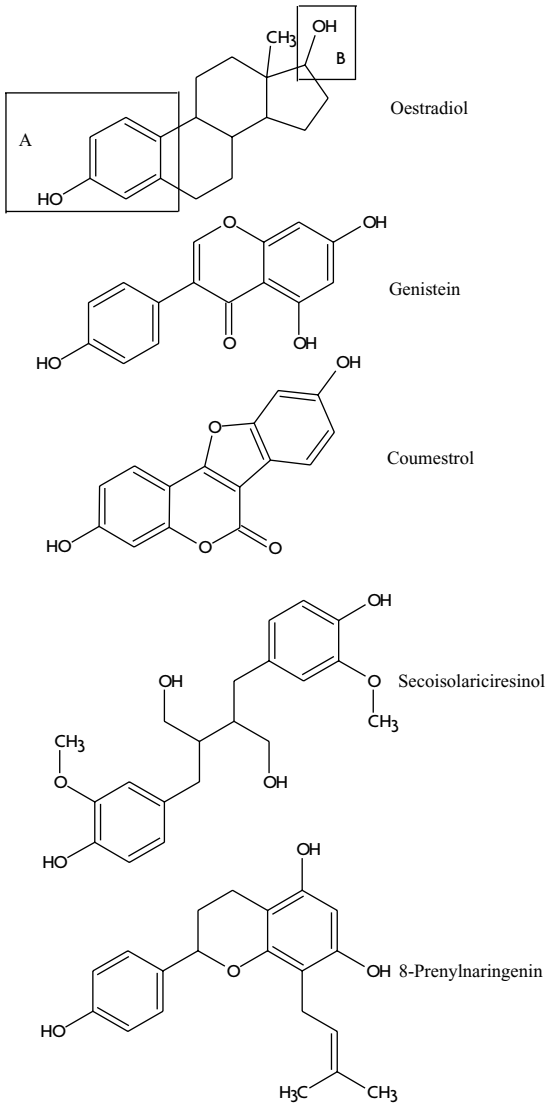


Figure 3.2 The structural similarities of phytoestrogens to oestradiol.
The similarity of the structure of the human hormone, oestradiol and examples from the four classes of phytoestrogen from Figure 3.1. All the structures possess the phenolic (box A) and hydroxyl (box B) moieties outlined on the oestradiol structure and the distances between the two groups in each compound are similar[7].



[7] Isoflavanones, isolated from the roots and bark of some plants and trees (Shirataki *et al*, 1999; Bojase *et al*, 2001), are compounds of similar structure to isoflavones (although they possess an asymmetric carbon and thus the central ring is not flat but buckled). Therefore, they may have oestrogenic properties. However, they have not been identified in food and their oestrogenic properties have not been established.

Isoflavones

3.5 The major isoflavones present in plant-based foods are:

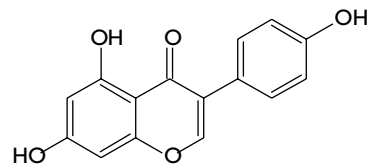
- genistein
- daidzein
- glycitein
- biochanin A
- formononetin

3.6 Their chemical structures are shown in Figure 3.3. Biochanin A and formononetin are derivatives of genistein and daidzein that have an additional methyl (-CH_3) group. Isoflavones are often present as glucoside conjugates (glucones) in plants and foods. In addition, the glucose group is often esterified with an acetyl- or malonyl group to form acetyl- or malonylglucosides (Barnes *et al*, 1994). The terminology used to describe isoflavones is summarised in Table 3.1.

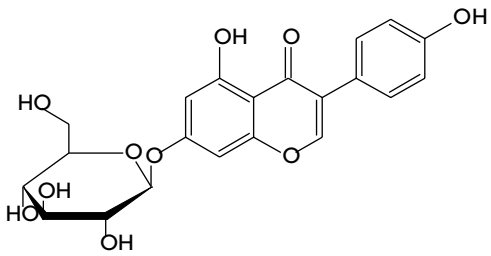
Table 3.1 Isoflavones most commonly found in foodstuffs.

Aglucones (unconjugated to glucose)	Daidzein
	Genistein
	Glycitein
	Formononetin
	Biochanin A
Glucosides (or glucones, conjugated to glucose)	Daidzin
	Genistin
	Glycitin
	Ononin
	Sissotrin
Acetylglucosides (acetylglucones)	Acetyldaidzin
	Acetylgenistin
	Acetylglycitin
Malonylglucosides (malonylglucones)	Malonyldaidzin
	Malonylgenistin
	Malonylglycitin

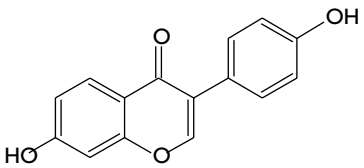
Figure 3.3 Chemical structures and names of the isoflavones, as aglucones, glucones, acetylglucones and malonylglucones, most commonly found in food.



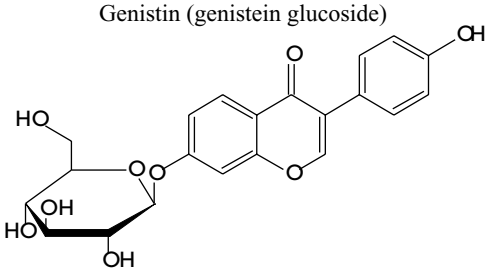
Genistein



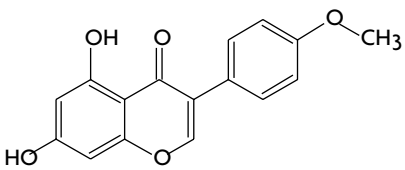
Genistin (genistein glucoside)



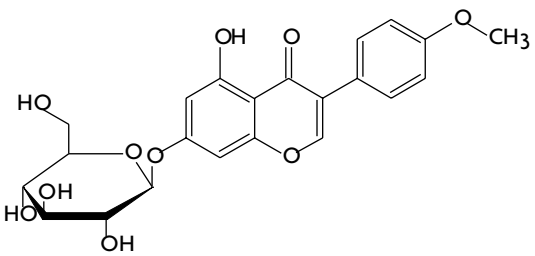
Daidzein



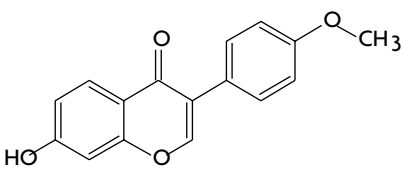
Daidzin (daidzein glucoside)



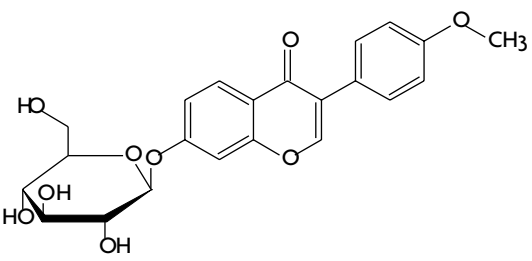
Biochanin A



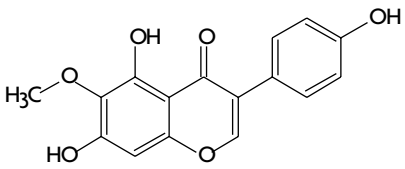
Ononin (biochanin A glucoside)



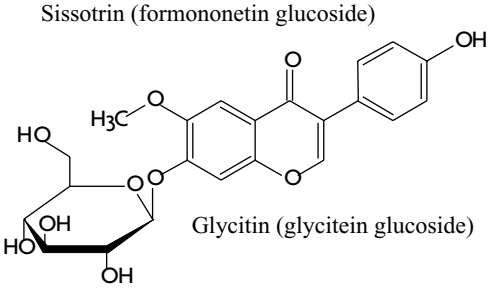
Formononetin



Sissotrin (formononetin glucoside)

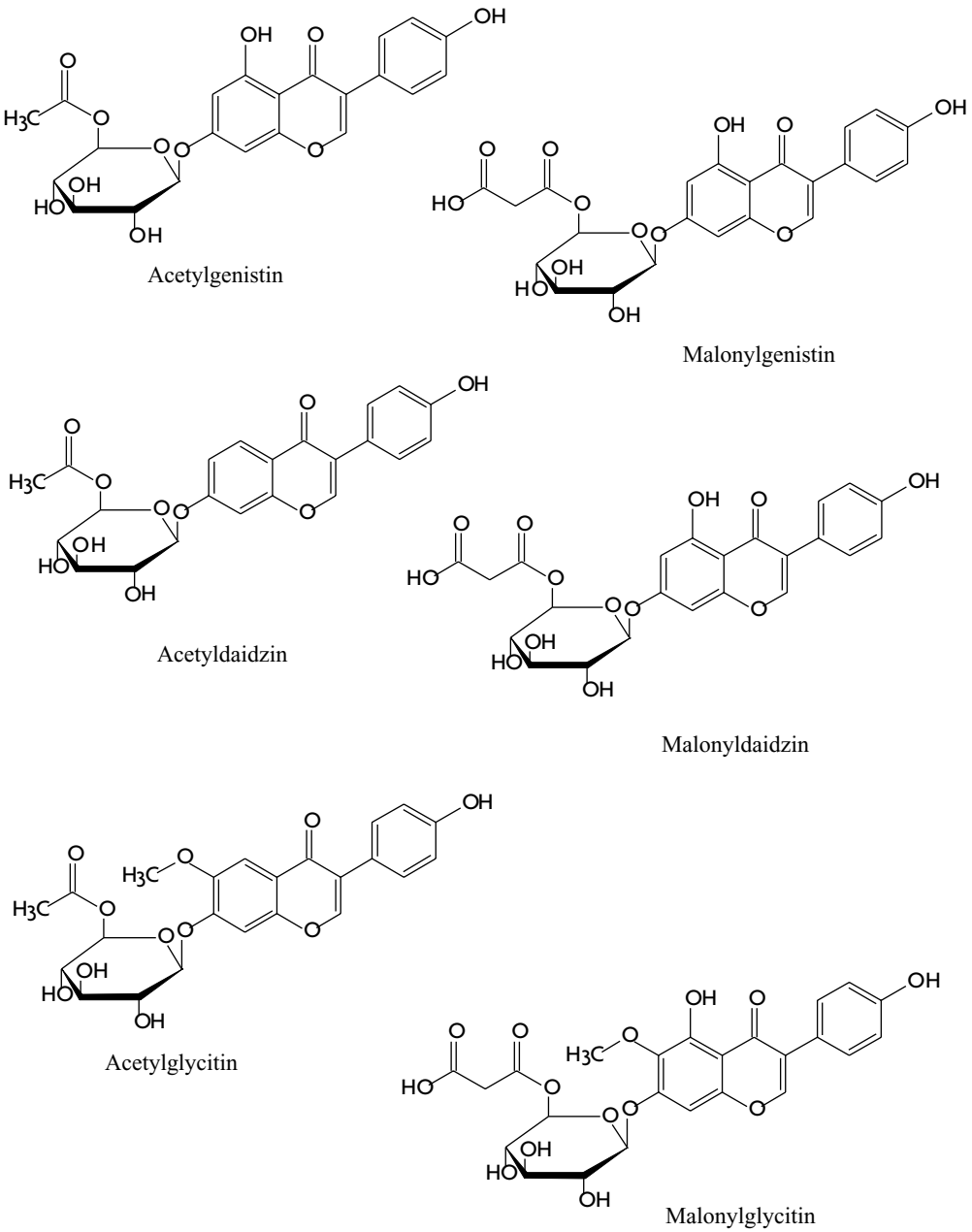


Glycitein



Glycitin (glycitein glucoside)

Figure 3.3 Chemical structures and names of the isoflavones, as aglucones, glucones, acetylglucones and malonylglucones, most commonly found in food. *(cont'd)*



Physical & chemical properties of isoflavones

Water solubility

3.7 Isoflavones are low molecular weight hydrophobic compounds. Conjugation to glucose, glucuronide or sulfate groups increases water solubility. Acetylation or malonylation of glucose conjugates and methylation of the isoflavone moiety will alter water solubility.

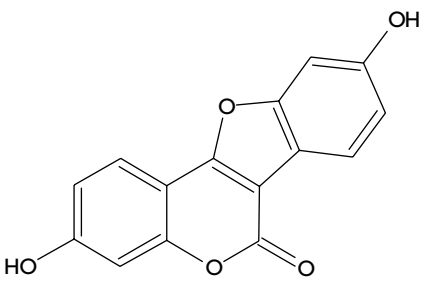
Chemical stability

3.8 Under acidic conditions, the glucones can be deconjugated to give aglucones. Whilst under acidic or basic conditions the acetyl- and malonyl groups can also be removed. In addition, malonyl groups can decarboxylate (lose CO_2) thus yielding acetyl groups. In the body, enzymes in the gut and liver can carry out these reactions during metabolism (see Chapter 5).

Coumestans

3.9 Compared with isoflavones, coumestans have been less well studied. They are structurally similar to isoflavones and possess similar physical and chemical properties (Humfrey, 1998). The structure of coumestrol, the coumestan most commonly found in foods, is shown in Figure 3.4.

Figure 3.4 The chemical structure of coumestrol, which has been identified in some foods.



Coumestrol

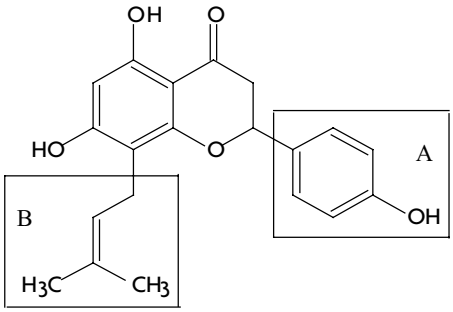
Prenylated flavonoids

3.10 A number of prenylated flavonoids with oestrogenic properties have also been identified (Kitaoka *et al*, 1998; Milligan *et al*, 1999; Miyamoto *et al*, 1998):

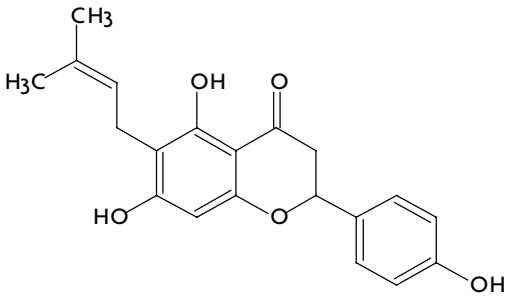
- 8-prenylnaringenin
- 6-prenylnaringenin
- xanthohumol
- isoxanthohumol

3.11 The structures of these compounds are shown in Figure 3.5. These compounds are also structurally similar to the isoflavones but substituted with a prenyl group (B) and the phenol ring (A) is orientated in a different direction. The presence of the prenyl group makes these compounds less water soluble than the isoflavones.

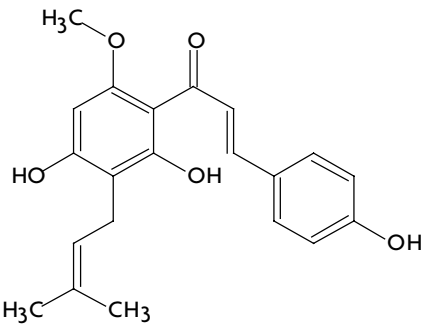
Figure 3.5 The chemical structures and names of the prenylated flavonoids that have been found in food: 8-prenylnaringenin, 6-prenylnaringenin, xanthohumol and isoxanthohumol.
The phenolic ring (box A) of these compounds is set in a different orientation to that found in isoflavones and in addition they possess a prenyl group (box B)

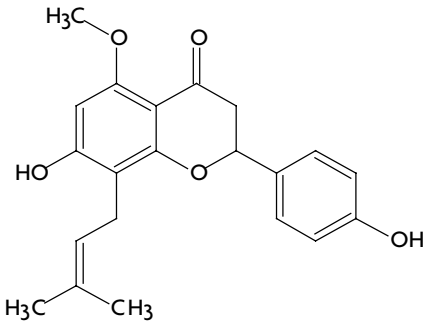


8-Prenylnaringenin



6-Prenylnaringenin





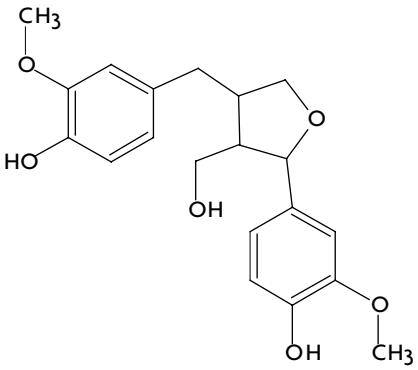
Lignans

3.12 Members of the lignan group of phytoestrogens are defined chemically as possessing the 2,3-substituted di-1,4-benzylbutane structure. The principal lignans identified in food are (Setchell & Adlercreutz, 1988):

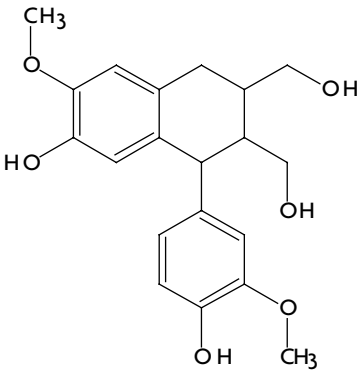
- lariciresinol
- isolariciresinol
- matairesinol
- secoisolariciresinol

3.13 The structures are shown in Figure 3.6. The form in which the lignans occur in foods is unknown but it has been suggested they are present as linked glucosides of differing chain length (Liggins *et al*, 2000). For this reason, isolation of these compounds from plants and foods requires chemical treatment after which they are in the form of aglucones or glucosides (Mazur *et al*, 1996; Liggins *et al*, 2000). Lignans are thought not to be oestrogenic themselves, but are converted to the oestrogenic compounds enterolactone and enterodiol by the gut microflora (Setchell & Adlercreutz, 1988).

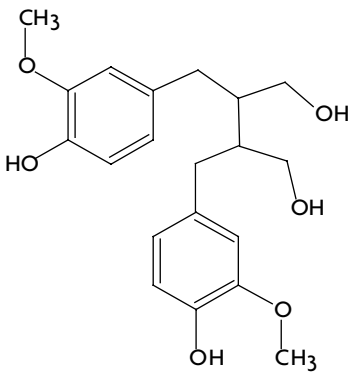
Figure 3.6 Structures and names of the lignans identified in food: lariciresinol, isolariciresinol, secoisolariciresinol and matairesinol and the metabolites enterodiol and enterolactone.



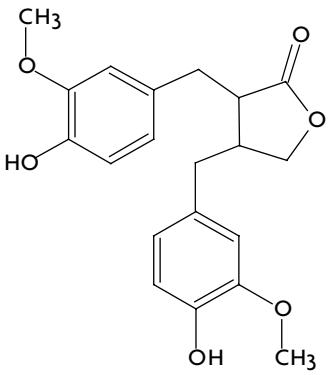
Lariciresinol



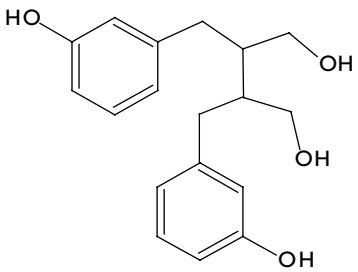
Isolariciresinol



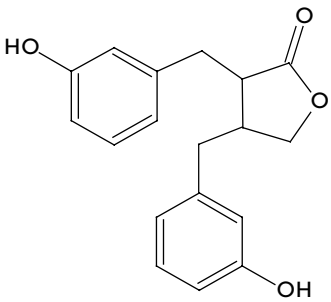
Secoisolariciresinol



Matairesinol



Enterodiol



Enterolactone

Analysis of Phytoestrogens

Introduction

3.14 The isolation and analysis of phytoestrogens and their metabolites is particularly difficult given the similarity of structures and chemical properties, as well as the range of matrices in which they are found. Initially, phytoestrogens were analysed using insensitive techniques such as thin-layer and paper chromatography. However, the development of increasingly sensitive technologies has advanced phytoestrogen analysis considerably. These developments have been important as quantification of phytoestrogens in foodstuffs as well as in pharmacological and toxicological studies are dependent on accurate and precise analytical methodology. As a result, the information available on phytoestrogen concentrations in foodstuffs and biological matrices has increased significantly in recent years and it may be considered that studies conducted more recently have produced results of greater reliability.

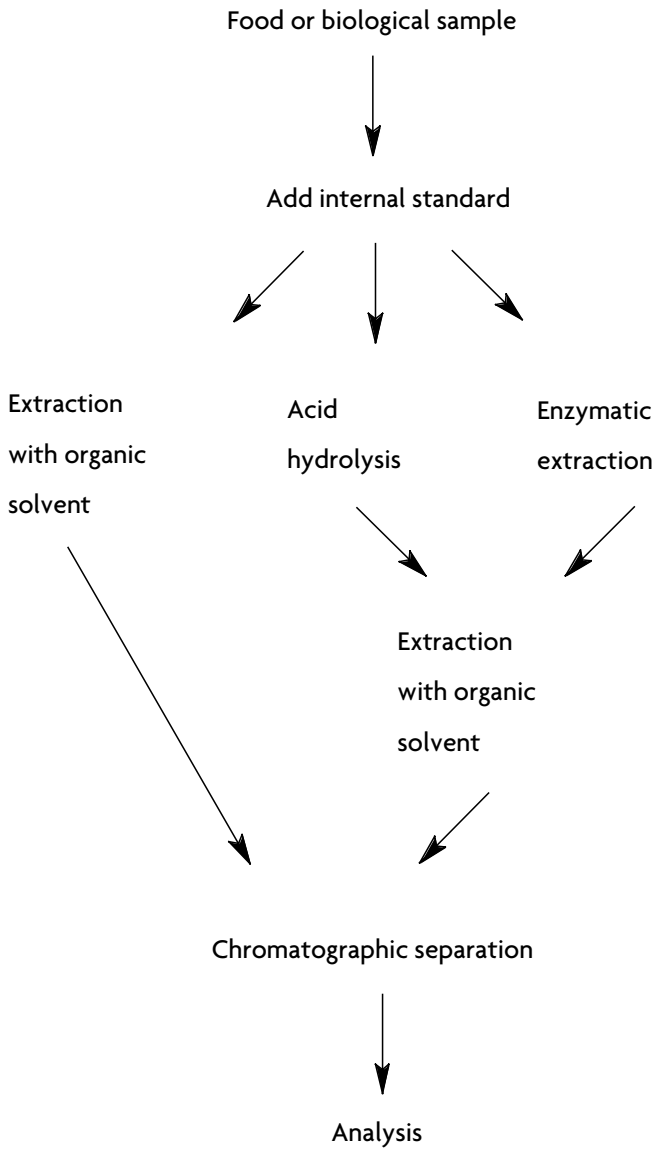
3.15 Prior to analysis by physico-chemical methods, compounds must first be identified as phytoestrogens i.e. as having potential oestrogenic activity. Assays to measure the oestrogenic activity of plant and food extracts have been developed and are discussed in Chapter 8. Physico-chemical methods can then separate and quantify these oestrogenic constituents present in the extract. The principal physico-chemical methods for separation and quantification of phytoestrogens, are described below.

Isolation of phytoestrogens

3.16 Phytoestrogens and their metabolites are generally present in parts per billion to parts per million concentrations in plants, solid and liquid foodstuffs as well as in biological matrices such as plasma, serum, urine and faeces. The concentrations of phytoestrogens present in plants and foods are described in Chapter 4 and concentrations in biological matrices are described in Chapter 5.

3.17 Phytoestrogens must be isolated from the major constituents of these matrices before quantification. The type of matrix, phytoestrogen and the analytical method determine which isolation or extraction procedures are required. Extraction of phytoestrogens is usually not 100% efficient. To correct for losses, internal standards that have similar chemical properties to the analytes are added to the sample in known quantities prior to extraction. The ratio of the internal standard to the analyte is measured during analysis to make this correction. A general scheme of the steps involved in preparing a sample for analysis is shown in Figure 3.7.

Figure 3.7 An outline of the principal steps involved in the extraction and analysis of phytoestrogens.



Analytical methods

3.18 The most widely used techniques for measurement of phytoestrogens are:

- reversed phase high performance liquid chromatography with ultraviolet detection (HPLC-UV)
- gas chromatography with mass spectrometric detection (GC-MS)
- liquid chromatography with mass spectrometric detection (LC-MS)

3.19 The most appropriate analytical method is dependent on the type of biological matrix and compound to be analysed. The analytical methods used in phytoestrogen analysis require reference standards, which are pure samples of the analyte and are used to validate and calibrate the analytical method (Thompson & Wood, 1993). As methods have developed, the limits of detection and quantification have decreased so that phytoestrogens can now be measured in foodstuffs and biological samples down to concentrations of parts per billion.

High performance liquid chromatography with ultraviolet detection (HPLC-UV)

3.20 HPLC-UV is a relatively rapid way of measuring phytoestrogens (Setchell & Welsh 1987; Wang *et al*, 1990; Franke *et al*, 1994; Saloniemi *et al*, 1995; Obermeyer *et al*, 1995; Franke & Custer, 1996; Coward *et al*, 1998; Murphy *et al*, 1999; Zhang *et al*, 1999; Nakamura *et al*, 2000; Griffith & Collison, 2001; Thomas *et al*, 2001). Following isolation of phytoestrogens from the matrix, they are directly separated and quantified. This method allows simultaneous purification and measurement of complex mixtures. UV detection is generally less sensitive than MS detection and the reported detection limits can be variable. The analytes are quantified by comparison with calibration curves derived from reference standards. However, substances present in the sample, but not in the reference standard, which may co-elute with the analyte during chromatography, may lead to falsely high measurements of the analyte. Reference standards are not available for many phytoestrogens, such as the acetyl- and malonyl isoflavone glucosides. Therefore, measurements of these compounds are based on calibration curves of isoflavone glucosides and aglucones. Research indicates that this approach could introduce errors in measurements (Coward *et al*, 1993). However, when reference materials of the acetyl- and malonylglucosides are available such assumptions can be tested and results re-calculated.

Gas chromatography with mass spectrometric detection (GC-MS)

3.21 GC-MS is sufficiently sensitive to measure concentrations of phytoestrogens of less than parts per million (Adlercreutz *et al*, 1991a and b; 1993; 1995; 1999; Morton *et al*, 1994; 1997; Joannou *et al*, 1995; Mazur *et al*, 1996; Liggins *et al*, 1998; 2000; Tekel *et al*, 1999; Nesbitt *et al*, 1999; Lampe *et al*, 1999; Heinonen *et al*, 1999; Foster *et al*, 2002). Measurement is done with the use of internal standards, preferably isotopically labelled analogues of the analytes. Samples must be treated to remove conjugating groups prior to analysis by GC-MS. As a result, phytoestrogens cannot be measured as conjugates as they may appear in the matrix and the method is more time consuming than HPLC-UV. As such, the analytical results obtained using this method are measurements of hydrolysed phytoestrogens, usually expressed as a quantity of 'total phytoestrogen'. Chemical and enzymatic methods have been developed to remove these groups (Liggins *et al*, 1998; Mazur *et al*, 1996). Enzymatic methods are preferable for isoflavones as they can be unstable under acidic conditions (Liggins *et al*, 1998). However, lignan glucosides are resistant to enzymatic hydrolysis and require strong acid to remove the sugars (Mazur *et al*, 1996).

Liquid chromatography with mass spectrometric detection (LC-MS)

3.22 LC-MS is also sufficiently sensitive to measure concentrations of phytoestrogens of less than parts per million (Setchell & Welsh, 1987; Barnes *et al*, 1994; Cimino *et al*, 1999; Doerge *et al*, 2000a and b; Rong *et al*, 2000; Chang *et al*, 2000). However, in contrast to GC-MS, removal of conjugating groups is not required prior to analysis. Therefore, different forms of phytoestrogens can be measured and expressed directly rather than as a value of 'total phytoestrogen'. However, measurement is dependent on the availability of standards for each analyte and many of these materials are unavailable at present. Laboratories using LC-MS have to use the available aglucone standards and hydrolyse samples prior to analysis. As such, the advantage of non-destructive sample preparation that LC-MS offers over GC-MS has not yet been realised. More recently, methods using LC coupled to mass spectrometers in tandem (MS/MS) have been developed for the analysis of phytoestrogens (Coward *et al*, 1996; Valentin-Blasini *et al*, 2000; Coldham *et al*, 2002; Fang *et al*, 2002). The specificity of tandem MS/MS systems allows direct injection of biological fluids such as urine, although plasma may need to be treated to remove protein before injection. Additionally, this method allows some structural characterisation of the analyte.

Importance of reference and internal standards

3.23 The analytical methods described above are dependent on reference standards and the accuracy of the MS-based methods may be improved by use of isotopically labelled internal standards. In the past, analysis of many phytoestrogens has been hindered by the lack of analytical standards (Song *et al*, 1998).

3.24 Reference standards have been isolated from natural sources (Farmakalidis & Murphy, 1985) or chemically synthesised (Adlercreutz *et al*, 1986; Wahala *et al*, 1995; Wahala & Rasku, 1997; Whalley *et al*, 1998). Synthetic routes to reference compounds are advantageous as pure, fully characterised compounds can be supplied in large quantities and used to incorporate isotopic labels, including heavier versions of hydrogen (deuterium [^{2}H]) or carbon (carbon-13 [^{13}C]).

3.25 Genistein, daidzein, equol and some of the lignans have been synthesised incorporating ^{2}H labels (Adlercreutz *et al*, 1986; Adlercreutz *et al*, 1991a; Wahala *et al*, 1995; Mazur *et al*, 1996; Wahala & Rasku, 1997). ^{2}H labels are introduced by exchanging hydrogens present in the compound with ^{2}H, but these are unstable and can re-exchange with hydrogen (^{1}H) during analysis, especially under acid conditions, which would lead to an overestimate of the analyte concentration (Adlercreutz *et al*, 1993; Wahala *et al*, 1995; Wahala & Rasku, 1997). The Food Standards Agency's research programme has funded projects to develop methods to synthesise phytoestrogens incorporating stable ^{13}C-labels. These labels have been introduced into genistein, daidzein and formononetin both as single (Whalley *et al*, 1998) and triple labels and supplied to the laboratories undertaking analysis in the Food Standards Agency research programme. The research programme also investigated the preparation of labelled standards by growing phytoestrogen-rich plants in an atmosphere enriched with $^{13}CO_2$ (Bluck *et al*, 2002). However, the synthetic approach has proved more successful and has produced large quantities of labelled and unlabelled material.

3.26 The lack of standards of phytoestrogen metabolites has limited quantification of these compounds in biological samples. Thus, little is known about the concentrations of conjugated and unconjugated phytoestrogens in plasma, urine and faeces. As such, much of the analysis of phytoestrogens in these matrices is preceded by deconjugation and concentrations are expressed as 'total phytoestrogen'. However, labelled and unlabelled standards of the glucones, lignans, prenyl flavonoids, coumestrol and phytoestrogen metabolic products are under development.

Immunoassays

3.27 Immunoassays using polyclonal antibodies raised against genistein, daidzein and enterolactone have been developed. Quantification is achieved using competitive assays incorporating fluorescent- or radio-labelled phytoestrogens (Adlercreutz *et al*, 1998; Lapcik *et al*, 1997; 1998; Uehara *et al*, 2000; Wang *et al*, 2000). These assays are equally as sensitive as, but less time consuming and expensive than, GC- or LC-MS based methods and allow rapid analysis of large numbers of samples (Uehara *et al*, 2000). However, the antibodies are not completely specific and cross-react with other phytoestrogens (Adlercreutz *et al*, 1998; Wang *et al*, 2000). They may also cross-react with other similar compounds, such as steroids, in biological samples.

Quality assurance (QA)

3.28 As phytoestrogen analysis is a difficult and developing area it was considered important to assess the various analytical methods employed in the Food Standards Agency's phytoestrogen research programme. The programme initiated a pilot QA scheme and the results from the participating laboratories were found to be in satisfactory agreement. The Food Standards Agency is currently supporting a further QA scheme.

Key points

- The four principal groups of phytoestrogens found in food are:
  - isoflavones
  - coumestans
  - prenylated flavonoids
  - lignans
- Phytoestrogens possess oestrogenic properties due to their structural similarities to the hormone, oestradiol.
- Measuring the concentrations of phytoestrogens in food and biological samples is difficult. Early studies have used inappropriate standards and may have reported inaccurate analytical results. Methods of phytoestrogen analysis have improved as interest in these compounds has grown. Consequently, it may be considered that studies conducted more recently have produced results of greater reliability.

- It will not be possible to quantify many of the different forms of phytoestrogens as they occur in food and biological samples until methods to synthesise internal and reference standards of these compounds have been developed.

- The Food Standards Agency's research programme has developed and improved analytical methods. This has included the synthesis of reference and internal standards and QA schemes for phytoestrogen analysis.

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4. Sources and concentrations of phytoestrogens in foods and estimated dietary intake

Sources of phytoestrogens in food

4.1 The principal phytoestrogens in food are the isoflavones, coumestans, prenylated flavonoids and lignans (see Chapter 3). The main classes of phytoestrogen and common dietary sources are shown in Table 4.1, which suggests only the isoflavones and lignans are commonly found in the UK diet.

4.2 The provision of accurate data on the phytoestrogen content of different foodstuffs has been hampered by the lack of suitable analytical methods and validation techniques (see Chapter 3). For this reason, it is possible that other phytoestrogen compounds present in food have not been detected.

4.3 Until recently most of the available information on dietary phytoestrogen concentrations related to isoflavone aglucones. This is due to the limitations in the analytical methods used (see Chapter 3). Data on the concentrations of isoflavone glucosides, coumestans and lignans in food are more limited (Reinli & Block, 1996).

Table 4.1 Classes of phytoestrogens and common dietary sources.

Phytoestrogen class	Example of dietary source
Isoflavones	Legumes, lentils, chickpeas, soybean
Genistein	
Daidzein	
Glycitein	
Formononetin	
Biochanin A	
Coumestans	Young sprouting legumes
Coumestrol	e.g. clover and alfalfa sprouts
Lignans	Most cereals, linseed, fruit & vegetables
Matairesinol	
Secoisolariciresinol	
Lariciresinol	
Isolariciresinol	
Prenylated flavonoids	Some beers (Hops)
6-prenylnaringenin	
8-prenylnaringenin	
Xanthohumol	
Isoxanthohumol	

Isoflavones

4.4 The most prevalent dietary isoflavones include genistein, daidzein, glycitein, biochanin A and formononetin. These compounds are primarily found in legumes where they occur as glucosides. Soybeans and soy-based foodstuffs are a particularly rich source of isoflavones, especially genistein and daidzein and to a lesser extent glycitein, whereas biochanin A and formononetin are generally less prevalent in soy and are found mostly in clover and alfalfa sprouts (Bingham *et al*, 1998).

Coumestans

4.5 The coumestans, of which coumestrol is the most common form, are structurally related to the isoflavones. They have been found in high concentrations in clover and fresh alfalfa sprouts (Humfrey *et al*, 1998).

Lignans

4.6 Lignans are a class of phytoestrogens that exist as minor constituents in many cereals, vegetables and fruit. The highest concentration of these compounds (such as matairesinol and secoisolariciresinol), are found in foodstuffs such as grains, seeds and other fibre rich foods. Linseed (flaxseed) is the richest known source of lignans (Morton *et al*, 1997; Meagher & Beecher, 2000).

Prenylated flavonoids

4.7 The prenylated flavonoids are a class of phytoestrogens also structurally related to the isoflavones. These compounds have been found in high concentrations in hops. The most common forms of these compounds are 6-prenylnaringenin, 8-prenylnaringenin, xanthohumol and isoxanthohumol (Milligan *et al*, 1999).

Variability of phytoestrogen concentrations in plants

4.8 There is considerable variation in the phytoestrogen concentrations of different plants. The concentrations of these compounds can be influenced by a number of factors including plant species, strain, crop year and geographical location (Eldrige & Kwolek, 1983; Wang & Murphy, 1994a). An example of how these factors can influence the concentration of isoflavones in soy plants is shown in Table 4.2.

Table 4.2 Factors affecting isoflavone concentrations in soy plants.

Factors	Typical ranges of total isoflavones (mg/kg)
Soy crop strain (n= 7)	2053–4216
Crop year (1989-91)	1176–3309
Location in which crop was grown (n= 4)	1176–1749

From Wang & Murphy (1994a)

Effect of plant species

4.9 Phytoestrogen concentrations can vary considerably between plant species. For example, concentrations of isoflavones varied from undetectable levels to g/kg concentrations in 37 species of plants used in foodstuffs (Franke *et al*, 1995). Variation in the concentrations of isoflavones present in different species of soybean seed (i.e. hulls removed) are shown in Table 4.3.

Table 4.3 Variability of isoflavone concentrations between soybean seeds and black soybean seeds.

Foodstuff	Isoflavone	Concentration (mg/kg)
Soybean seed (n=11)	Daidzein	68–1006
	Genistein	18–1382
Black soybean seed (n=3)	Daidzein	269–698
	Genistein	277–612

Modified after Franke *et al* (1995)

Effect of plant strain

4.10 Concentrations of phytoestrogens also vary significantly between strains of the same plant. Differences in total isoflavone concentration of 2- to 3-fold have been observed between soybean strains grown under similar conditions (Eldrige & Kwolek, 1983; Wang & Murphy, 1994a).

4.11 The chemical form of phytoestrogen is also variable within soybean strains. However, the order of compound prevalence in the strains remains the same. Of eleven varieties of soybean analysed the order of isoflavone concentration was shown to be: genistein derivatives > daidzein derivatives > glycitein derivatives. Of these the malonylgenistin, malonyldaidzin, genistin and daidzin derivatives were the most prevalent forms. Comparisons between strains of Japanese and American soybeans have shown that the Japanese variety have higher ratios of malonylgenistin: genistein and malonyldaidzin: daidzein (see Table 4.4). However, American strains have significantly higher total isoflavone content.

Table 4.4 Ratios of malonylglucoside: glucoside concentration in American and Japanese soybean strains.

Soybean strains analysed, Japanese: Keburi, Kuro diazu, Raiden; American: Pioneer 9111, Pioneer 9202, Prize, HP204, LS301, XL72, Strayer 2233.

	Soybean strain	
Malonylglucoside : glucoside ratio	American (n= 7)	Japanese (n= 3)
Malonyldaidzin : daidzein	0.4–1.8	3.4–6.2
Malonylgenistin : genistein	0.9–2.9	4.9–6.9
Total isoflavone concentration (mg/kg)	2053 – 4216	1261 – 2343

Modified after Wang & Murphy (1994a).

Environmental effects

4.12 Environmental conditions can influence the concentrations of phytoestrogens present in plants (Eldrige & Kwolek, 1983; Wang & Murphy, 1994a). It has been shown that isoflavone concentrations can vary by 2-fold when a particular strain of soybean is grown in different locations in a single year and up to 3-fold when grown in the same location over a number of years. However, while the concentration of individual isoflavones is variable, there appears to be little fluctuation in the ratios of genistein, daidzein and glycitein (Wang & Murphy, 1994a).

Concentrations of phytoestrogens in foods

4.13 The United States Department of Agriculture and Iowa State University have established a database of isoflavone concentrations in foodstuffs. The database is a compilation of internationally published scientific literature on the concentrations of isoflavones in food and is updated when new studies are published. Before new information is included in the database, the analytical methodology is evaluated and compared to a reference method (Murphy *et al*, 1997). A confidence code (a-c) is then assigned which provides an indication of the data quality ('a' indicates considerable reliability). The concentrations of isoflavones are presented as 'total aglucone'. This is obtained by converting the glucoside figures into aglucone values. The dry weight values are converted into wet weight either by using given moisture content or by assuming a commonly expected moisture content for that particular food. The database is published on the Internet at the website address: http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html

4.14 The information from this database indicates that the most prevalent isoflavones present in foodstuffs are genistein and daidzein. Formononetin, biochanin A and glycitein are also present in foodstuffs but to a lower extent and often at undetectable levels.

4.15 Genistein and daidzein were also the most prevalent phytoestrogens in a database of the phytoestrogen content of foodstuffs based on data from the Health Habits and History Questionnaire (HHHQ), a dietary assessment questionnaire widely used in cancer research (Pillow *et al*, 1999).

4.16 Soy is recognised as the major dietary source of phytoestrogens (Bingham *et al*, 1998; Mazur & Adlercreutz, 1998; USDA-Iowa State University Isoflavone Database, 2002, Fukutake *et al*, 1996). Soy-based products have been shown to contain significant quantities of total isoflavones with soybeans and soy flour containing the highest quantities (14-153 mg/100g and 131-198 mg/100g, respectively). Fermented soy products including miso and tempeh also contain high concentrations of total isoflavones (29 – 53 mg/100g) (see Table 4.5).

4.17 Fruits, vegetables, nuts and cereals are all minor sources of isoflavones (Liggins *et al*, 2000a and b; 2002).

4.18 Of the other common phytoestrogens, the highest concentrations of coumestrol have been found in clover (5611 mg/kg) and alfalfa sprouts (720 mg/kg) (Franke *et al* 1995). The highest concentrations of lignans have been found in grains and seeds, in particular linseed (600 – 3700 mg/kg), and other fibre rich foods (Bingham *et al*, 1998; Mazur & Adlercreutz, 1998; Morton *et al*, 1997; Meagher & Beecher, 2000).

Table 4.5 Approximate concentrations of total isoflavones, coumestrol and lignans in various foods.

Food	Isoflavones (mg/100 g)	Coumestrol (mg/100 g)	Lignans (mg/100 g)
Beans i.e. kidney, pinto, green,			
broad, lima, and mung	0–1.5[a]	0–3.6[a]	0.05–0.18[e]
Bean sprouts, alfalfa	–	4.7[a]	–
Biscuits	<0.1[d]	–	–
Bread, brown, white	0–0.8[d]	0[a]	–
Breakfast cereals	<0.1[d]	–	–
Brown rice	–	–	0.3[e]
Cereals i.e. wheat bran, cornflour, maize, oatmeal	<0.1[d]	–	0.5[e]
Cherries, raspberries, strawberries	<0[b]	–	–
Cranberries, currants, dates, figs, prunes, raisins	0-0.2[b]	–	–
Flour, brown, plain, granary	<0.1[d]	–	–
Fruit i.e. apples, banana, grapes, Kiwi fruit, mango, melon, oranges, pears, pineapple, plum, watermelon	<0.1[b]	–	0.1[e]
Legumes	0–0.58[g]	–	–
Linseeds (flaxseed)	–	–	60–370[e]

Food	Isoflavones (mg/100 g)	Coumestrol (mg/100 g)	Lignans (mg/100 g)
Meatless (soy-based) burgers,			
bacon, chicken and sausages	8–15[a]	–	–
Miso	43–60[a]	–	–
Mushrooms, raw	0.02[d]	–	–
Noodles	<0.1[d]	–	–
Oils e.g. soybean, canola	0[a]	–	–
Peanuts, brazil nuts, chestnuts, hazel nuts, almonds, coconuts	<0.1[b]	0[a]	0.02–0.33[e]
Pastas	<0.1[d]	–	–
Peas i.e. lentil and split	0.1–2.5[a]	0[a]	0–0.20[e]
Potatoes	<0.1[c]	–	–
Seeds i.e. sunflower, alfalfa, clover	0.01–0.6[a]	0[a]	–
Soy cheeses	6–31[a]	–	–
Soy milk	5–10[a]	–	–
Soy protein concentrate/isolate	12–102[d]	–	–
Soy noodles	8.5[a]	–	–
Soy yoghurt	16[a]	–	–
Soy sauces	0.1–1.6[a]	–	0.03[e]
Soy flour	131–198[a]	0[a]	0.13[e]
Soy beans	14–153[a]	0.05[a]	0.01 – 0.27[e]
Teas i.e. black, green	0.04–0.05[a]	0[a]	2.04[e]
Tempeh	29–53[a]	–	–
Tofu	13.5–67[a]	–	–
Vegetables i.e. asparagus, aubergine, beetroot, broccoli, brussels sprouts, cabbage, carrots, cauliflower, celery, courgette, cucumber, leek, lettuce, marrow, okra, onion, parsnip, pumpkin, radish, spinach, spring onion, swede, sweetcorn, tomato	<0.1[c]	–	–

Adapted from: [a]USDA-Iowa State University isoflavones database (2002); [b]Liggins *et al* (2000a); [c]Liggins et al (2000b); [d]Liggins *et al* (2002); [e]Mazur *et al* (1998). – signifies not determined.

Effect of food processing and cooking

4.19 In general, commercial processing of soy into food products reduces phytoestrogen concentrations and can lead to decarboxylation, deacetylation or deglucosylation of the isoflavones glucosides present (Coward *et al*, 1993, 1998; Wang & Murphy, 1994b). For example, fermentation of soy into products such as tempeh, miso and bean paste reduces the isoflavone content by between 2- to 3-fold when compared to non-fermented soy foods (Wang & Murphy, 1994b). Also, the aglucone is the predominant form of isoflavone present in fermented products whereas in non-fermented foods glucosides are the most prevalent form (see Table 4.6).

Table 4.6 Isoflavone concentrations in fermented, non-fermented and second-generation food products.

Food	Glucoside (mg/g dry weight)		Aglucone (mg/g dry weight)	
	Genistin	Daidzin	Genistein	Daidzein
Soy milk	1.68	1.34	0.10	0.14
Tofu	1.22	0.59	0.15	0.08
Tempeh	0.30	0.10	0.43	0.30
Miso	0.06	0.05	0.75	0.52
Soy sauce	nd	nd	0.04	0.05
Soy cheese	0.06	0.04	0.005	0.001

Modified after Coward *et al* (1993)

nd – not detected

4.20 Cooking has also been shown to reduce phytoestrogen concentrations and alter the chemical form of phytoestrogens present in foodstuffs. For example, boiling vegetables in water causes a decrease in the daidzein and genistein content, presumably due to loss in the water, and during roasting of soybeans, malonylglucosides are rapidly converted into acetylglucosides (Wang & Murphy, 1994b; Coward *et al*, 1998; Liggins *et al*, 2000b). However, baking or frying does not appear to alter the total isoflavone content of foodstuffs (Coward *et al*, 1998).

4.21 The isoflavone content of 'second-generation' soy foods, i.e. foods made by the addition of soy ingredients (primarily soy protein isolate), is further decreased as only a percentage of the product comes from soy (see Table 4.7). Soy ingredients are often used to replace animal protein and/or to reduce the fat content of foods. Hence the phytoestrogen content of these products is higher when compared with the traditional meat based products.

Table 4.7 Factors affecting isoflavone concentrations in soy derived foods.

Factors	Typical ranges of total isoflavones (mg/kg)
Processing i.e. heating and extraction	1001 – 1313
Processing i.e. fermentation	294 – 625
Dilution into 'second generation' foods (i.e. soy-based foods)	34 – 289

Calculated from Wang & Murphy (1994b)

Concentrations of isoflavones in infant foods

Concentrations of isoflavones in infant formula

4.22 The concentration of isoflavones present in various types of infant formulae was determined. Samples of cows' milk formula (n=3), soy-based powder (n=5) and liquid (n=1) infant formulae were analysed using HPLC-UV. Isoflavones were detected in all samples of soy-based infant formula. The concentration of total isoflavones present, expressed as aglucone/L formula as fed, ranged from between 18-41 mg. Isoflavones were not detected in any of the brands of cows' milk formula analysed (MAFF Food Surveillance Information Sheet 167, 1998). There have been no reports of changes to the isoflavone content of soy-based infant formulae. Thus, these concentrations probably reflect the levels of isoflavones in the formulae currently used.

Concentrations of isoflavones in weaning and soy-containing family foods

4.23 Isoflavone concentrations have been analysed in samples of commercial weaning foods (n= 6) and samples of soy-containing family food (n= 11) 'as consumed' (see Table 4.8). The commercial soy-containing weaning foods contained 18-78 mg total isoflavones/kg food whereas in soy-containing family foods the concentrations were in the range of 29-275 mg total isoflavones/kg food (MAFF report FS2829).

Table 4.8 Isoflavone content of soy-containing weaning foods and other soy-containing foodstuffs.

Food product	Total isoflavone content (mg/kg food)
Instant weaning foods	22–66
Ready to eat weaning foods	18–78
Soy yoghurts	29–83
Soy milk	130–200
Soy dessert	104
Firm tofu	275

Concentrations of isoflavones in human milk

4.24 The breast milk of mothers following an omnivorous, vegetarian or vegan diet has been analysed (see Table 4.9). Generally, the highest concentrations of total isoflavones were detected in the breast milk of mothers who followed a vegetarian or vegan diet. The highest concentration detected, (~32 ng total isoflavone/g), was from an individual following a vegan diet, (corresponding to approximately 0.032 mg total isoflavone/L breast milk). This figure is orders of magnitude less than the concentrations present in soy-based infant formula (18-41 mg total isoflavones/L made up formula) (MAFF report FS2829). Similar findings have been reported by Franke *et al* (1998) and Irvine *et al* (1998).

Table 4.9 Total isoflavones in milk from breast feeding mothers.

Breast Feeding	Total isoflavones in breast milk (μg aglucone/kg)	
Mother's Diet	Mean	Range
Omnivorous (n= 14)	1	0-2
Vegetarian (n= 14)	4	1-10
Vegan (n= 11)	11	2-32

Adapted from MAFF report FS2829

Phytoestrogen intakes from the UK diet

4.25 Estimates of mean phytoestrogen intake from the UK diet are available from a total diet study (TDS) carried out in 1982 and 1987. The estimates were based on twenty dietary groups of foodstuffs and included: carcass meat, offal, meat products, poultry, fish, oils and fats, potatoes, milk, dairy products, eggs, bread, miscellaneous cereals, nuts, sugar and preserves, fruit products, beverages, green vegetables, other vegetables, canned vegetables and fresh fruit.

4.26 The foodstuffs were analysed 'as consumed' for isoflavones and coumestans using reversed phase HPLC-UV (LOD = 0.02 mg/kg). None of the dietary groups had a detectable isoflavone content so it was estimated that the average total isoflavone consumption in the UK was likely to be less than 1 mg/person/day. This is in agreement with estimated isoflavone intake (< 1 mg/day) reported by Jones *et al* (1989). However, given that industry sources have estimated approximately 60% of processed foods currently in British supermarkets contain soy products, it is speculated that the 1987 TDS underestimates the mean intake of phytoestrogens in the UK (ENDS Report, 1996).

4.27 Wiseman *et al* (2002) investigated the isoflavone content of soy containing foods. The findings indicated that isoflavone concentrations correlated with soy content of the foods.

4.28 Typical weight and portion size data are available for the average weights of both individual food items and average portion sizes of food eaten in the UK. The energy and nutrients in portions of foods and therefore the intake in a typical meal can be calculated. Table 4.10 shows approximate concentrations of isoflavones in some soy-based foods in commonly eaten portion sizes.

4.29 Certain sections of the UK population, including infants fed soy-based infant formulae, different ethnic populations, vegetarians and vegans or those consuming diets rich in soy foods may be exposed to higher concentrations of dietary phytoestrogens than the average consumer.

4.30 In 2001, the Phytoestrogen Working Group Secretariat, in collaboration with the FSA Consumer Exposure Analysis Team (CERT), prepared an assessment of isoflavone intake in the UK. The assessment was based on data collated from National Diet and Nutrition Survey (NDNS) 1986-87 and the Dietary Survey of Vegetarians (DSV) 1994-95. The DSV included people who ate fish. The mean exposure to total isoflavones was estimated for consumers of soy and soy-containing foods in UK adults (n= 2197) and UK adult vegetarians (n= 415). Mean exposure was estimated at 0.6 and 2.6 mg/person/day for the total population and vegetarians, respectively.

Table 4.10 Examples of dietary sources of isoflavones and lignans in average portion sizes*.

Food	Total isoflavones (mg)	Food	Lignans (mg)
Soybeans	25–143	Linseed (flaxseed)	13.5
Soybean flour	0.8–59	Oat bran	0.2
TVP	29–67	Asparagus	0.5
Tofu	19	Carrot	0.2
Tofu yoghurt	5–85	Broccoli	0.2
Tempeh	4–38	Lentils	0.7
Miso	4–16	Pear	0.3
Soy sauce	–	Sweet potato	0.4
Soy cheese	1–24	Kidney bean	0.2
Soy milk	3–53	Leek	0.2

*Adapted from the USDA-Iowa State University Isoflavone Database (1999); Thompson *et al* (1991); Cassidy & Faughnan (2000), using average portion sizes from Crawley (1992).

4.31 It should be noted that the estimate was based on dietary surveys that were conducted several years ago and the general eating patterns of the UK population have changed and the availability and use of soy and soy-containing foods has increased in recent years. Analysis of the 1998 Total Diet Study shows soy isoflavones are present in foods such as bread, meat and fish products. The ratio of isoflavone concentrations in these products suggests that soy is introduced during processing. This suggests that consumption of soy and therefore, exposure to isoflavones, for both the whole population and for

vegetarians may now be significantly higher than estimated by the 1986-7 and 1994-5 dietary surveys (ENDS Report, 1996; Clarke *et al*, 2003).

Estimated dietary intake of isoflavones by vegetarians

4.32 It has been estimated that up to 5% of the UK population does not eat fish or meat and follows a vegetarian diet. A 7-day duplicate dietary study was carried out in UK vegetarians to determine the dietary intake of a number of inherent natural toxicants, metals and other chemical contaminants. The samples were analysed by LC-MS (Clarke *et al*, 2003).

4.33 The study participants were asked to collect an exact duplicate of all food consumed over a 7-day period. Dietary exposures were calculated from the concentrations of isoflavones in the duplicate diet samples, the weights of the samples and the weights of the study participants.

4.34 The UK vegetarian duplicate diet study gave an estimated intake of 12 mg isoflavone/day, which is considerably higher than the mean value calculated for vegetarians in the 2001 assessment.

4.35 The results may be taken as an indication of isoflavone intake by vegetarians, however the figures should be treated with caution, as the number of subjects used for intake estimation was small (n= 35). The study set out to gather data from 100 subjects but only data from 35 subjects were used in the final intake estimate because diets were poorly characterised by many of the participants (see Table 4.11).

Table 4.11 Estimate of dietary daidzein and genistein intake from an UK vegetarian diet.

Isoflavone	Intake		
	Total isoflavone (mg/kg bw/day)		Average 60 kg adult (mg/day)
	Mean	Range	
Total daidzein	0.1	0–0.3	4
Total genistein	0.1	0–0.6	8
Total daidzein and genistein	0.2	0–0.9	12

Adapted from Clarke *et al* (2003)

Estimated dietary intake of isoflavones by vegans

4.36 Only one estimate of isoflavone intake by vegans is available (see Table 4.12). The estimate is from breast-feeding vegan mothers in the UK (n= 11) and the mean intake is estimated to be 75 mg isoflavones/day (MAFF report FS2829).

Estimated dietary intake of isoflavones by breast feeding mothers

4.37 The diet of omnivorous, vegetarian and vegan mothers was analysed to determine to what extent it could provide an indirect source of isoflavones. The mothers were requested to record both their infant's food intake and their own consumption of isoflavone rich foods. A checklist of isoflavone rich foods was provided (MAFF report FS2829).

4.38 An estimation of the intake of isoflavones by breast feeding mothers on the various diets is shown in Table 4.12. It should be noted however, that these figures are only an indication of intake as the isoflavone content of the foodstuffs were derived mainly from calculated estimates rather than analytical data. Furthermore, restricted dietary details were provided (i.e. only foods rich in isoflavones were recorded) therefore the intake figures may underestimate the intake of dietary isoflavones by these mothers.

Table 4.12 Intake of isoflavones by breast feeding mothers in the UK.

Mother's Diet	Total isoflavone intake (mg/day)	
	Mean	Range
Omnivorous (n= 13)	0	0
Vegetarian (n= 13)	7	0–70
Vegan (n= 11)	75	30–150

Adapted from MAFF report FS2829

Estimated dietary intake of isoflavones by infants

4.39 The estimated mean intake of total isoflavones from soy-based infant formula has been estimated to range from 5 mg isoflavone/kg bw/day for 1-2 month old infants to 4.5 mg isoflavone/kg bw/day for 4-6 month old infants (MAFF Food Surveillance Information Sheet 167, 1998).

4.40 Other studies, however, have indicated that isoflavone consumption by infants could potentially be higher. Setchell *et al* (1998) estimated that 4-month old infants (n= 7) fed soy-based infant formula would be exposed to between 4.5 – 8 mg isoflavones/kg bw/day of total (free and conjugated) isoflavones. A Swiss study by Rupp *et al* (2000) estimated that infants up to 5 months of age would be exposed to between 3-13 mg isoflavones/kg bw/day whilst infants older than 5 months were estimated to be exposed to a maximum of 20 mg isoflavones/kg bw/day. In addition, Murphy *et al* (1997) reported consumption in the range of 5-12 mg isoflavones/kg bw/day.

Phytoestrogen intake in consumers of health food supplements

4.41 A large number of health food supplements that contain phytoestrogens are marketed in the UK. They are marketed for a wide range of conditions such as 'natural alternatives to hormone replacement therapy', breast enhancement and osteoporosis.

4.42 In response to concerns raised by the Food Commission, the Food Standards Agency commissioned an analysis of the phytoestrogen content of a dietary supplement that is claimed to promote 'natural breast enhancement'. The analysis did not detect the presence of genistein, daidzein, coumestrol, matairesinol, secoisolariciresinol or anhydrosecoisolariciresinol. However, the supplement was shown to contain 8-prenylnaringenin and possibly 6-prenylnaringenin, xanthohumol, isoxanthohumol and trace levels of glycitein. These phytoestrogens are found in hops, a stated ingredient on the supplement label (Coldham & Sauer, 2001).

4.43 A report from New Zealand has shown that the concentrations of phytoestrogens in dietary supplements vary according to the food source. The highest concentrations recorded were approximately 29 mg isoflavones/day at the manufacturers' recommended daily dose (Taylor & Burlingame, 1998).

4.44 The isoflavone content of fifteen different soy-based supplements available on the market was analysed using HPLC (Nurmi *et al*, 2002). The report found only one supplement actually contained the same amount of isoflavones as stated by manufacturers on the product label. In general isoflavone content was found to be lower than the value given on the product labels and total isoflavone content ranged from 0.1-201 mg/g per tablet (Nurmi *et al*, 2002). A study by Setchell *et al* (2001) also reported considerable differences in the phytoestrogen content of dietary supplements (n= 33) from that stated by the manufacturers. Similar findings were reported by Howes & Howes (2002) who reported 6/10 supplements analysed had concentrations significantly below that stated on the label.

4.45 The Food Standards Agency carried out analysis of a range of phytoestrogen dietary supplements available in the UK (n= 51). This analysis also showed that phytoestrogen content varied widely between products with many of the supplements analysed containing less than the stated content of total phytoestrogens (unpublished results). Concentrations of phytoestrogens ranged from 0.01-35 mg/g supplement. If the manufacturers' recommended daily dosage is taken, the highest isoflavone concentrations consumed could be approximately 88 mg isoflavones/day. From this value, the estimated exposure of isoflavones from this supplement for a 60 kg adult is 1.5 mg/kg bw/day.

Phytoestrogen intake in other countries

4.46 Dietary phytoestrogen intake is dependent on the type and composition of the foodstuffs consumed by different populations. Soybeans have, for many centuries, played an integral part in some Asian cultures both as a foodstuff and as a medicine (Messina, 1995). However, feeding of soy, including soy-based infant formula, to infants in the first 4-6 months of life has not been traditional in Chinese and Japanese cultures (Tso, 1928; Guy & Yeh, 1938; Takeuchi, 1992; Ping-Chen, 1995).

4.47 The daily intake of isoflavones in the Japanese population has been estimated to be in the range 150-200 mg (Cassidy *et al*, 1994) although other estimates of 25-100 mg/day have been suggested to represent a more likely intake for this population (Coward *et al*, 1993). Nagata *et al* (2000) studied the dietary intake of isoflavones in 69 Japanese men residing in Japan. Semi-quantitative food frequency questionnaires (FFQ) indicated an intake of 22 mg isoflavones/day following consumption of 51 g of soy products per day in this population.

4.48 This is in agreement with studies by Kimira *et al* (1998), Wakai *et al* (1999), and Arai *et al* (2000) where Japanese adults residing in Japan were reported to consume isoflavones in the range of 30-50 mg/day.

4.49 Kikuchi *et al* (2001) reported a daily intake of 35 mg isoflavones from consumption of soy products (8-40 g soybean products/day) for Japanese adults. Data collated from the Japanese National Nutritional Survey was also used to calculate isoflavone intake from the general diet. Daily intake of isoflavones for Japanese school children, adults and the elderly was reported to be 18, 38 and 43 mg isoflavones, respectively.

4.50 Chinese populations are reported to have isoflavone intakes similar to the Japanese. Intake of isoflavones for healthy Chinese women in Shanghai was calculated to be 39 mg/day from consumption of soy (approximately 100 g soy/day). This calculation was based on data from 60 Shanghai women (aged 37-61 years) asked to complete a 24-hour food frequency questionnaire (FFQ) and report on the frequency of soy consumption over the previous five years (Chen *et al,* 1999).

4.51 In a survey of Hong Kong Chinese women (n= 650), total intake of phytoestrogens was reported to be 60.5 mg/day based on consumption data. Subjects (aged $<$40 years) were asked to complete 24 hour, weekly and monthly FFQs of soy-based foods. The phytoestrogen content of each food was estimated using published values. Mean daily intakes for isoflavones and lignans were reported to be 25 $\pm$ 35 and 2 $\pm$ 1 mg/day, respectively (Mei *et al*, 2001).

4.52 In a Singapore study on diet and cancer, the dietary habits of Chinese men (n= 76) and Chinese women (n= 71) aged 45-74 years were assessed using FFQ's. Mean isoflavone intake from the total diet was calculated at 61 mg/day (Seow *et al*, 1998).

4.53 Dietary isoflavone intake of a Korean population was estimated to be 15 mg/day. This was based on data collated from the Korean National Nutritional Survey and published literature values on the isoflavone content of soy-based foods. Soybeans and soy foods, tofu, soybean paste and soybean sprouts contributed to more than 94% of the total isoflavone intake of the Korean population (Kim & Kwon, 2001).

4.54 The mean dietary soy isoflavone intake of elderly Japanese-American women (aged over 65 years) was estimated to be 10 mg/day (Rice *et al*, 2001).

4.55 In contrast, the estimated dietary isoflavone intake reported for the UK and other Western populations is significantly lower than that reported for Eastern populations (see Table 4.13). The mean daily dietary intake of isoflavones in postmenopausal Caucasian women in the United States (n= 964) was calculated to be 0.8 mg/day (mainly from beans and peas). Fruit derived lignans and coumestans from vegetables were calculated to contribute 0.6 and approximately 0.3 mg/day, respectively to the diet (De Kleijn *et al*, 2001).

4.56 In a study investigating phytoestrogen intake and cancer, 190 Caucasian American men were asked to complete questionnaires on their lifestyle and diet for the previous year. The mean isoflavone intake was estimated at 1.2 mg/day (Strom *et al*, 1999). This is in contrast to findings by Kirk *et al* (1999) where a smaller sample of staff (n= 29 omnivores and n= 22 vegetarians) from an American university were asked to report on frequency of consumption of soy foods. Mean soy-derived isoflavone intakes were reported to be 10 and 15 mg/day for omnivores and vegetarians, respectively.

4.57 In Australian-born women aged 51-62 years, the mean intake was estimated to be 17 mg isoflavones/day. This was calculated from a cohort of Australian women (n= 354) of which 62% reported consuming isoflavone rich foods such as soy breads, soymilk and linseed bread. Although reported isoflavone consumption ranged from 0-340 mg isoflavones/day, only 14% of the women surveyed consumed more than 40 mg isoflavones/day (Gutherie *et al*, 2002).

4.58 Average consumption of isoflavones in New Zealand is estimated at 0.8 mg isoflavones/day for individuals following an omnivorous diet and 140 mg isoflavones/day for those following a vegan diet. These estimates were based on calculations from two sample, three-day diets for the omnivorous diets and the same diets with soy substitutions for the vegan diet (Taylor & Burlingame, 1998).

Table 4.13 Comparison of estimated dietary isoflavone intakes for Western and Eastern populations.
Studies presented have used different methodologies in deriving daily intakes. C- calculated values; E-estimated values; n/r – not reported. 1= direct measurement of consumption using food frequency questionnaires; 2= direct measurement of serum and/or urinary excretion of phytoestrogens; 3= direct analysis of phytoestrogen content of foods; 4= intakes based on consumption of specific food groups only (e.g. soy foods, beans, seeds) and not total diet; 5= no direct analysis of phytoestrogen content of foods, published literature values used.

Study	Study group size (n)	Country	Mean Isoflavone intake (mg isoflavone aglucone/day)
Jones *et al* (1989)	n/r	UK	1 [E, 3, 4]
FSA Intake Assessment (2001)	2197 (population) 415 (vegetarians)	UK	0.6 [C,4,5] (total population) 2.6 [C,4,5] (vegetarians)
De Kleijn *et al* (2001)	964 women	USA	0.76 [E,1,5]
Strom *et al* (1999)	190	USA	1.2 [C,1,5]
Kirk *et al* (1999)	51	USA	10 [E,1,4] (omnivores) 15 [E,1,4] (vegetarians)
Rice *et al* (2001)	274 women	USA	10 [E,1,4,5] (Japanese-American)
Gutherie *et al* (2000)	354 women	Australia	17 [E,1,4,5]
Taylor & Burlinghame (1998)	n/r	New Zealand	0.8 (omnivores) [E,1,3] 140 (vegan) [E,1,3]
Kim & Kwon (2001)	3224 men	Korea	15 [E,1,4,5]
	3475 women		
Chen *et al* (1999)	60	China	39 [C,1,2,3,4]
Mei *et al* (2001)	650	China	25 [E,1,2,5]
Seow *et al* (1998)	147	Singapore	61 [C] (Singapore -Chinese)
Kikuchi *et al* (2001)	n/r	Japan	18 [C,1,3,4] (school children) 38 [C,1,3,4] (adult) 43 [C,1,3,4] (advanced age)
Nagata *et al* (2000)	69	Japan	22 [E,1,2,4]
Coward *et al* (1993)	n/r	Japan	25-100 [E,3,4]
Kimira *et al* (1998)	50 women	Japan	39.5 [C,1,2,3]
Arai *et al* (2000)	106	Japan	46.5 [C,1,2]
Wakai *et al* (1999)	1274	Japan	31.5-51.4 [E,1,5]
Cassidy *et al* (1994)	n/r	Japan	150-200 [E,1,2,5]

4.59 Maskarenic *et al* (1998) investigated the dietary isoflavone intake among women of different ethnicity residing in Hawaii. The study group consisted of women of Asian ancestry (aged 36-80 years) who had maintained some of their traditional dietary habits. Self-administered food questionnaires indicated dietary isoflavone intake differed significantly among ethnic groups, with Japanese women consuming highest levels of dietary isoflavones 18.9mg isoflavones/day following consumption of 6.1g soy protein per day (see Table 4.14).

Table 4.14 Mean soy protein and isoflavone intake among Hawaiian women by ethnicity.
Estimated intakes based on the usual eating habits during both the previous 24 hrs and the previous 12 months.

Soy intake/ Phytoestrogen excretion	Chinese (n=13)	Filipino (n=7)	Hawaiian (n=11)	Japanese (n=25)	Caucasian (n=42)
Soy protein intake during previous 24 h (g/day)	13	2	8	9	3
Soy protein intake during previous year (g/day)	5	2	5	6	9
Dietary isoflavone intake during previous 24 h (mg/day)	38	5	22	31	7
Dietary isoflavone intake during previous year (mg/day)	12	5	12	19	5

Modified after Maskarinec *et al* (1998)

Key points

- Phytoestrogens are natural constituents of many plants, seeds and grains. There is considerable variation of phytoestrogen concentrations in different plants. A number of factors can influence the phytoestrogen content including, species and strain differences and environmental conditions. Processing can also alter the phytoestrogen content of foodstuffs.

- Studies indicate that the most prevalent isoflavones in the UK diet are genistein and daidzein. Formononetin, biochanin A and glycitein are also present but to a lesser extent and often at undetectable levels.

- In general, commercial processing of soy into food products reduces phytoestrogen concentrations and can alter the chemical form of isoflavones present. Hence, isoflavone concentrations in fermented and soy-based food products are usually lower.

- Soy is recognised as a major dietary source of isoflavones. There is a paucity of data on the forms and concentrations of lignans in plants and foods. Vegan or vegetarian diets may have higher intakes of phytoestrogens compared with the general population, due to the consumption of soy-based meat and dairy replacement foods.

- Estimated exposure of infants to phytoestrogens is dependent on whether the infant is fed breast or formula milk. Soy-based infant formulae have been shown to contain significantly higher concentrations of isoflavones than human breast milk. Cows' milk-based formula contains no detectable isoflavones. Concentrations of isoflavones have been shown to be generally higher in the breast milk of mothers following a vegetarian or vegan diet, (corresponding to approximately 2-32 μg isoflavones/L). However, these concentrations were substantially lower than those present in soy-based infant formulae (18-41 mg isoflavones/L made up formula) and weaning foods (18-78 mg isoflavone/kg). Mean intakes of soy-based infant formula are estimated at 4.5-5 mg/kg bw/day, although other studies have indicated that consumption may be higher.

- A range of phytoestrogen food supplements is now widely available in the UK. Analysis has shown that the actual phytoestrogen content varied between products and was often less than the content declared on the label.

- Soybeans have, for many centuries, played an integral part in some Eastern cultures (e.g. Japanese and Chinese) as a foodstuff, although soy is not generally fed to infants in the first 4-6 months of life. Comparison of estimated dietary isoflavone intakes in Western and Eastern populations illustrate that Eastern populations have a significantly higher intake of phytoestrogens. These differences are generally attributed to the usage and higher consumption of soy and soy-based foods.

- Due to the large variability in isoflavone concentrations in plants and foods it is difficult to provide direct comparisons between different exposure groups. Broadly speaking however, a rank order may be determined of daily isoflavone intake: soy formula fed infant (approximately 40 mg/day) > average Japanese consumer (approximately 25-100 mg/day) > vegetarian consumer (approximately 3 mg/day) > average UK consumer (approximately 1 mg/day). There are few data on the intake of consumers of dietary supplements and 'functional foods'. It is possible that exposures from these sources may equate to those of infants fed soy-based formula for products that are particularly rich in isoflavones.

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5. Absorption, distribution, metabolism and excretion of phytoestrogens

Introduction

5.1 The absorption, distribution, metabolism and excretion (ADME) of phytoestrogens have not been fully elucidated in human adults or infants. Most of the information concerns the isoflavones daidzein and genistein and to a lesser extent, the lignans enterodiol and enterolactone. This chapter reviews the ADME studies carried out in humans and, where indicated, provides relevant additional information gained from animal studies. There are no data available on the ADME of prenylated flavonoids or coumestans. Other gaps in the knowledge in this area are highlighted in this chapter.

Absorption

Uptake

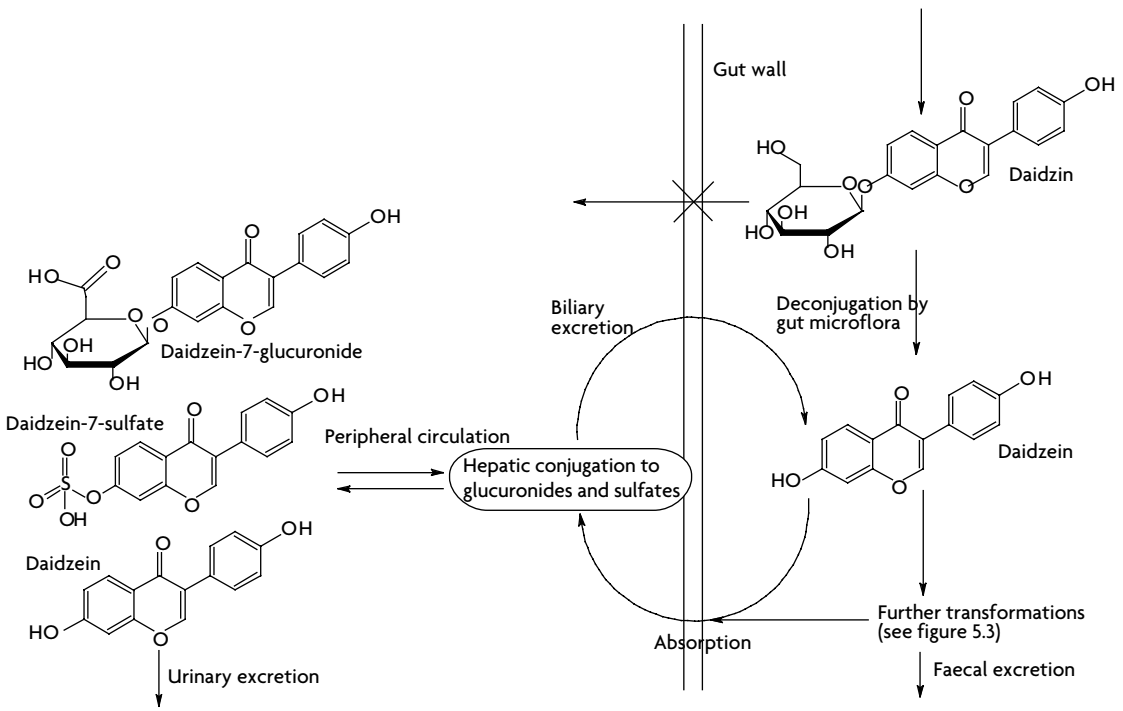
5.2 Isoflavones are present in food mainly as glucosides. Although there is evidence to suggest that particular members of a related class of flavonoids are absorbed in their naturally occurring glucosidic forms (Hollman & Katan, 1997), this does not appear to be the case for the isoflavones. It is thought that isoflavones are absorbed as aglucones, which are more readily absorbed than the parent glucosides due to their higher hydrophobicity and lower molecular weight. Glucosides of isoflavones have not been identified in plasma. This is supported by recent evidence from Setchell *et al* (2002), which indicates that isoflavone glucosides are not absorbed intact across the enterocyte of healthy adults and shows that uptake requires hydrolysis of the isoflavone glucosides to their aglucone form. Absorption of aglucones takes place mainly in the small and large intestine (see Figure 5.1).

Transfer

5.3 It has been suggested that acid hydrolysis of glucosides occurs in the stomach (Kelly *et al*, 1993) although there is some disagreement about this (Piskula *et al*, 1999). There is evidence that the liver and enterocytes of the human small intestine contain ß-glucosidase enzymes capable of efficiently hydrolysing some, but not all, naturally occurring flavone and isoflavone glucosides (Day *et al*, 1998). ß-Glucosidases associated with the gut microflora (including *Lactobacilli*, *Bifidobacteria* and *Bacteroides*) also play a role in glucoside hydrolysis (Xu *et al*, 1995; Barnes *et al*, 1996). A study has also suggested that isoflavone glucosides can be converted to aglucones by enzymes in saliva (Allred *et al*, 2001). In humans, prior to absorption, the isoflavones may be further metabolised by the gut microflora, with genistein being converted to the hormonally inert *p*-ethyl-phenol and daidzein reduced to the oestrogenically active isoflavone equol and the non-oestrogenic *O*-demethylangolensin (*O*- DMA).

Figure 5.1 Schematic representation of absorption of daidzein from the gut.

Prior to absorption from the gut, daidzin is converted to daidzein by gut microfloral enzymes. It is partially converted to glucuronide and sulphate conjugates by enzymes in the liver before entering the peripheral circulation. These conjugates can be excreted back into the gut from the liver via the bile duct (enterohepatic circulation) where they can be deconjugated by gut microfloral enzymes. They may then be re-absorbed or further transformed in the gut and absorbed.



5.4 Little information on the processing of lignans and coumestans prior to absorption has been reported. However, ingested lignans have been shown to undergo bacterial hydrolysis and metabolism. Colonic fermentation results in the removal of glucose residues, demethylation and dehydroxylation to the diphenol compounds, enterolactone and enterodiol, which are absorbed. Enterodiol may be further metabolised to enterolactone in the gut (Setchell & Adlercreutz, 1998; Kurzer & Xu, 1997) (see Figure 5.2).

5.5 Studies have suggested that isoflavones are more bioavailable in food if present as aglucones (as in fermented soy) than when present as glucosides (as in unprocessed soy). Hutchins *et al* (1995) reported that the recovery of urinary daidzein and genistein was higher when subjects consumed a diet consisting of tempeh (fermented soy) compared to a similar diet containing conjugated isoflavones in the form of unfermented soy pieces. In addition, Slavin *et al* (1998) reported that while fermentation decreased the isoflavone content of soy, the increased recovery of urinary isoflavones observed suggested fermentation (conversion to aglucones) increased the bioavailability of isoflavones. However, the bioavailability of isoflavones reported in these studies may be attributable to differences in the food matrix rather than the chemical form of the isoflavones.

5.6 More recent studies suggest that isoflavone bioavailability is independent of form (i.e. glucoside or aglucone). Richelle *et al* (2002) showed that plasma concentrations of isoflavones were similar in subjects (n= 6) after consumption of a soy drink which had been enzymatically treated to convert the isoflavone glucosides to their aglucone form, compared with subjects that had ingested the untreated drink. In a study by Tsunoda *et al* (2002), similar concentrations of isoflavones were found in the urine of subjects (n= 14) consuming equal quantities of isoflavones either as glucosides or aglucones.

5.7 Data from animal studies indicates that, although the initial rates of absorption and excretion for the aglucone forms were greater than for the conjugated forms, the total percentage recoveries in urine and faeces does not differ significantly. Thus, the extent of absorption and the bioavailability of aglucone and glucosidic forms are considered to be similar (King *et al*, 1996). An FSA project is currently examining what effect food matrix and isoflavone form has on the bioavailability of isoflavones in humans.

Metabolism

Bacterial activity in the human intestine[8]

5.8 Metabolism of isoflavones and lignans is mediated both by tissue enzymes and gut microflora, either prior to absorption or during enterohepatic circulation (see Figure 5.1).

[8] The reader is referred to the Institute of Environmental Health Report: Assessment of phytoestrogens in the diet (1997) for a detailed treatise on gut floral composition, inter-individual variations and implications for health.

5.9 The importance of the gut microflora in the metabolism of phytoestrogens has been clearly established. Setchell *et al* (1984) showed that incubation of textured vegetable protein with cultured human faecal bacteria resulted in the formation of equol, a metabolic reduction product of daidzein (see Figure 5.3). Chang & Nair (1995) demonstrated the metabolism of daidzein to dihydrodaidzein, benzopyran-4,7-diol,3-(4-hydroxyphenol) and equol and of genistein to dihydrogenistein, when fermented with human faecal bacteria under anaerobic conditions. Antibiotic treatment in humans has been shown to inhibit the formation and excretion of enterolactone and enterodiol, which are bacterial metabolites of the lignans (Setchell *et al*, 1981).

5.10 Several groups of bacteria are known to possess ß-glucosidase activity, including *Lactobacilli, Bacteroides* and *Bifidiobacteria* (Hawkesworth *et al*, 1971). However, little research has been carried out to identify the types of bacteria specifically involved in the metabolism of isoflavones or lignans.

5.11 In humans, the upper third of the small intestine contains very low levels of bacteria, but this changes to a colon-like flora in the lower third (Heneghen, 1988). The distal part of the small intestine and the large intestine contain substantial numbers (10^4-10^7 bacteria/g wet weight) of *Lactobacilli, Bacteroides* and *Bifodiobacteria* (Gorbach *et al*, 1967; Mitsuoka 1982) whereas the proximal end of the small intestine contains very few of these bacteria.

5.12 Some bacteria present in the large intestine also possess ß-glucuronidase and arylsulfatase activity, which can liberate aglucones from conjugates excreted in the bile and render them available for reabsorption (Heneghan, 1988). Incubation studies with human faeces suggest that human intestinal bacteria from some, but not all, individuals can further metabolise and degrade isoflavones (Xu *et al*, 1995), thus preventing their reabsorption from the lower bowel. Consequently, the composition of intestinal microflora can have a profound effect both on the magnitude and pattern of isoflavone bioavailability.

5.13 Genistein and biochanin A, are much more susceptible to cleavage of the central ring system by rat intestinal bacteria than daidzein or formononetin (Griffiths & Smith, 1972). Whether the same is true with human faecal bacteria is unclear, although selective central ring cleavage of certain flavonoid compounds by certain strains of *Clostridium* isolated from human faecal flora has been shown (Winter *et al*, 1989; Hur *et al,* 2002). Less faecal degradation should result in greater exposure to lignans and isoflavones in the circulation and increased urinary isoflavone excretion, although specific data are lacking.

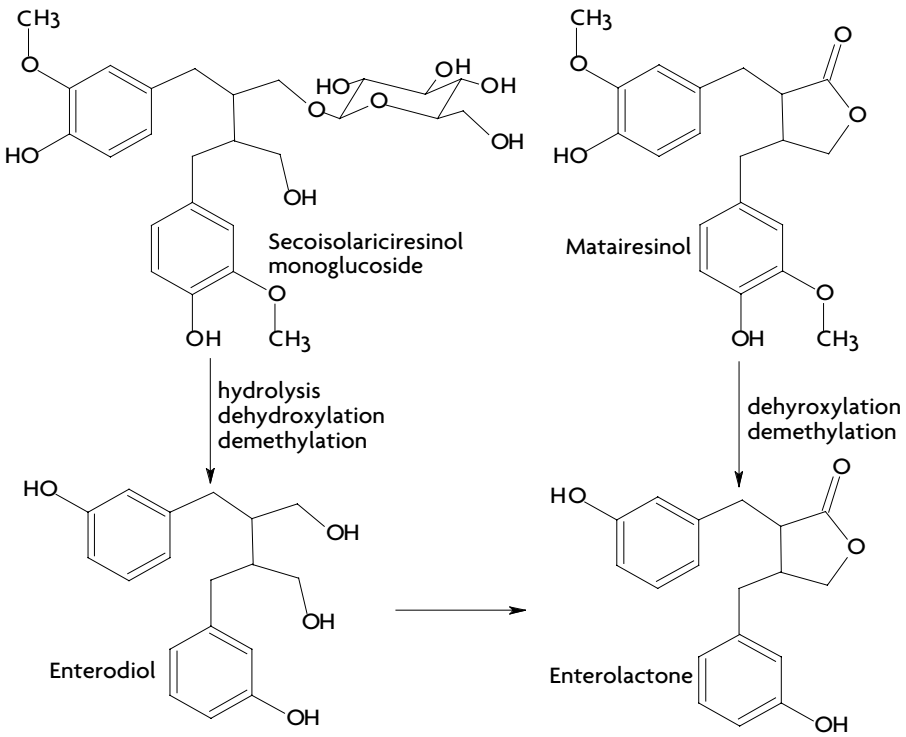
5.14 Lignans have also been shown to produce enterodiol and enterolactone in humans via microfloral metabolism (Setchell & Adlercreutz, 1998) (see Figure 5.2).

Conjugation

5.15 Once absorbed, isoflavones and lignans are efficiently reconjugated, either with glucuronic acid or, to a lesser extent, sulfate. In addition, some sulfoglucuronides may be formed. Conjugation takes place either in the liver with hepatic UDP-glucuronosyl transferase or sulfotransferase enzymes (Knight & Eden, 1996; Bingham *et al*, 1998; Setchell, 1998), or within the intestinal epithelium, which has also been shown to possess glucuronosyl transferase and sulfotransferase activity (Sfakianos *et al*, 1997). As a consequence, isoflavones and lignans are present in the circulation in predominantly conjugated forms.

5.16 Zhang *et al* (1999) have shown that rat microsomal UDP-glucuronosyl transferase has a greater affinity for genistein than daidzein *in vitro.* However, it remains unclear as to the relevance of this finding to humans.

Figure 5.2 Example conversion of lignans to enterolactone and enterodiol by human faecal flora.
For example, secoisolariciresinol monoglucoside is metabolised to enterodiol through hydrolysis of the sugar moiety, dehydroxylation and demethylation. Enterodiol can then be further converted to enterolactone. Matairesinol is converted to enterolactone by gut bacteria through dehydroxylation and demethylation (adapted from Kurzer & Xu, 1997).



Further metabolism

Isoflavones

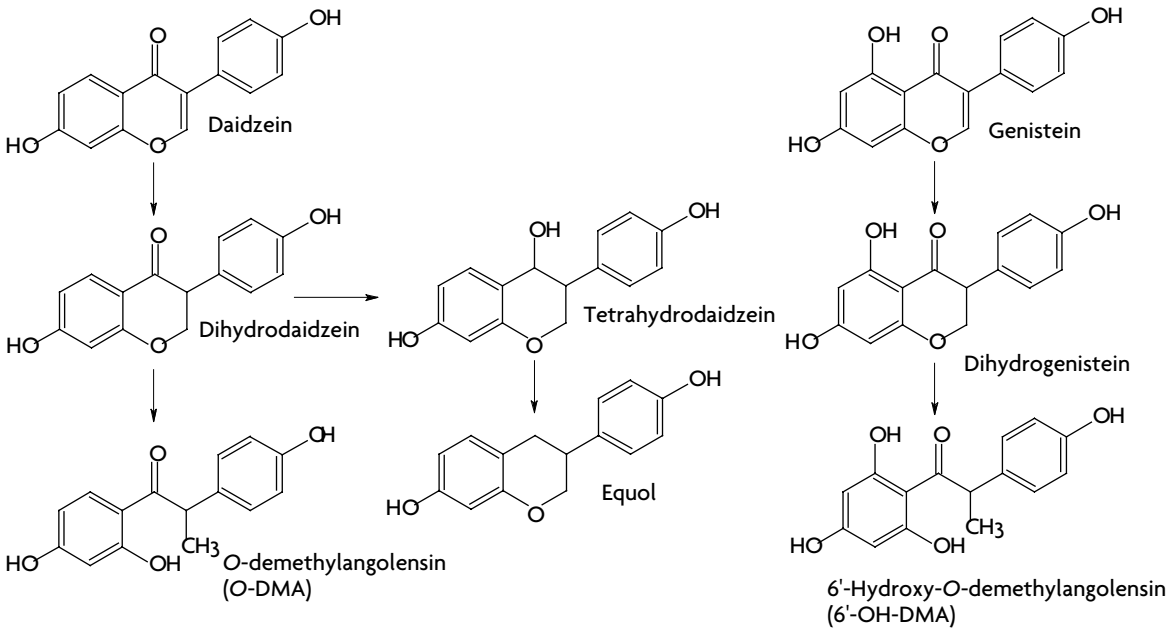
5.17 The metabolism of unconjugated isoflavones is extensive (see Figure 5.3). Studies have revealed several diphenolic metabolites can be produced during the intermediary metabolism of daidzein and genistein. The intermediate metabolites can be produced either from microfloral or liver metabolism and include 6'-OH-*O*-desmethylangolensin (6'OH-DMA), dihydrogenistein, *O*-demethylangolensin (*O*-DMA), di and tetrahydrodaidzein and equol (Adlercreutz *et al*, 1987; Kelly *et al*, 1993; Joannou *et al*, 1995; Wahala *et al*, 1998; Yasuda & Ohsawa, 1998; Heinonen *et al*, 1999). It has been shown that some aglucones may undergo metabolism mediated by cytochrome P450 (CYP) enzymes (Roberts-Kirchhoff *et al*, 1999). Incubations with genistein in the presence of human recombinant CYP1A1[9], 1A2, 1B1[2], or 2E1 isoforms resulted in the formation of one predominant and two minor unidentified metabolites. Incubation with CYP3A4 catalysed the formation of two unidentified products. All metabolites were considered to be hydroxylation products. Similarly, transformation of the flavonoid galangin to kaempferol then to quercetin in rat liver microsomes is thought to be CYP-mediated, with the latter step being attributed specifically to CYP1A1 (Silva *et al*, 1997). Additionally, metabolism studies with cultured (T47D breast cancer) cells have shown that biochanin A and genistein undergo methylation and hydroxylation reactions as well as sulfate ester formation (Peterson *et al*, 1998).

5.18 Following incubation of multiply labelled ^{3}H-genistein with rat caecal and faecal or human faecal cultures, the genistein metabolites, dihydrogenistein and 6'- OH-*O*-DMA were detected by radioactive isotope detection-HPLC. Further hydrolysis by the gut microflora of human and rat, resulted in the formation of 4-hydroxyphenol-2-propionic acid (Coldham *et al,* 2002). Previous work has suggested that 4-*p*-ethylphenol was the final metabolite in the plasma and urine of rats and humans after administration of unlabelled genistein (Barnes *et al*, 1998; King, 1998; Setchell, 1998).

5.19 Five metabolites of genistein were identified in rats following an oral dose of 4 mg genistein/kg bw. The metabolites were identified as genistein glucuronide, dihydrogenistein glucuronide, genistein sulphate, dihydro-genistein and 4-hydroxyphenyl-2-propionic acid (Coldham *et al*, 1999). The last of these metabolites is thought to be a microfloral product of dihydrogenistein. The major metabolites of genistein identified in the rat are therefore the same as those identified in man.

[9] CYP1A1 and CYP1B1 activities are low or absent in human liver.

Figure 5.3 Proposed phase I metabolism of daidzein and genistein based on human urinary metabolites.
Adapted from Joannou *et al*, 1995.



Lignans

5.20 A range of lignan metabolites have been identified in human urine: enterolactone, enterodiol, matairesinol, lariciresinol, isolariciresinol, secoisolariciresinol and the tentatively identified as hydroxylated forms of matairesinol and enterolactone (Adlercreutz *et al*, 1995).

5.21 Oxidative metabolites of enterodiol and enterolactone have been identified in the urine and bile of female rats (Niemeyer *et al*, 2000). Following intraduodenal administration of enterodiol or enterolactone to bile duct-catheterised female rats, 5 hydroxylated biliary metabolites were produced from enterodiol and 11 were produced from enterolactone. The precise identity of these compounds was not determined.

Distribution

5.22 Isoflavone and lignan phytoestrogens have been detected in a number of body fluids such as urine, plasma, faeces, prostatic fluid, semen, bile, saliva, breast milk, breast aspirate and cyst fluid. The major isoflavones and their metabolites detected in the blood and urine of humans and animals are daidzein, genistein, equol and *O*-DMA (Adlercreutz *et al*, 1995; Knight & Eden, 1996). Lignans identified in human plasma and urine include enterolactone, enterodiol, lariciresinol and isolariciresinol (Adlercreutz *et al*, 1987; Jacob *et al,* 2002).

5.23 In rats, relatively high levels of daidzein in the plasma, liver, lung and kidney were observed 15 min after intravenous injection. Lower levels were found in skeletal muscle, spleen, heart, testis and brain (Yueh & Chu, 1997).

5.24 Following intraperitoneal administration to rats, genistein rapidly appears in brain and then in microdialysate fluid from the corpus striata together with its metabolite *p*-ethyl-phenol (Setchell, 1998). Following oral administration of ^{14}C-genistein to rats, the levels in reproductive organs (vagina, uterus, ovary and prostate) were higher than in other peripheral organs. The major plasma-derived compounds were genistein glucuronides and 4-hydroxyphenyl-2-propionic acid with only trace amounts of parent compound (Coldham & Sauer, 2000).

5.25 The concentrations of genistein in plasma and selected tissues in weaning (plasma only) and adult rats exposed to genistein *in utero*, through maternal milk from dietary administration to the mothers and to the offspring after weaning (5, 100 or 500 mg genistein/kg diet) were measured (Chang *et al*, 2000). Plasma and tissue concentrations of genistein increased dose dependently. Genistein was predominantly (95-99%) present in conjugated form in plasma but to a much lesser extent in tissues, unconjugated genistein ranged from 18-100% (see Table 5.1). Gender differences in the tissue concentrations of genistein and the proportion of genistein in conjugated form were evident particularly in the liver, thyroid and mammary glands. Little genistein was detected in the brain.

5.26 Holder *et al* (1999) demonstrated that genistein was predominantly conjugated (90%) to glucuronic acid with minor quantities of sulfate conjugates in the plasma of rats. Janning *et al* (2000) also demonstrated that following a single dose of daidzein (100 mg/kg bw) administered to adult female rats, daidzein was detected in plasma almost exclusively in its conjugated form.

Table 5.1 Plasma and tissue concentrations of genistein in male and female rats.
Concentrations and percentage unconjugated genistein shown are from animals (n= 6) exposed to 500mg genistein/kg diet. Adapted from Chang *et al* (2000).

Tissue	Genistein Concentration pmol/mg (% aglucone)	
	Male	Female
Plasma[a] (adult)	6 μmol/L (<5%)	7.9 μmol/L (<5%)
Plasma[a] (weaning)	1.9 μmol/L (<5%)	2.1 μmol/L (<5%)
Mammary glands	0.8 (24%)	2.4 (49%)
Thyroid	0.4 (25%)	1.2 (18%)
Liver	0.7 (34%)	7.33 (77%)
Brain[b]	Lod	Lod
Prostate	1.1 (45%)	
Testes	0.6 (11%)	
Ovary		1.1 (80%)
Uterus		1.4 (100%)

[a] Concentrations in plasma are given in μmol/L.

[b] Concentrations in brain were at or below the limit of detection (0.5 pmol/mg).

Pharmacokinetics

5.27 Many studies have examined the excretion profiles of phytoestrogens. However, few have examined the pharmacokinetics of these compounds in plasma.

Isoflavones

5.28 In a study, by Watanabe *et al* (1998), seven adult males (previously receiving an otherwise low isoflavone diets for 6 days) received a single dose of baked soybean powder (26 mg daidzein [103 μmol] and 30 mg genistein [112 μmol]). The isoflavone concentrations in plasma, urine and faeces are shown in Table 5.2. Plasma genistein concentrations had significantly increased 2 hours after ingestion and reached maximum concentrations by 8 hours. The plasma concentration of daidzein peaked at approximately the same time but was lower than genistein. Plasma levels of *O*-DMA and equol peaked later than genistein and daidzein in 2/7 and 4/7 subjects, respectively.

5.29 In contrast to plasma, daidzein was the major compound present in urine. Recoveries of ingested daidzein and genistein in urine were 36% and 18%, respectively. Equol was detectable in the urine of 2/7 subjects and was observed after the peak of daidzein excretion.

Table 5.2 Total recovery of isoflavones, *O*-DMA and equol in urine and faeces of men after ingestion of 60g kinako (baked soybean powder).
Data adapted from Watanabe *et al*, 1998.

Subject	Daidzein (μmol)		*O*-DMA (μmol)		Equol (μmol)		Genistein (μmol)	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
1	27	1	12	1	<1	<1	10	<1
2	47	5	1	2	<1	<1	27	6
3	28	5	4	2	<1	<1	11	3
4	30	5	6	6	<1	<1	12	3
5	65	13	2	2	<1	<1	48	7
6	32	1	1	<1	34	8	20	<1
7	30	2	8	1	19	3	10	<1
Mean	37	4.5	4.9	2.1	8.3	2.3	19.7	3.1
SD	14.1	4.2	4.1	1.8	13.2	2.6	14.0	2.5
Recovery (%)	36	4	4[a]	2[a]	7[a]	2[a]	18	2

[a]These values are the percentage of recovery of daidzein ingested. *O*-DMA = *O*-desmethylangolensin.

5.30 Most of the isoflavones were recovered in the faeces 2-3 days after ingestion. Excretion of total diphenolics, including equol, was often higher in the second and third days than in the 24 hours after ingestion. This suggests that faecal isoflavones and equol were derived from biliary excretion. Total recovery of daidzein and its metabolites, *O*-DMA and equol, from urine and faeces was ~55% whereas ~20% of administered genistein was recovered unchanged. Plasma half-lives for genistein and daidzein were 8.4 and 5.8 hours, respectively.

5.31 Significant inter-individual variation was observed in the plasma and urinary levels of equol and *O*-DMA. In addition, a biphasic peak in plasma and urine genistein and daidzein concentrations was observed in a number of subjects, suggesting enterohepatic circulation.

5.32 A recently completed study investigated the pharmacokinetic profile of ^{13}C-labelled daidzein and genistein administered to pre-menopausal women (n=20). Four separate experiments were conducted. In the first, subjects received a single oral dose of 0.4 mg/kg bw of each compound. This was repeated in the second experiment to assess intra-individual variability in metabolism. To assess the effects of dose on pharmacokinetics 0.8 mg/kg bw doses were administered in the third experiment and in the fourth 0.4 mg/kg bw doses were administered following ingestion of 50 mg/kg bw isoflavones in food for 7 days (Setchell *et al*, 2003).

5.33 No significant differences in serum and urinary profiles were observed between repeat doses demonstrating little intra-individual variability in metabolism of daidzein and genistein. Mean plasma half-lives for both compounds were 7.7 hours. Peak plasma concentrations were greater for genistein than daidzein at the doses used, suggesting genistein is the more bioavailable isoflavone. The bioavailability of both compounds, as assessed by AUC (area under the curve) increased with dose. Both compounds had a large volume of distribution. The mean recoveries of daidzein and genistein were 30% and 9%, respectively, suggesting significant metabolism to other compounds (Setchell *et al*, 2003).

5.34 A mean half-life of 9.2 hours was reported for total genistein in a study of men (n= 30) ingesting a single dose of isoflavones (1-16 mg isoflavones/kg bw, > 90% genistein) (Busby *et al*, 2002). At the highest dose the plasma C_{max} for total and free genistein was 7.7 and 0.07 μM, respectively, indicating extensive conjugation by the liver.

5.35 Howes *et al* (2002) reported mean half-lives for the isoflavones genistein, daidzein, formononetin and biochanin A of 13, 16, 23 and 18 hours, respectively, after chronic ingestion of a red clover supplement (1.5, 1.5, 16 and 24.5 mg genistein, daidzein, formononetin and biochanin A, respectively) in 14 subjects. Mean plasma C_{max} ranged from 0.04 (formononetin) to 0.5 μM (genistein).

5.36 In a study by King & Bursill (1998) the plasma and urine concentrations of daidzein and genistein peaked 7 to 8 hours after ingestion of a single soybean based meal by adult men (n= 6). Elimination half-lives were approximately 5 and 6 hours for daidzein and genistein, respectively. Although the urinary excretion of daidzein was greater than that for genistein, the ratios of the plasma AUCs for the respective isoflavones were similar to the ratio of concentrations present in the soybean meal, indicating similar bioavailabilities for the two isoflavones (King & Bursill, 1998). Similar findings were reported by Xu *et al* (2000). These studies apparently conflict with those of Watanabe *et al* (1998) and Setchell *et al* (2003). However, a study by Setchell *et al* (1998) employing pure isoflavones administered as a single bolus dose, has also demonstrated that the bioavailability of daidzein and genistein (determined from plasma appearance and disappearance curves) are similar. Peak plasma concentrations were usually attained between 6-8 hours after ingestion and plasma half-lives were approximately 8 hours.

5.37 In a study reported by Xu *et al* (1994), the plasma concentrations of daidzein and genistein in women (n= 12) were both significantly increased 6.5 hours after the consumption of a soy milk powder drink (containing a daidzein:genistein ratio of 44:56). Faecal excretion was low (1-2% of isoflavones ingested). On the basis that urinary excretion of daidzein (21%) was much higher than that of genistein (9%), the authors concluded that daidzein had a greater bioavailability.

5.38 Coward *et al* (1996) reported that the ratios of genistein to daidzein concentrations in plasma did not vary significantly from that present in a soy beverage, thus suggesting bioavailability of the two isoflavones in humans was similar. However, daidzein conjugates were reported to be more bioavailable than genistein conjugates, when administered to rats as a soy extract (King, 1998).

5.39 The pharmacokinetics of genistein and daidzein was compared with that of their glucosides in premenopausal women (n= 19) fed a 50 mg single bolus dose of each compound (Setchell *et al*, 2001). All compounds were efficiently absorbed from the intestinal tract however, the glucosides took longer to achieve maximum plasma concentrations (9.3 and 9.0 hours compared with 5.2 and 6.6 hours for genistein and daidzein, respectively). The bioavailability of the isoflavones was greater when ingested in the glucoside form.

5.40 Gender differences in bioavailability were reported in rats (Coldham & Sauer, 2000). The mean total excretion of ^{14}C-genistein, after an oral dose of 4 mg/kg bw for male and female rats, was approximately 67% and 33% in faeces and urine, respectively, within 168 hours of dosing. Mean and maximal concentrations in the plasma were higher in male than in female with half-lives of 12.4 hours and 8.5 hours, respectively.

5.41 In a study by Janning *et al* (2000), the plasma concentration-time curve following intravenous administration of daidzein to female rats could be fitted to a triexponential model with a final half-life of 4 hours. The oral bioavailability was 9.7% and 2.2% when 10 and 100 mg daidzein/kg bw, respectively, were administered, which suggests that absorption is saturable. Multiple peaks in the plasma concentration-time profile, which is suggestive of enterohepatic circulation.

5.42 Supko & Malspeis (1995) reported a pharmacokinetic profile for genistein in the male mouse. Plasma profiles of genistein after intravenous injection showed a prominent secondary peak 1-hour after administration followed by decay phase with a mean half-life of 40 minutes. The bioavailability of genistein (180 mg/kg bw) after oral gavage was 12% producing a peak plasma concentration of 3.7 μM. Plasma levels after intraperitoneal injection of genistein (185 mg/kg bw) were approximately 5- fold higher than achieved by the oral route.

Lignans

5.43 Nesbitt *et al* (1999) examined the urinary and plasma profiles of the lignans, enterolactone and enterodiol following single (day 1) and repeated (days 1-7 or 8) administration of flaxseed. Dosages of 5, 15 or 25 mg of raw or processed flaxseed (in the form of bread or a muffin) were given to nine women. The subjects were dosed during the follicular phase of their menstrual cycle and otherwise maintained on a low-lignan, low-fibre diet. The urinary excretion of lignans was dose-dependent and unaffected by the form in which the flaxseed was ingested. Enterodiol was the predominant lignan in urine and plasma, although considerable variation in the ratio of enterolactone:enterodiol was evident among individuals. Significant increases in plasma concentrations of lignans were observed within 9 hours of the first dose. These plasma levels were maintained at 12 and 24 hours[10] post administration. Significantly higher concentrations of lignans were excreted within 12-24 hours after dosing when compared to the first 12 hours. The plasma AUC after repeated administration was higher than after single dose administration indicating bioaccumulation.

[10] Plasma half-lives for the lignans could not be determined from the data. However, the data did indicate a slow absorption and/or excretion of these compounds.

5.44 Morton *et al* (1997) also reported comparatively slower plasma accumulation of lignans compared to isoflavones. An increase in serum concentrations of daidzein and genistein were observed within 30 minutes following consumption of a cake containing soybean flour and cracked linseed, in male subjects reaching a maximum within 5.5-8.5 hours after ingestion. In contrast, increases in the plasma levels of enterolactone and enterodiol were not observed until 8.5 hours after ingestion.

5.45 Jacobs *et al* (2002) examined the plasma profile of enterolactone following consumption of wholegrain foods or refined-grain foods in adults aged between 26-54 years. In this crossover feeding study men (n= 5) and women (n= 6) were assigned to a 6 week diet, with an intervening washout period of 6-9 weeks. Despite considerable variation in baseline serum enterolactone between and within subjects, differences in serum enterolactone concentrations in subjects on the two diets was evident after 2 weeks and continued to increase over the 6-week period. Concurrent antibiotic treatment, administered in two subjects only, was found to reduce serum enterolactone concentrations. However, concentrations recovered after 2 weeks in the subject on whole grain diet whereas, concentrations remained lowered in the subject on the refined-grain diet.

5.46 A crossover intervention trial by Juntunen *et al* (2000), studied the effect of rye bread on serum and urine enterolactone in healthy men (n= 18) and women (n= 21) all aged 43 years. Groups consumed wholemeal rye bread for 4 weeks followed by washout period. The mean concentration of serum enterolactone was significantly lower in men following consumption of rye bread compared with women (26 versus 40 nM) suggesting pharmacokinetic differences in lignan metabolism. However, mean concentrations of urinary enterolactone were similar between men and women (5.1 versus 4.9 μM).

5.47 A single meal of lignan-rich berries resulted in elevated plasma levels ($p<0.05$) and increased urinary excretion of the mammalian lignan enterolactone 24 hours post challenge (Mazur *et al*, 2000). Adults aged 23-55 (n= 5) were maintained on a phytoestrogen free regime for 72 hours before consuming a single meal of strawberries (500 g). The strawberries contained secoisolariciresinol (12 mg) and matairesinol (1 mg). In subjects, 24 hour urinary enterolactone concentrations ranged from 0.9-6.6 μmol enterolactone suggestive of inter-individual variations in metabolism and gut microflora.

Summary of pharmacokinetic findings

5.48 There is a paucity of data regarding the pharmacokinetics of phytoestrogens, particularly with respect to the lignans. To date, only one published study has concurrently determined the appearance and elimination of soy-derived isoflavones and their major metabolites in plasma, urine and faeces. In most of the studies reported to date, the total recovery of dose is not stated and unlikely to be 100% since the complete metabolic fate of the dose is never established.

5.49 In general, peak concentrations of daidzein and genistein are achieved within 5-8 hours after ingestion. Plasma concentrations of genistein and daidzein begin to rise within 2 hours of an ingested dose and can occur as early as 15 minutes after ingestion. It has been observed that a number of individuals exhibit more than one plasma peak, which probably reflects enterohepatic circulation of the isoflavones. The plasma half-lives for genistein and daidzein have been estimated at 5-8 hours.

5.50 There is debate as to whether aglucones are more bioavailable than the glucosides. This may ultimately be attributed to inter-individual variation and the gut microflora population (see paragraphs 5.76-5.78). Studies in rats have shown the bioavailability of aglucones and glucosides to be similar. The majority of isoflavones are excreted in urine, either as parent compound or metabolites with only a small percentage of absorbed isoflavones appearing in the faeces either as parent compounds or metabolites. The data indicate considerable inter-individual variation in plasma and excretion profiles for daidzein and genistein and their metabolites. Some individuals produced little or no *O*-DMA and equol (Kelly *et al*, 1995; Setchell *et al*, 1984; Morton *et al*, 1994; Slavin *et al,* 1998; Lampe, 1998; Rowland *et al*, 1999). Repeat dosing of isoflavones may lead to accumulation.

5.51 Similarly, there is conjecture over the comparative bioavailability of genistein and daidzein. The bioavailability of these isoflavones appears to be similar when administered in a pure form. However, it differs when administered as part of foodstuffs, suggesting the bioavailability may be dependent upon the form in which they are ingested.

5.52 Inter-study differences in the various pharmacokinetic parameters for genistein and daidzein probably reflect the differing matrices in which the isoflavones were administered (e.g. soyflour-based meal versus powder drink or fermented soy versus purified isoflavones). Inter-individual differences such as gender, age and dietary habits may have also contributed to the differences observed between studies of isoflavones and lignans.

Excretion

5.53 Urine and bile are both important excretory routes for phytoestrogens. Several bacterial metabolites of isoflavones and lignans have been detected in urine and faeces. Conjugates excreted in the bile can undergo deconjugation by gut bacteria and undergo enterohepatic circulation (see Figure 1). Elimination via the faeces is thus largely determined by the degree of enterohepatic circulation, which may result in prolonged exposure to these compounds.

5.54 Short-term studies have shown that no more than 30% of an ingested dose of isoflavone is recovered from the urine and faeces in a diphenolic form. It has been suggested that this low recovery is the result of extensive microbial degradation of isoflavones, producing simpler phenols such as *p*-ethyl-phenol (Coldham *et al*, 1999; Setchell, 1998).

5.55 The pattern of phytoestrogen excretion reflects that of food intake. Thus, in Western populations, where the typical diet is relatively low in isoflavones, urinary levels of isoflavones tend to be lower than lignan levels. Concentrations of total phytoestrogens are relatively low in most subjects consuming omnivorous diets not containing soy-based foods. In contrast, vegetarians and individuals consuming macrobiotic diets tend to have higher urinary excretion of phytoestrogens, particularly lignans (Adlercreutz *et al*, 1987). Intervention studies suggest that urinary excretion of isoflavones increases with increased soy intake, but absorption, as reflected by urinary excretion, may be saturable at high doses (Karr *et al*, 1997; Bingham *et al*, 1998; Setchell, 1998).

5.56 The urinary concentration of total lignans and isoflavones and their metabolites in a group of Japanese women (age= 47 ± 12 years) consuming a traditional diet was compared to different ethnic populations. These included macrobiotic lactovegetarian (n= 11) and omnivorous (n= 10) women from USA (age= 56 ± 3 years), and lactovegetarian (n= 11) and omnivorous (n= 12) women from Finland (age= 33 ± 6 years) (Adlercreutz *et al*, 1989; 1991). In comparison to the Japanese, lignan (enterolactone and enterodiol) excretion was greater in Finnish and American women. Omnivorous women had a relatively low excretion of daidzein, *O*-DMA and equol. However, the excretion of these compounds by vegetarian and macrobiotic women was comparable to that of the Japanese (see Table 5.3).

Table 5.3 Excretion of lignans and isoflavones and their metabolites in different populations.
Adapted from (Adlercreutz *et al*, 1986; 1989; 1991).

	Total lignans[a] (μmol/day)	Total isoflavones and metabolites[b] (μmol/day)
Japanese Women		
(n= 10)	1.4	4.7
Finnish women		
Omnivores (n= 12)	2.4	0.2
Lactovegetarians (n= 11)	6.1	0.5
American women		
Omnivores (n= 10)	2.1	0.3
Lactovegetarians (n= 11)	4.3	1.6
Macrobiotics (n= 13)	26.6	6.2

[a] Total lignans is the sum of enterolactone and enterodiol.

[b] Total isoflavone and metabolites is the sum of daidzein, *O*-DMA and equol.

5.57 Roach *et al* (1998) reported the mean urinary excretion of total isoflavones was 5.4 μmol/day in a study of postmenopausal Chinese women (n= 21, mean age= 46 years).

5.58 Maskarenic *et al* (1998) investigated the association between dietary isoflavone intake and urinary excretion of isoflavones and metabolites among women of different ethnicity residing in Hawaii. The study group consisted of women of Asian ancestry (aged 36-80 years) who had maintained some of their traditional dietary habits. Self-administered food questionnaires indicated dietary isoflavone intake differed significantly among ethnic groups, with Japanese and Chinese women consuming highest levels of isoflavones (see Table 5.4). The data illustrate possible ethnic differences in excretion of isoflavones. For example, although the dietary intake of isoflavones for Chinese and Japanese was similar, Japanese women had greater isoflavone excretion rates. However, this could also be due to a number of other factors such as overall diet, food matrix and transit time, which are discussed later in this chapter. Nevertheless, there was good correlation between urinary isoflavone excretion rates and dietary intake over 24 hours between the respective ethnic groups.

5.59 Lampe *et al* (1999) found that the urinary isoflavones and their metabolites as well as lignans in an USA population were significantly higher in "high" compared to "low" fruit and vegetable consumers. However, there was also a positive correlation between high fat and processed meat intake and isoflavone and metabolite excretion that could not be explained.

Table 5.4 Mean dietary isoflavone intake and urinary isoflavone and metabolite excretion among Hawaiian women by ethnicity.

Isoflavone intakes estimated from self-administered questionnaires on usual eating habits over 24 hours. Age of study population 36-80 years. Modified after Maskarinec *et al* (1998).

Phytoestrogen intake/ excretion	Chinese (n=13)	Filipino (n=7)	Hawaiian (n=11)	Japanese (n=25)	Caucasian (n=42)
Dietary isoflavone intake during previous 24 h (mg/day)	38.2	5.0	22.2	31.3	6.9
Total urinary phytoestrogen excretion (nmol/h)	307.6	77.6	293.7	724.7	138.9
Daidzein (nmol/h)	187	44	179	442	79
Genistein (nmol/h)	65	12	72	134	28
Glycitein (nmol/h)	24	4	20	88	14
Coumestrol (nmol/h)	0	3	0	0.1	13

5.60 Atkinson *et al* (2002) found that urinary isoflavones were higher in soy consumers than non-consumers ($p < 0.001$). In this study, 312 American women, aged 25-59 years, completed food frequency questionnaires on their dietary habits of the previous 3.5 years. Women from Asian backgrounds reported greater frequency of soy consumption (75% of Asian women) compared with non-Asian women (28% of non-Asian women). Excretion rates also correlated with dietary intakes as determined by 1- day food frequency questionnaires completed 24 hours prior to urine collection ($p<0.01$).

5.61 The excretion of daidzein in the rat was investigated after a single oral dose of 100 mg daidzein/kg bw (Bayer *et al*, 2001). Total urinary excretion accounted for < 10% of the dose. In males, the urinary metabolites were identified as daidzein, daidzein-sulfate and daidzein-glucuronide. Only daidzein and daidzein-glucuronide were identified in the females. The major pathway of excretion of daidzein in males and females was as unchanged compound in the faeces. Other faecal metabolites such as equol and *O*-DMA accounted for < 5% of the dose. Elimination half-lives for daidzein and its metabolites was < 12 hours. Gender differences in daidzein metabolism in rats may be attributed to the differential expression of hepatic phenolsulfotransferases (Dunn & Klaassen, 1998).

5.62 Coldham & Sauer (2000) found, following an oral dose of ^{14}C-labelled genistein (4 mg/kg bw) to male and female rats, that 66% and 33% of the dose was excreted in urine and faeces, respectively. In addition, 4-hydroxyphenyl-2-propionic acid was the exclusive urinary and faecal metabolite excreted by males but in addition females excreted genistein and dihydrogenistein glucuronides and sulfates in urine and faeces (Coldham *et al,* 1999; Coldham & Sauer, 2000).

5.63 Barnes *et al* (1996) examined the enterohepatic recirculation of ^{14}C-labelled genistein (0.17 mg) in bile duct cannulated female rats. Genistein (70-80% of the administered dose) appeared in the bile as the glucuronide metabolite. Re-administration of this metabolite into the upper-small intestine resulted in delayed recovery in the bile. Urinary recovery following infusions with ^{14}C-labelled genistein and ^{14}C- labelled genistein glucuronide was 2.4 and 7.4%, respectively.

Inter-individual variation in ADME

5.64 Human metabolism and excretion of isoflavones and lignans are subject to considerable inter-individual variation (Setchell, 1998). Variation in the urinary excretion of daidzein, genistein and glycitein and more marked variation in the excretion of *O*-DMA, 6'OH-DMA, and in particular equol, has been shown following an oral soy challenge (Kelly *et al,* 1993). Several studies have suggested that approximately one third of the population are capable of equol production (Kelly *et al*, 1995; Setchell *et al*, 1984; Morton *et al*, 1994; Slavin *et al*, 1998; Lampe, 1998). An example is illustrated in Figure 5.4 (Rowland *et al*, 1999). As a consequence, higher concentrations of precursor compounds appear in the urine of low equol excretors and inverse relationships have been established between equol and daidzein (Setchell, 1998) and equol and *O*-DMA and 6'OH-*O*-DMA excretion (Kelly *et al*, 1993). It has been suggested that low equol excretors will also have increased plasma levels of precursor compounds.

5.65 A recently completed study has also shown inter-individual variation in the urinary excretion of isoflavones and their metabolites following soy challenge in adults (Wiseman *et al*, 2003). In this study, 76 volunteers were fed either a high (104 ± 24 mg total isoflavones/day) or low (0.5 ± 0.5 mg total isoflavones/day) soya diet for 10 weeks. Volunteers on the "high" soya diet showed extensive urinary excretion of daidzein, genistein and their metabolites. Of the volunteers on the high soya diet 34% were identified as good equol excretors (> 1000 nmol/24 hours). Comparative analysis of the faecal flora between equol and non-equol producers was investigated, however, the microflora (bacteria) responsible for equol production could not be isolated and therefore, were not be identified.

5.66 Inter-individual variation in the ADME of lignans has also been reported. Nesbitt *et al* (1999) showed that 2/9 female subjects produced little or no enterolactone following ingestion of lignans from flaxseed. Lampe *et al* (1994) also reported considerable differences in the ratios of urinary enterolactone and enterodiol in 30 women, following ingestion of flaxseed. These data suggest inter-individual variation in the ability to oxidise enterodiol to enterolactone.

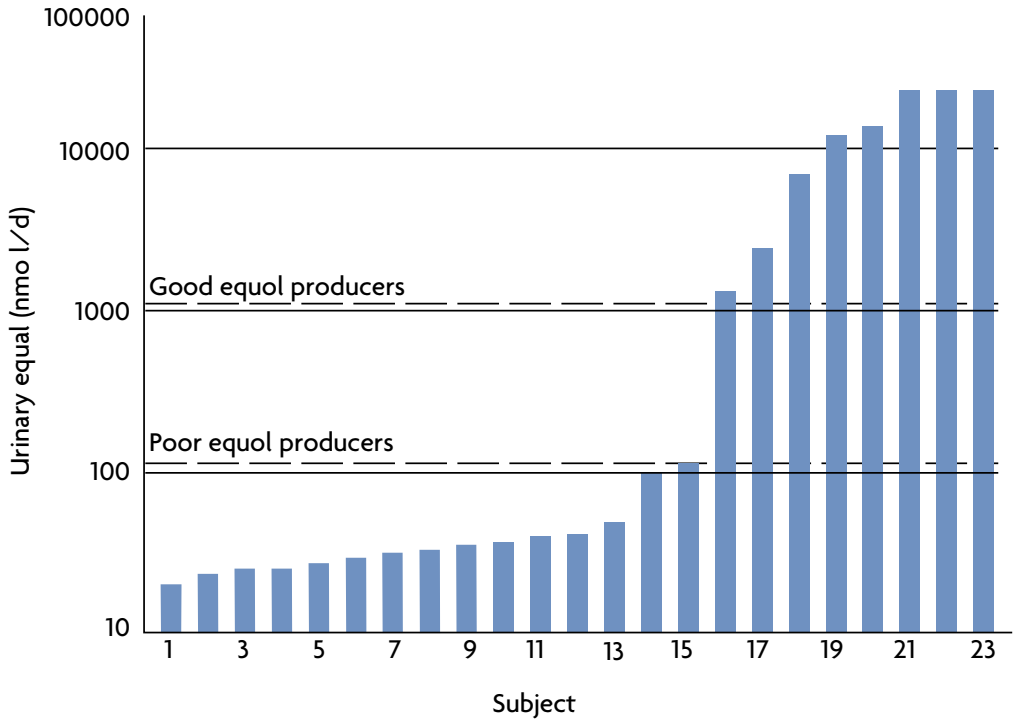
Diet

5.67 Studies in humans have shown that diet can influence metabolism of phytoestrogens mediated by the gut microflora. For example, consumption of less fat and more carbohydrate, as a proportion of total energy intake, has been correlated with greater equol production, particularly in women (Slavin *et al*, 1998; Lampe *et al*, 1998). The reason for this is unclear but it is possible that complex carbohydrates stimulate fermentation in the large bowel, resulting in an increased breakdown of daidzein to equol (Bingham *et al*, 1998; Rowland *et al,* 1999; 2000). Co-administration of dietary fructo-oligosaccharides with intragastric administration of isoflavone glucones has been shown to modify the absorption and enterohepatic circulation in male rats (Uehara *et al*, 2001). Furthermore, low-fat diets are associated with decreased ß-glucuronidase activity in the intestinal contents, which may impede absorption of more fat-soluble deconjugated compounds (Adlercreutz *et al*, 1987).

5.68 Dietary fibre has been shown to affect the absorption, reabsorption and excretion of oestrogens and phytoestrogens by influencing the ß-glucosidase and ß-glucuronidase activities of the intestinal microflora. The bulking effect of dietary fibre, which results in the dilution of gut microflora activity, and the hydrophobic bonding, particularly of non-conjugated compounds, are thought to contribute to a reduction in absorption and reabsorption of isoflavones (Tew *et al*, 1996; Tham *et al* 1998). Vegetarians generally have higher faecal weights than omnivores, and a lower faecal bacterial ß-glucuronidase activity (Adlercreutz *et al*, 1987). The implication is that high dietary fibre could result in the partial disruption of enterohepatic circulation of phytoestrogens and endogenous oestrogens. Increased dietary fibre intakes have also been associated with equol excretion, in females (Lampe *et al*, 1998).

5.69 The effect of diet on the metabolism of phytoestrogens, both from the influence on gut microflora and differences in hepatic enzyme activities, may in part explain, any ethnic differences in the metabolic, and perhaps biological, response to phytoestrogens.

Figure 5.4 Equol excretion in 23 subjects consuming soy isoflavones from extruded soy protein flour (56 mg isoflavones/day) for 17 days.
(Adapted from Rowland *et al*, 1999)



Gender

5.70 It has been claimed that there are gender differences in the metabolism and urinary excretion of isoflavones. Data reported by Lu & Anderson (1998) showed that during a one-month trial in which soymilk was ingested (80-210 mg each of genistein and daidzein/day) the excretion half-life progressively shortened in women but progressively lengthened in men throughout the trial.

5.71 Zhang *et al* (1999) reported gender differences in urinary glycitein, but not daidzein and genistein, following ingestion of soymilk. However, the sample size of these studies was small and these findings have not been reported in other studies (Setchell, 1998). It has been reported that men excrete a higher ratio of enterolactone:enterodiol than women, suggesting a gender difference in the colonic bacterial metabolism of lignans (Kirkman *et al,* 1995). No difference in the urinary excretion of isoflavones was observed in this study, however the number of subjects in this study was small which limits the validity of this conclusion.

5.72 Gender differences have also been reported in humans for the serum concentrations of the lignan enterolactone. Jacobs *et al* (2002), reported higher serum enterolactone in women than in men (20 versus 6 nM) fed on wholegrain and refined grain diets. Although this was a small study (n= 11), this gender-based difference remained apparent throughout a 6 week treatment period.

5.73 A smaller gender difference in the serum enterolactone concentrations was reported in a large study of Finnish adults (n= 2753) following normal consumption of lignan-containing foods (Kilkkinen *et al,* 2002). Men and women were reported to have baseline serum enterolactone concentrations of 17.7 and 20.9 nM, respectively.

5.74 Gender differences in the ADME of genistein (Coldham *et al*; 1999; Coldham & Sauer, 2000) and daidzein (Chang *et al*, 2000) have been reported in rats. Dihydrogenistein was identified as the major product in the faeces of female rats at 48 hours in contrast to the male where 4-hydroxyphenyl-2-propionic acid was identified as the major metabolite. Furthermore, genistein appeared to be more extensively sulfated in the liver of males compared to females (Coldham *et al*, 1999; Coldham & Sauer, 2000).

Age

5.75 With the exception of the data from neonates and infants (see paragraphs 5.82-5.86), there have been no reports of age related differences in the metabolism of phytoestrogens (Adlercreutz *et al*, 1993).

Inter-individual variability in gut microflora mediated metabolism

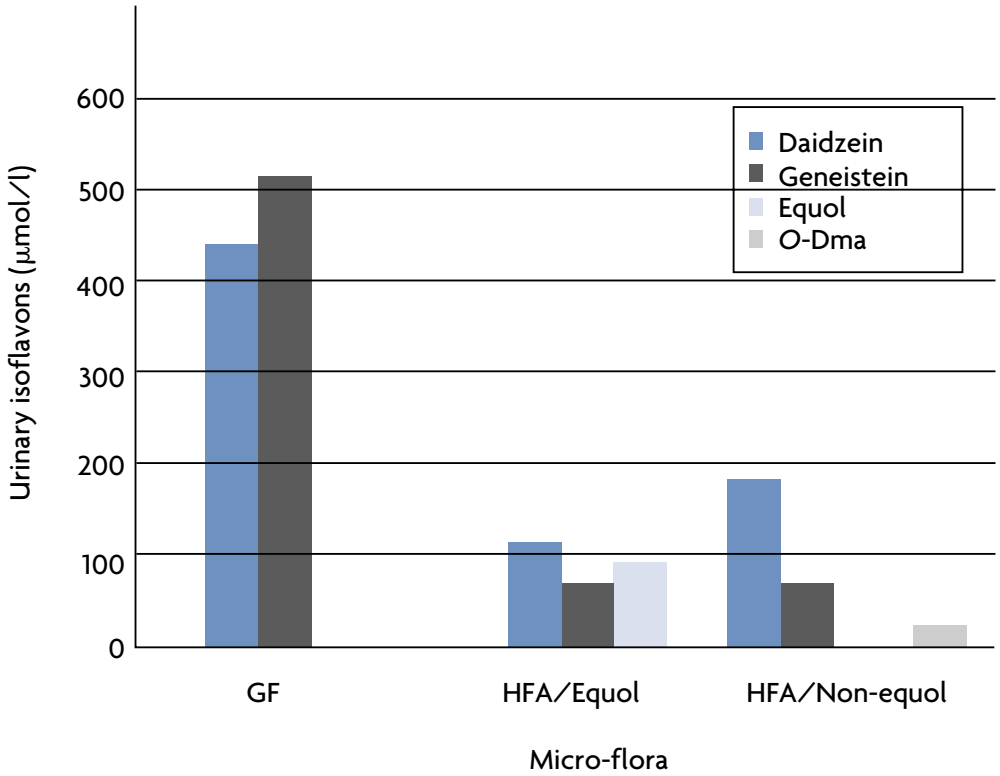
5.76 The large inter-individual variation in phytoestrogen metabolism is likely to be largely a consequence of inter-individual differences in the gut microflora involved in metabolising these compounds. Various physiological, pathological and environmental factors are likely to influence gut bacterial profile, including hygiene, antibiotic use, bowel disease, stress, gut motility, gastric pH, mucin secretion, bile secretion, diet and intestinal transit time. Gender, genetics and ethnicity may have a role in influencing gut bacterial profile although this remains to be clearly established (Kirijavainen & Gibson, 1999; Rowland *et al*, 1999).

5.77 In a study by Kilkkinen *et al* (2002), the use of oral microbial medicines was found to decrease serum enterolactone concentrations in a study of Finnish adults (n= 2753). Serum enterolactone levels were found to be lower in subjects that had used antimicrobials up to 12-16 months before sampling than in non-users (mean 16 versus 19 nmol/L). Enterolactone concentrations were also reduced with increasing number of antimicrobial treatments, although concentrations recovered with length of time from the last administration.

5.78 The influence of gut microflora on inter-individual variability has been demonstrated in studies carried out in germ-free rats, which are devoid of gut microflora. The animals were fed soy protein and found to excrete daidzein and genistein in urine but not the daidzein metabolites, equol or *O*-DMA. Colonisation of the same rats with bacterial flora from a human subject capable of converting daidzein

to equol resulted in the substantial excretion of equol, but not *O*-DMA. Germ-free rats, colonised with flora from a non-equol-producing human, produced no detectable urinary equol and only trace amounts of *O*-DMA, when fed with soy protein (see Figure 5.5).

Figure 5.5 Excretion of soy isoflavones by germ-free (GF) and human-flora-associated rats (HFA) rats. GF rats were associated with a faecal flora either from an equol-producing subject (HFA/equol) or a non-equol-producing subject (HFA/non-equol). The rats were then fed a diet containing soy protein. Germ-free rats fed soy excreted only daidzein and genistein and the putative bacterial metabolites equol and *O*-DMA were not detected. HFA/equol rats excreted substantial amounts of equol. In comparison, equol was not detected in HFA/non-equol producing animals although small amounts of *O*-DMA were formed (adapted from Rowland *et al*, 1999).



Food matrix and transit time

5.79 The effects of the food matrix and intestinal transit time on phytoestrogen metabolism have yet to be investigated.

Plasma protein binding

5.80 In the plasma, the extent of free phytoestrogen available for biological interaction (e.g. with oestrogen receptors) is determined by the extent to which they are bound to plasma proteins. Binding to plasma proteins renders them unavailable to interact with other molecules (plasma protein binding is discussed further in Chapter 7).

Hepatic metabolism

5.81 Biotransformation of phytoestrogens in the liver, involving phase I and II metabolism may be subject to the influence of genetic polymorphisms and influenced by environmental factors, including exposure to drugs and dietary components (for a review, see Pelkonen *et al*, 1998). These factors will all contribute to variability between individuals and the non-genetic factors will also contribute to variability within an individual. It is also important to recognise that should phytoestrogens inhibit or induce xenobiotic metabolising systems then they have the potential to alter the metabolism of other compounds including drugs. The implications of this are discussed by Evans (2000).

Bioavailability and metabolism of phytoestrogens in the new-born and infants

5.82 On a body weight basis, some of the highest intakes of isoflavones can occur in very young babies (4 months old) fed soy-based infant formulae (see Chapter 4). It has been suggested that the plasma levels achieved may be high enough to elicit biological effects (Setchell *et al*, 1997). However, several factors may influence age-related differences in the pharmacokinetics and metabolism of phytoestrogens in adults and babies. These include the nature of the gut microflora, which can influence the absorption, metabolism and reabsorption of isoflavones. Gut microflora can, to a limited extent, also influence body water content, which is higher in neonates and could result in an altered distribution of hydrophilic compounds. However, clearance of foreign compounds in infants can be relatively high when compared to adults for some foreign compounds on the basis of a relatively greater liver weight:body weight ratio (Renwick, 1998).

Development of the gut microflora in the new-born and infant

5.83 It is unclear at what age an infant acquires the gut microflora necessary for the metabolism and absorption of isoflavones. Setchell *et al* (1997; 1998) reported that 4-month-old infants fed soy-based infant formula (approximately 28-47 mg isoflavones/day) absorb isoflavones efficiently. Plasma concentrations of isoflavones in these infants were approximately 4 μM and were judged to be 2-5 fold higher than peak plasma concentrations in adults consuming isoflavones (50 mg/day). However, it appeared that the gut microflora in these infants were not fully developed beyond the initial hydrolysis of the glucosidic moiety since plasma levels of the isoflavone metabolite, equol, were found to be very low. Cruz *et al* (1994) found low concentrations of equol in infants fed soy-based formulae. However, a study reported by Irvine (1998), suggested that infants can absorb and excrete daidzein and genistein derived from soy- based infant formulae as efficiently as adults consuming soy products from the age of

4 weeks did. However, there were only four infants in this study who received soy- based formula thus the small numbers make it difficult to generalise on these findings.

5.84 A recently completed study in human infants (4-6 months) and children (> 6 months) showed that infants fed soy-based infant formula excreted significant quantities of genistein, daidzein and glycitein in their urine. *O*-DMA was detected in 3/6 infants and a small quantity of equol in one infant in the 4-6 month group (Rowland *et al*, 2003). However, in age groups > 6 months, *O*-DMA and low levels of equol were found in urine from 12/16 and 3/16 subjects, respectively. This suggests that the ability to convert daidzein to *O*-DMA develops early in infancy, while the ability to produce equol develops later.

5.85 The gut is sterile at birth, but within a week of birth a microflora begins to develop and the profile continues to change from infancy into adulthood (Klein, 1998). Factors such as the composition of maternal gut flora, the mode of delivery (conventional or caesarean birth), hygiene, environment and genetics determine initial colonisation. The intestine of the new-born has a higher redox potential compared to adults, therefore the gut microflora which first colonises the gut must be capable of oxidative metabolism and typically include *Enterobacteria*, *Streptococci* and *Staphylococci*. These facultative bacteria rapidly metabolise oxygen to provide a lower redox potential, thereafter allowing strictly anaerobic bacteria, such as *Bifidobacteria*, *Clostridia* and *Bacteroides,* to flourish.

5.86 The type of feed the infant is given also influences the microflora. Faecal populations diversify more rapidly in formula-fed babies than in breast-fed babies (Kirjavainen and Gibson, 1999). Bacterial enzyme activities increase with age and are greatly influenced by the adoption of an adult diet (Mykkanen *et al*, 1997). The influence of the diet is greater on the gut microflora of babies who were breast-fed than those who were fed infant formula.

Isoflavones from soy-based formulae compared with human breast or cows milk

5.87 Soy-based formulae contain isoflavones largely in the form of glucosides and malonyl derivatives. In contrast, human breast milk and cows' milk contains much lower levels of total isoflavones. The proportion of free to conjugated isoflavones in breast milk has not been reported. Human and cow's milk also contains equol, and the levels present are determined by maternal diet (Setchell *et al*, 1997). Equol is not present in soy formula (see Chapter 4).

Transfer of isoflavones to breast milk

5.88 A rapid and dose dependent increase in isoflavones was observed in breast milk following ingestion of 5, 10 or 20 g roasted soybeans (containing 0.08, 0.15 or 0.30 mg daidzein/kg and 0.08, 0.17 or 0.33 mg genistein/kg respectively). Isoflavones were detected in breast milk slightly later than they appeared in urine. The maximum concentrations in breast milk were attained 10-14 hours after ingestion and were followed by a smaller secondary peak. This biphasic pattern was also evident in both plasma and urine and is thought to be a reflection of enterohepatic circulation. A return to baseline levels was complete within 2-4 days. The pattern of metabolites found in the breast milk reflected that observed in plasma (Franke & Custer, 1996; Franke *et al*, 1998).

5.89 No studies have measured the transfer of lignans, prenylated isoflavonoids and coumestrol to breast milk.

Placental transfer of phytoestrogens

5.90 Genistein (20, 34 and 75 mg/kg bw) was administered by oral gavage to pregnant rats (n= 3) 20-21 days after confirmation of pregnancy (Doerge *et al*, 2001). Genistein concentrations were measured in maternal and fetal plasma and fetal brain 2 hours after administration. Genistein concentrations were lower in fetal plasma compared to maternal plasma and unlike maternal plasma, concentrations did not increase dose dependently (see Table 5.4). However, a greater proportion of genistein was present in fetal plasma in unconjugated form. Concentrations of genistein in fetal brain were much lower than in fetal plasma but were predominantly in unconjugated form.

Table 5.4 Concentrations of genistein in maternal plasma and fetal plasma and brain.
Adapted from Doerge *et al* (2001).

Dose mg/kg bw	Maternal plasma µmol/L (% aglucone)	Fetal plasma µmol/L (% aglucone)	Fetal brain Pmol/mg (% aglucone)
20	3.5 (8%)	0.3 (31%)	0.2 (91%)
34	5.5 (5%)	0.2 (34%)	nd
75	4.4 (18%)	0.2 (27%)	0.2 (90%)

nd: Not determined.

5.91 In a study by Degen *et al* (2002), 10 mg daidzein/kg bw was administered intravenously to rats on day 18 of gestation. The fetal liver contained approximately 3% of the concentration found in maternal liver.

5.92 Little is known about trans-placental transfer of phytoestrogens in humans. To investigate this, Adlercreutz *et al* (1999) measured the level of isoflavones (daidzein, genistein, equol and *O*-DMA) and lignans (enterodiol and enterolactone) at delivery in the plasma of pregnant Japanese women (n=7) consuming soy-rich diets. Concentrations were also measured in umbilical cord plasma and amniotic fluid.

5.93 Daidzein, genistein, *O*-DMA, equol (metabolites of genistein were not measured) and lignans (enterolactone and enterodiol) were found in the amniotic fluid, maternal and cord plasma (Adlercreutz *et al*, 1999). Total concentrations of isoflavones and their metabolites ranged from 19-744 nM (mean 232 nM), 568-831 nM (299 nM) and 52-799 nM (mean 223 nM) in maternal plasma, cord plasma and amniotic fluid, respectively, indicating that phytoestrogens can cross from the maternal to the fetal compartment (Adlercreutz *et al*, 1999). The metabolic clearance of phytoestrogens may be slower in the fetus or neonate due to a relatively low glucuronidation capacity compared to adults. However, the concentrations detected in this study show *in utero* concentrations of phytoestrogens approximately 10-fold below those detected in the plasma of infants fed soy infant formula (genistein 2.5 µM; daidzein 1.2 µM (Setchell *et al*, 1997)).

5.94 Foster *et al* (2002) analysed samples of human amniotic fluid in women in their second trimester (15-23 weeks). Dietary phytoestrogens were detected in amniotic fluid of 50 of the 53 women tested. Mean concentrations of daidzein and genistein were reported to be 5.2 nM (max 20 nM) and 6.7 nM (max 26 nM), respectively. Biochanin A was also found at concentrations of 1.8 nM in 8 out of 12 women tested. None of the samples analysed contained quantifiable levels of formononetin or coumestrol.

Key points

- The isoflavones and lignans are ingested mainly as glucosides, which undergo hydrolysis most probably in the small intestine through the action of ß-glucosidase enzymes associated with the intestinal mucosa and in the lower bowel by the gut microflora. There is uncertainty on whether the bioavailability of isoflavones ingested as glucosides differs from that of the aglucones. The deglycosylated (aglucone) compounds may be further metabolised by the gut bacteria and/or absorbed. It is clear that the gut microflora play a crucial role in determining the absorption, metabolism, re-absorption (enterohepatic circulation), degradation and excretion of ingested isoflavones and lignans and their metabolites.

- Once absorbed, these compounds are rapidly and extensively re-conjugated, largely with glucuronic acid, but also with sulfate, and excreted in the bile or urine. Biliary conjugates are hydrolysed by the gut bacteria and/or excreted in the faeces or further metabolised and/or reabsorbed (enterohepatic circulation) or degraded.

- As a consequence, the activities of gut microflora influence the metabolic profile of phytoestrogens and may influence the bioavailability of phytoestrogens and/or their metabolites. Since biological activities/potencies of parent molecules and metabolites differ, this could have implications for the determination of any ensuing biological response, beneficial or adverse.

- Few studies have been undertaken that comprehensively describe the pharmacokinetics of ingested isoflavones and lignans. Data indicate considerable inter-individual variation in the pharmacokinetic and metabolic handling of ingested phytoestrogens. It is clear that the gut microflora greatly influences the exposure to ingested phytoestrogens and their metabolites by determining the extent to which they are absorbed, metabolised, reabsorbed and degraded. This seems to be particularly marked for the daidzein to equol and enterodiol to enterolactone conversions. Such differences may be largely attributed to an individual's unique gut microflora, which is influenced by factors including diet, particularly fibre content, and intestinal transit time, hygiene, antibiotic use, bowel disease, stress, gut motility, gastric pH, mucin secretion and bile secretion. Gender, age, genetics, food matrix and ethnicity may also be determining factors.

- There is limited information on how phytoestrogens are handled in the newborn and infants. The pharmacokinetics of absorption in the neonate is unclear but it is likely to differ considerably from that of the adult, particularly as the gut microflora in neonates is not fully developed. Data on the levels of isoflavones in the blood of infants fed soy-based formula suggest that they can absorb isoflavones from such formula.

- Isoflavones and their metabolites are widely distributed within body fluids, although definitive tissue distribution studies have not been performed in man. There is evidence of transfer of isoflavones and their metabolites to the fetal compartment as concentrations similar to those in maternal plasma have been detected in umbilical cord plasma and amniotic fluid. There is evidence for transfer of isoflavones to breast milk *via* the maternal diet. No studies on the transfer of lignans to breast milk have been conducted.

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6. Cellular and molecular mechanisms of oestrogen action

Introduction

6.1 Oestrogens are hormones involved in regulating the development, growth and function of many tissues in both males and females. Oestrogens act through proteins known as oestrogen receptors. Oestrogen receptor biology is an area of intense research interest and is far from being completely understood. As a consequence, the full extent of oestrogen interactions with oestrogen receptors and the resulting biological responses have yet to be fully elucidated. This chapter summarises what is currently known about oestrogen receptors and how oestrogens interact with them.

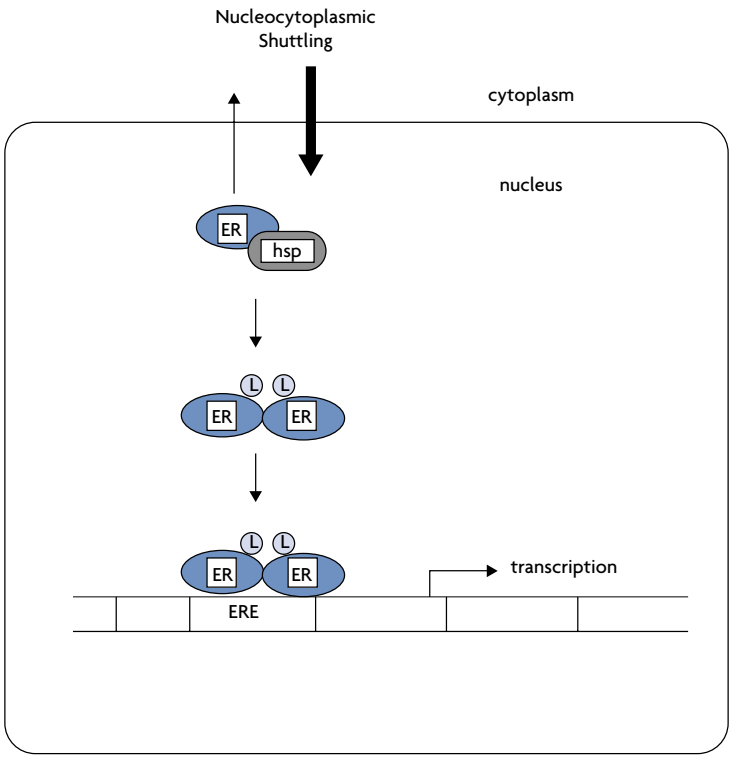
6.2 As shown in Chapter 3, phytoestrogens have similar chemical structures to the oestrogens and have been found to bind to oestrogen receptors. However, it should be acknowledged that most investigations contributing to the cellular and molecular action of oestrogens have not employed phytoestrogens, although there is reason to believe they will elicit oestrogenic effects using identical mechanisms.

Receptor mediated mechanisms

6.3 Oestrogens are involved in the growth, development and homeostasis of a number of tissues. The physiological effects of these steroids are mediated by ligand-activated nuclear transcription factors, the oestrogen receptors (ERs). The ERs and other steroid hormone receptors are members of the nuclear receptor family of transcription factors that exhibit common structural domains. ERs are predominantly located in the nucleus, although they can move between the nucleus and the cytoplasm, where they are complexed with heat shock proteins (hsp56, hsp90 and possibly hsp70) until activated by an appropriate ligand.

6.4 Bound ERs dissociate from hsps and subsequently dimerise and undergo a conformational change. The activated dimers have high binding affinities for specific DNA-binding sites called oestrogen receptor response elements (ERE) (see Figure 6.1), which are situated in promoter regions upstream of oestrogen-sensitive genes. Proteins known as coactivators or corepressors, are believed to be essential for ER action and influence the level of expression of oestrogen-responsive genes. Binding to the ERE results in the initiation or repression of target gene transcription and ultimately elicits a biological response (Clarke *et al*, 1996; Diel *et al*, 1999; Fitzpatrick, 1999; Gillesby & Zacharewski, 1998).

Figure 6.1 A mechanism for the receptor mediated induction of oestrogen responsive genes.
Oestrogen receptors can move between the nucleus and the cytoplasm, but under normal conditions they are predominantly located in the nucleus. Upon entering the cell, oestrogens bind to oestrogen receptors (ER) which are complexed with heat shock proteins (hsp). Upon ligand binding (L), the hsps dissociate from the receptor, allowing two ERs to dimerise and bind to DNA containing the oestrogen responsive elements (ERE) found within genes responsive to oestrogen. Receptor and ERE binding promotes gene transcription and the synthesis of protein which produces an oestrogenic response such as cell proliferation (Adapted from Parker, 1995).



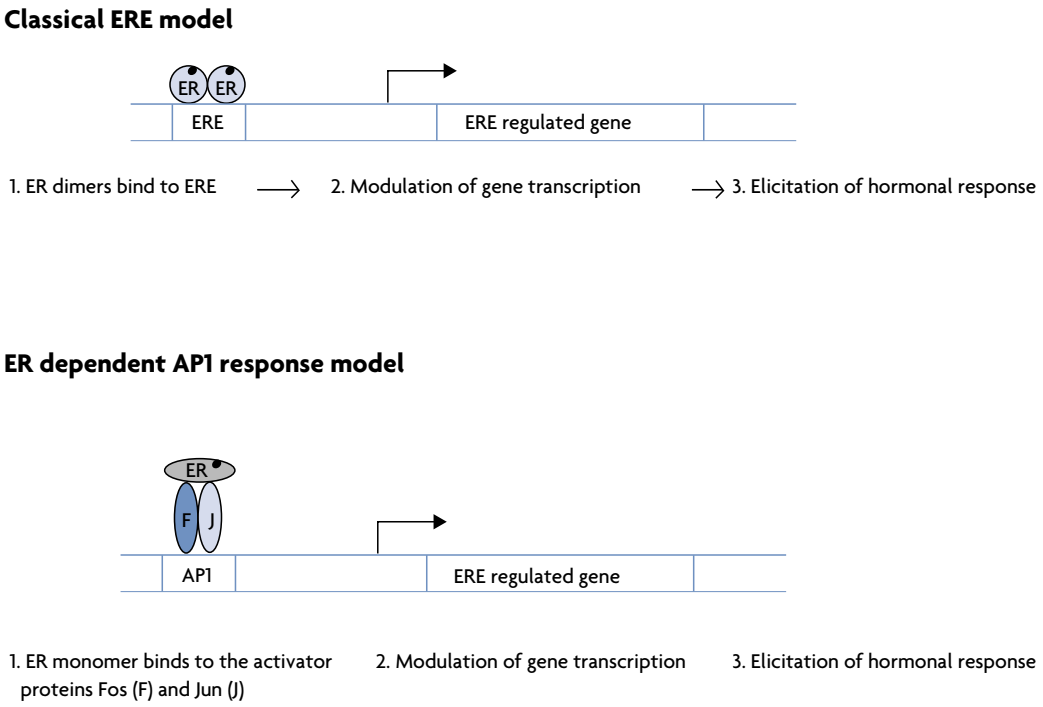
Oestrogen response elements (EREs)

6.5 It has been shown that the consensus ERE is a 13-base pair inverted repeat motif consisting of 2 AGGTCA (or minimally GGTCA) inverted repeats separated by 3 nucleotides (Parker, 1995). The symmetry of the sequence facilitates the binding of ER as a homodimer (see Figure 6.2). However, only a small number of oestrogen-responsive genes contain the consensus sequence. Sequences flanking the ERE may also affect the relative binding of an ER to different EREs. Many genes that contain functional EREs have been identified and most of these response elements contain one or more changes from the consensus sequence (see Table 6.1). A number of genes have multiple copies of these imperfect EREs. An ERE with

a single change from the consensus sequence may be sufficient to prevent binding of the ER (Driscoll *et al*, 1998). However, an ERE with two changes from the consensus may be capable of binding avidly to ERs in the context of certain flanking sequences. Changes in the sequences flanking a non-consensus ERE can alter ER-ERE interactions, positively or negatively. Different combinations of ERs and ERE sequences could lead to the association of the receptor with different transcription factors resulting in the differential modulation of oestrogen-responsive genes (Wood *et al*, 1998).

Figure 6.2 Models of ER action at a classical ERE and ER-dependent AP1 (activator protein-1) response element.

To initiate transcriptional activation, the ligand bound-ER binds to the classical ERE as a homodimer. The ER can also mediate transcription from an AP1 enhancer element that requires the ligand and the AP1 transcription factors Fos and Jun. The filled circles represent ligand-bound to the ER. The AP1 proteins, Jun and Fos are labelled J and F, respectively. Adapted from (Paech *et al*, 1997).



Activator protein-1 (AP1) response element

6.6 ERs also mediate gene transcription from an activator protein-1 (AP1) enhancer element that requires ligand and the AP1 transcription factors Fos and Jun for transcriptional activation (see Figure 6.2). Particular ER ligands can exert apparently opposing effects in different target tissues. Experiments have shown that tamoxifen, a drug used in the management of breast cancer, inhibits cell proliferation in breast tissue but can stimulate cell proliferation in the endometrium. A possible explanation is that ligand-bound ER may have differing transactivation properties when complexed with coactivator or corepressor proteins. Transactivation experiments lend support to this hypothesis by demonstrating that tamoxifen inhibits the transcription of genes regulated by a classical ERE, but activates the transcription of genes under the control of an AP1 element (Paech *et al*, 1997).

Table 6.1 Genes containing the oestrogen response element.
This table illustrates variations from the consensus sequence. Changes in the sequences flanking a non-consensus ERE can greatly alter ER-ERE affinity. Adapted from Driscoll *et al* (1998).

ERE containing gene	Function	ERE sequence
Extended consensus inverted repeat	Theoretical sequence	5'-CAGGTCAgagTGACCTG-3'
Frog vitellogenin B1 gene	Gene encoding vitellogenin required for yolk formation in developing ova	5'-CCAGTCActgTGACCCA-3'
Frog vitellogenin B2 gene	Gene encoding vitellogenin required for yolk formation in developing ova	5'-CAAGTTAtcaTGACCTC-3'
Rodent hsp70-related gene	Heat shock protein	5'-CTGGTCActcCGACCAG -3'
Rabbit uteroglobin gene	Progesterone binding protein	5'-CAGGTCAccaTGCCCTC-3'
Human pS2	Oestrogen responsive gene	5'-CAGGTCActgTGGCCCT-3'
Mouse Lactoferrin	Iron binding protein	5'-CAGGTCAAGGTAACCCA-3'
Human Lactoferrin	Iron binding protein	5'-CAGGTCAAGGCGATCTT-3'
Mouse c-jun	Proto-oncogene	5'-AAGCAGAgcaTGACCTT-3'
Rat creatinine kinase B	Catalyses ATP to ADP	5'-AGGTCAgaaCACCCT-3'
Rat c-fos	Proto-oncogene	5'-CAGGTCAccaCAGCCCA-3'

Oestrogen receptor subtypes

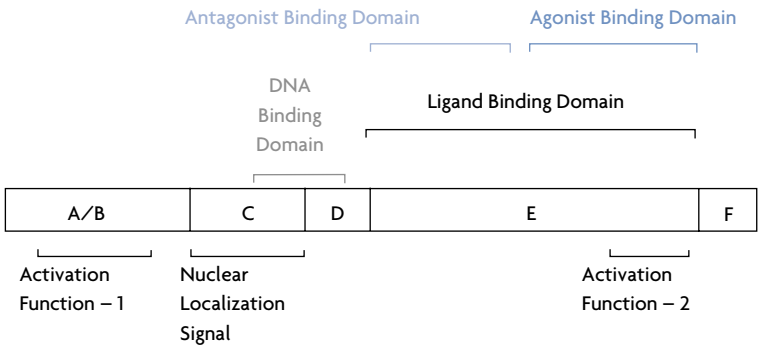
6.7 Until recently, a single ER had been isolated. However, a second oestrogen receptor (ERß) has been identified in rat, mouse, primate and man (Enmark *et al*, 1997; Kuiper *et al*, 1996; Mosselman *et al*, 1996; Tremblay *et al*, 1997; Saunders *et al* 2000; 2001). A third ER (ERγ) has also been identified. The ERγ was isolated from teleosts (Hawkins *et al*, 2000), but the significance of this receptor is not yet clear. Compared to the ERα subtype, ERß may mediate different biological effects and display different intracellular and tissue distribution patterns. ERß also has a different expression pattern during development. The ERα and ERß subtypes can interact with a wide variety of different compounds although some ligands appear to have different relative affinities for the subtypes. Indeed, although the phytoestrogens can bind to the ERα, they appear to bind preferentially to the ERß (see paragraph 6.20-6.23).

ERα and ERß: structure and functions

6.8 While the protein sequences of the ERα and ERß are highly homologous, they represent two distinct gene products. In humans, the gene for ERα is located on chromosome 6 while that for ERß is on chromosome 14 (Enmark *et al*, 1997; Couse & Korach, 1999). The structure of the oestrogen receptors, and other steroid hormone receptors, can be subdivided into several functional domains (see Figure 6.3). The DNA-binding domain (C) contains two zinc fingers involved in specific DNA-binding and receptor dimerisation. This domain is highly conserved between receptors having greater than 94% amino acid identity (3 differing amino acids residues). This suggests the ERα and ERß receptors may interact with similar response elements. The ligand-binding domain (E) contains regions that are important for ligand binding, receptor dimerisation, nuclear localisation and interactions with transcriptional coactivators and corepressors (such as AF-2; there may also be an AF-2a domain within the ligand-binding domain (LBD) of human ERα). The LBD is partially conserved, having an amino acid identity of approximately 55% (see Figure 6.4), and both receptors are known to bind oestradiol and other (but not all) ligands with similar affinity. The N-terminal domain (A/B) is highly variable and usually contains a transactivation function (AF-1). This is thought to be a region of site-specific phosphorylation involved in ligand independent activity of the receptor. It is possible, therefore, that transcriptional activation of different oestrogen-responsive genes may show variable patterns. The activation of target genes occurs by direct interaction with components of core transcriptional machinery or with coactivators that mediate signalling to other proteins.

6.9 The hinge domain (D) contributes flexibility to the DNA- versus ligand-binding domain and may influence DNA-binding of individual receptors or serve to anchor certain corepressor proteins. The C-terminal domain (F) contributes to the transactivation capacity of the receptor but may have other functions also (Couse & Korach, 1999; Enmark & Gustafsson, 1999). It is now understood that gene activation is influenced by both promoter and cell-specific factors and by synergistic interaction between the N- and C-terminal receptor activation domains (Enmark & Gustafsson, 1999; Katzenellenbogen & Korach, 1997; Kuiper *et al*, 1996). It has been proposed that the α and ß receptors may be to some extent mutually antagonistic since ERß often appears to quench activities up-regulated by ERα, particularly in the context of cell proliferation but this has only been shown in *in vitro* cell transfection studies (Reynolds, 1999).

Figure 6.3 Structural domains of a generic member of the nuclear receptor family.
Five to six distinct domains have been identified in oestrogen receptors, which are responsible for specific functions. The functions associated with each of these domains are indicated. Adapted from Gillesby & Zacharewski (1998).



Tissue distribution of ERα and ERß

6.10 It is clear that the distribution of ER subtypes varies markedly both between and within the tissues of both humans and rodents (see Table 6.2). In rat and mouse, different regions and cell-populations in the brain, including the hypothalamus and pituitary, appear to express either ERα or ERß, exclusively, although one minor cell population within the rat pituitary co-expresses both receptors (Couse *et al*, 1997; Mitchner *et al*, 1998; Osterlund *et al*, 1998). The different cell types within the rat ovary have also been found to exhibit differences in receptor expression and these may be influenced by the stage of development or maturation of the animal (Sar & Welsch, 1999; Shughrue *et al*, 1998). Variable expression profiles of the two receptor types occur within tissues of the developing human fetus (Brandenberger *et al*, 1997). Sex differences occur in embryonic brain development in the mouse (Karolczak & Beyer, 1998). Stage-specific changes in expression of oestrogen receptor subtypes have been shown to occur in the development of pre-implantation mouse embryos (Hiroi *et al*, 1999).

6.11 Expression of both ERα and ERß has been found in all major human uterine cell types at every menstrual stage. However, expression varies from cell-type to cell-type and expression of ERα mRNA is generally greater than that of ERß (Matsuzaki *et al*, 1999). Altered expression of oestrogen receptors has been found in certain tumour types. For example, normal human mammary tissue has been shown to predominantly express ERß mRNA, whereas most ER-positive breast tumours appear to exhibit increased ratios of ERα:ERß (Leygue *et al*, 1998; Speirs *et al*, 1999). Likewise, an increased ratio in ERα:ERß mRNA has been demonstrated in ovarian carcinoma compared with normal tissue or cysts (Pujol *et al*, 1998). High levels of ERß have also been found within the gut of humans (Enmark *et al*, 1997).

Figure 6.4 Percentage amino acid identity between human ERß and other oestrogen receptors: rat ERß (rERß), rat rERß$_2$ (a splice variant), mouse ERß (mERß), human ERα (hERα).
Numbers above each box refer to the amino acid number from the protein sequence, whereas numbers inside each box refer to the percentage amino acid identity to hERß. The letters within each box refer to the N-terminal (A/B), DNA-binding (C), hinge (D), ligand-binding (E) and C-terminal (F) domains respectively. ERß$_2$, the most prevalent isoform in the rat contains 54 additional nucleotides within its ligand-binding domain, generating an 18 amino acid residue insertion in the resulting protein. Adapted from Enmark & Gustafsson (1999), Maruyama *et al* (1998) and Petersen *et al*, 1998).

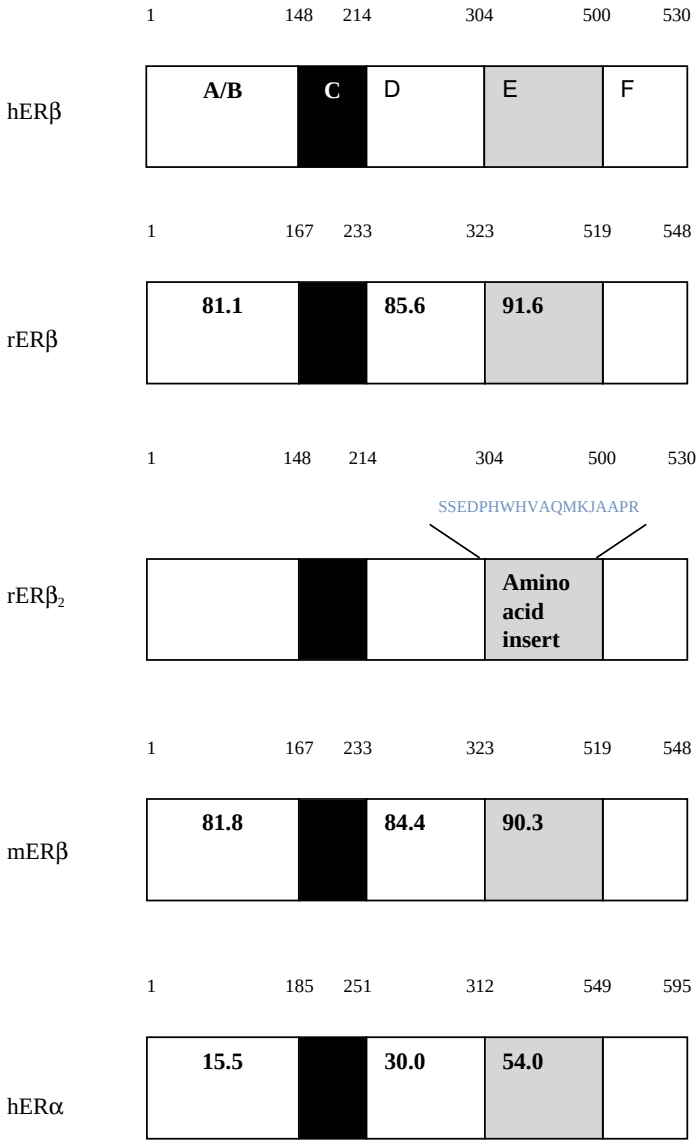


Table 6.2 Tissue distribution of ER subtypes in humans and rodents.
A list of what is currently known about the tissue distribution of oestrogen receptors. The (-) symbol denotes that either the receptor has not been detected or it has not been determined whether the receptor is present in that tissue.

Organ/Tissue	Human		Rodent		
	ERα	ERß	ERα	ERß	ERß2
Lung	–	✔	–	✔	–
Vascular	✔	✔	–	–	–
Adrenal	✔	–	✔	–	–
Kidney	✔	✔	✔	–	✔
Prostate	–	✔	–	✔	✔
Testes	–	✔	✔	✔	–
Heart	✔	✔	✔	✔	–
Brain	✔	✔	–	✔	✔
Thymus	–	✔	–	✔	–
Breast	✔	✔	–	–	–
Uterus	✔	✔	✔	✔	✔
Endometrium	✔	✔	–	–	–
Vagina	✔	–	–	–	–
Fallopian tube	–	✔	–	–	–
Ovary	✔	✔	✔	✔	✔
Bladder	–	✔	–	✔	–
Epididymus	–	✔	✔	✔	–
Pituitary	–	✔	✔	–	–
Liver	✔	–	✔	✔	✔
Muscle	–	–	✔	✔	✔
Fat	–	–	✔	✔	✔
Gastrointestinal tract	–	✔	–	✔	–
Colon	–	✔	–	✔	–
Small intestine	–	✔	–	✔	–
Bone	✔	✔	✔	✔	✔

Variants of ER isoforms

6.12 A number of different isoforms of human ERs have been reported in the literature. Inoue *et al* (2000) described a spliced isoform of ERß (ERßΔ5) co-expressed with the wild type in normal human testes. ERßΔ5 contains a deletion of exon 5, predicted to encode the ligand-binding domain in the wild-type receptor. Transient expression of ERßΔ5, in COS-7 cells, had no effect on the basal transactivation activity of an oestrogen-responsive luciferase reporter gene. However, when co-transfected with either wild-type ERß or ERα, behaved as a dominant negative receptor inhibiting oestradiol-stimulated transactivation by both ERß and ERα. Moore *et al* (1998) identified five ER transcripts (hERß 1-5) from a human testis cDNA library and from MDA-MB 435 cells. The isoforms show differential expression in human tissues and tumour cell lines at the RNA level, and are predicted to form DNA-binding homo- and heterodimers when co-expressed.

6.13 Ogawa *et al* (1998a) identified the novel human ERß isoform, ERßcx, from a human testis cDNA library. ERßcx is identical to ERß except that the C-terminal domain has been replaced by a 26 amino acid sequence. Northern blot analysis showed ERßcx mRNA expression in testis, ovary, thymus and prostate as well as in the human cell lines HEC-1, HOS-TE85 and Saos-2. Radioligand binding assays using whole cell extracts of transfected COS-7 cells showed that ERßcx has no ligand binding activity. ERßcx does not show any ligand-dependent transactivation ability of a basal promoter and cannot interact with TIF1α, a cofactor, in the presence or absence of oestradiol. The novel isoform was found to preferentially form a heterodimer with ERα rather than ERß, thus inhibiting DNA binding by ERα. ERßcx also shows significant dominant negative activity against ERα transactivation.

6.14 Vladusic *et al* (1998) described a variant form of ERß mRNA co-expressed with the wild-type form in a human ERα-negative MDA-MB-231 oestrogen-dependent breast cancer cell line and in malignant breast tumour specimens, but not in samples of normal breast tissue. This ERß variant contained a deletion predicted to be within the hormone-binding domain, and which, it was suggested, may alter the binding affinity for oestradiol.

6.15 Novel isoforms of the ERß receptor have also been identified in the rat (Maruyama *et al*, 1998; Petersen *et al*, 1998). The major form, referred to as rERß$_2$, is putatively a splice variant of ERß which contains an additional 54 base pair insertion in the ligand-binding domain that generates an 18 amino acid residue insertion in the protein (see Figure 6.4). The insertion resulted in reduced ligand-binding activity for oestradiol, although the ability to bind to the ERE was retained. However, rERß$_2$ failed to activate oestradiol-dependent transcription in an ERE-dependent reporter gene assay. Furthermore, rERß$_2$ suppressed ERα- and ERß-mediated transcriptional activation in a dose-dependent manner. It was suggested that rERß$_2$ may therefore operate as a negative regulator of oestrogen action. Petersen *et al* (1998) also detected novel isoforms of both ERß and rERß$_2$ mRNAs. Several truncated oestrogen receptor products (TERPs) have been identified in rat pituitary (Demay *et al*, 1996; Friend *et al*, 1995; 1997; Shupnik *et al*, 1989) and their expression is also detected in uterus, testes, heart, hypothalamus and liver (Friend *et al*, 1997).

6.16 Several ERα isoforms have been identified, mainly in human cancer cells (Lemieux & Fuqua, 1996; Pfeffer *et al*, 1996; Karas *et al*, 1995), but their function is uncertain. Hodges *et al* (1999) identified 5 variant ERα transcripts, in addition to the wild-type, in human vascular smooth muscle. All variants contained deletions of exons encoding regions of the hormone-binding domain. Recently, a further ERα isoform has been identified in humans. This isoform lacks the transactivation function AF-1 and, may regulate cell proliferation (Flouriot *et al*, 2000). An ERα isoform lacking exon 4 has also been reported in the brain (Skipper *et al*, 1993). However at this point in time, the implications of these variants for phytoestrogen binding and receptor function is unclear.

Homodimers, heterodimers and auxiliary binding proteins

6.17 Differential tissue distribution of ERα and ERß and their variant subtypes may only partly explain the tissue specific effects of oestrogens. Tissue specificity may in fact be attributable to the nature of the dimers formed by receptors and their interaction with accessory proteins. Several groups have reported that ERα and ERß can form functional heterodimers (Cowley *et al*, 1997; Ogawa *et al*, 1998b; Pettersson *et al*, 1997). Furthermore, Cowley *et al* (1997) has demonstrated that heterodimers bind to the consensus ERE sequence with an affinity similar to that of ERα homodimers (Kd 2 nM), but greater than that for ERß homodimers. Consequently there are at least three possible pathways through which oestrogens may activate target genes by dimerisation. This gives rise to the possibility that the ratio of different receptor types present in any particular tissue may be an important determinant of a biological response. It remains uncertain whether specific response elements exist that are recognised selectively by dimer types (Enmark & Gustafsson, 1999). However, Hyder *et al* (1999) reported that ERß bound to several different endogenous EREs (vitellogenin, c-fos, c-jun, pS2, cathepsin D, and choline acetyltransferase) which were already known to bind ERα and confer oestrogen inducibility in reporter constructs. It was established that the binding characteristics of ERα and ERß were similar for some EREs but different for others.

6.18 In addition to these possibilities, the auxiliary proteins (coactivators or corepressors), which are required for ER-mediated activation or repression, may also be tissue or receptor specific (Paige *et al*, 1999).

Differential responses from interaction of ERα or ERß with AP1

6.19 It has been shown that ERα and ERß signal in opposite ways from the AP1 site when complexed with the natural hormone oestradiol. With ERß, oestradiol has been found to activate transcription, whereas with ERß, oestradiol inhibits transcription. Moreover, anti-oestrogens such as tamoxifen and raloxifene are potent activators of transcription with ERß at an AP1 site. Thus the two ERs signal in different ways, depending on both ligand and response element (Paech *et al*, 1997).

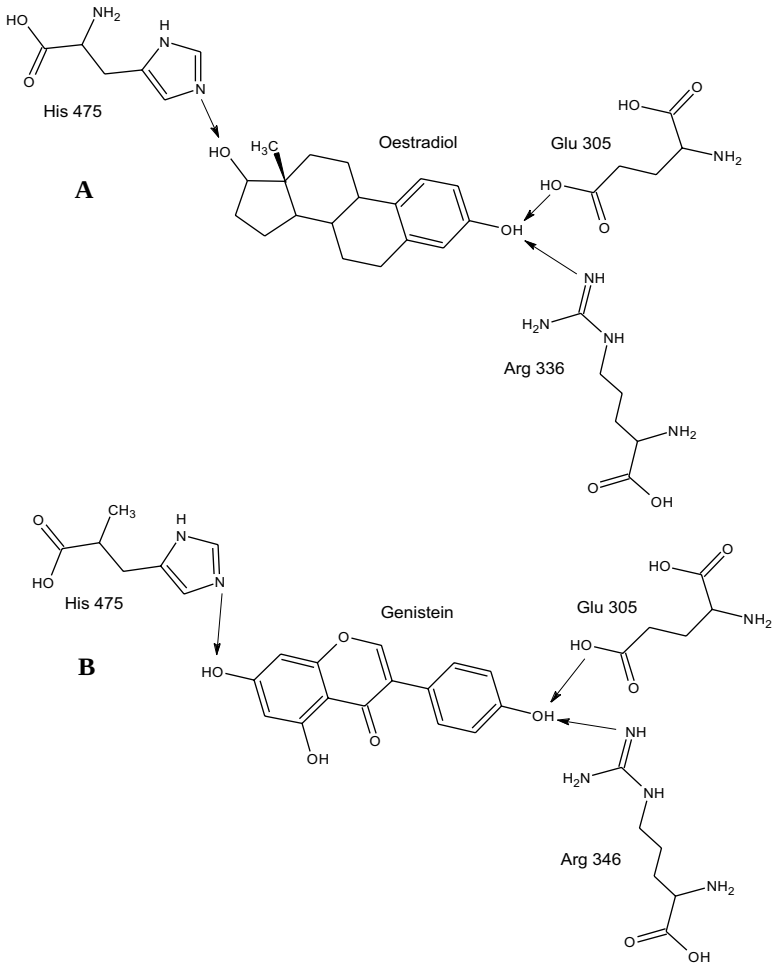
Binding of phytoestrogens to oestrogen receptors

6.20 The structure of the oestrogen binding sites of ERs and how oestrogenic ligands bind to ERα and ERß has been determined (Anstead *et al*, 1997; Brzozowski *et al*, 1997; Pike *et al*, 1999). The amino acids within the binding site interact directly with the oestrogen molecule. The strength of these interactions determines the affinity of the compound for the receptor. Oestradiol fits into the oestrogen binding pocket well (see Figure 6.5) thus it binds to the ER with high affinity.

6.21 The oestrogen binding site is flexible and can accommodate a wide variety of compounds with structural similarities to oestradiol, such as the phytoestrogens (Brzozowski *et al*, 1997; Pike *et al*, 1999; Fang *et al*, 2001; Blair *et al*, 2000), even those with substituents such as the alkyl group of 8-prenylnaringenin (Milligan *et al,* 2000). Receptor binding experiments have not been reported for lignans.

6.22 The affinity of phytoestrogens for the ERs is related to their stereochemical structure i.e. the arrangement of the ring structures and the presence and position of chemical groups such as hydroxyl groups (Miksicek, 1993, 1995; Collins *et al*, 1997; Zava & Duwe, 1997; Breinholt & Larsen, 1998). For example, genistein binds to both oestrogen receptors as it is a similar size to oestradiol and has hydroxyl groups which are appropriately positioned to interact with the amino acids in the binding pockets (see Figure 6.5) (Brzozowski *et al*, 1997; Pike *et al*, 1999).

Figure 6.5 Oestradiol and genistein binding to the ligand-binding domain of the oestrogen receptor. The figure shows selected interactions (marked with arrows) between oestradiol (A) and genistein (B) and specific amino acids in the ligand-binding pocket of ERß. (Adapted from Brzozowski et al (1997); Pike *et al* (1999)).



6.23 Oestradiol binds with equal affinity to both ERß and ß (Kuiper *et al*, 1997). However, the phytoestrogens, coumestrol, genistein and daidzein show greater selectivity towards binding to ERß (see Table 6.3) (Kuiper *et al*, 1997). The difference in the amino acid sequence between ERα and ß accounts for this selectivity (Pike *et al*, 1999). 8-Prenylnaringenin does not show this selectivity (Milligan *et al*, 2000). It is unknown whether the lignans have selectivity for one receptor subtype.

Table 6.3 The selectivity of oestradiol and phytoestrogen binding to ERα and ERß
The table shows the binding affinities of phytoestrogens to each receptor subtype relative to the binding affinity of oestradiol (arbitrarily set at 100).

Compound	Binding at ERα (%)	Binding at ERß (%)
Oestradiol[a]	100	100
Coumestrol[a]	34	100
Genistein[a]	0.7	13
Daidzein[a]	0.2	1
8-Prenylnaringenin[b]	10	10

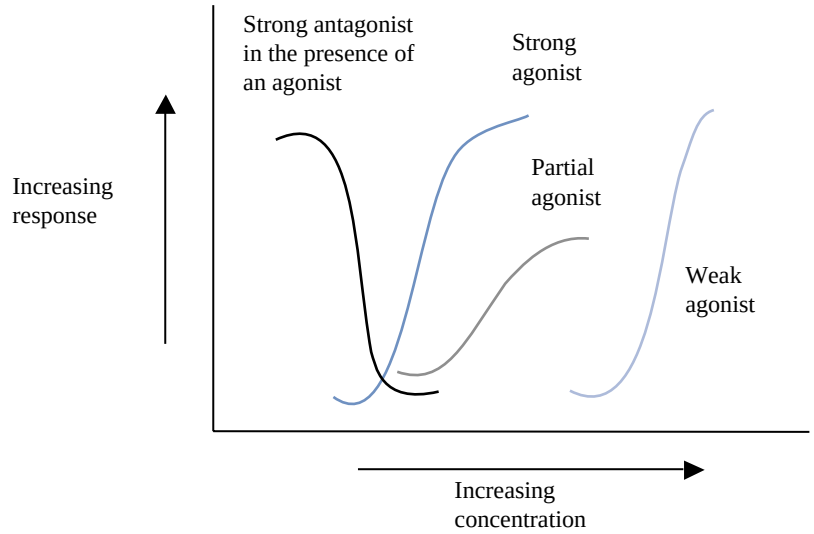
a Kuiper *et al* (1997)

b Milligan *et al* (2000)

Phytoestrogen agonist/antagonist activity

6.24 Different biological responses may occur when a phytoestrogen binds to the oestrogen receptors. If phytoestrogens bind to and activate the receptor this is referred to as agonist activity (Paige *et al*, 1999). However, a phytoestrogen may bind to the receptor but block a further biological response and this is termed antagonist activity. A major determinant of the potency of agonists or antagonists is their binding affinity for the receptor (Clarke *et al*, 1996). If phytoestrogens induce a biological response at concentrations similar to that of oestradiol they are considered as "potent" agonists. Conversely, compounds deemed "weak" agonists require much higher concentrations, to produce responses of a similar magnitude to oestradiol. The same is true for antagonists. Some phytoestrogens are very weak agonists and cannot activate the oestrogen receptors to the same extent as agonists even at high concentrations and may even have antagonist activity. These compounds are known as partial agonists (see Figure 6.6) (Collins *et al*, 1997; Mousavi & Adlercreutz, 1992). The oestrogenic potency of phytoestrogens i.e. the ability to elicit an oestrogenic response is discussed in detail in Chapter 8. Additionally, despite binding to oestrogen receptors in a similar way it is possible that oestrogenic compounds may have somewhat differing biological properties. A recent study by Naciff *et al* (2002) compared the transcription profile induced in the ovary and uterus of rats after administration of either the synthetic oestrogen, ethinyl oestradiol or genistein. Although the two compounds influenced the expression of many of the same genes in a similar way, there were some marked differences suggesting that there may be differences in the biological properties of two oestrogens.

Figure 6.6 The relationship between agonists, antagonists and partial agonists.
Agonists all produce greater responses with increasing concentration of compound with strong agonists requiring lower concentrations to achieve the same effect relative to weak agonists. Partial agonists cannot achieve the maximal effect of agonists no matter how much their concentration is increased. Increasing concentrations of antagonists reduce the effect of an agonist.



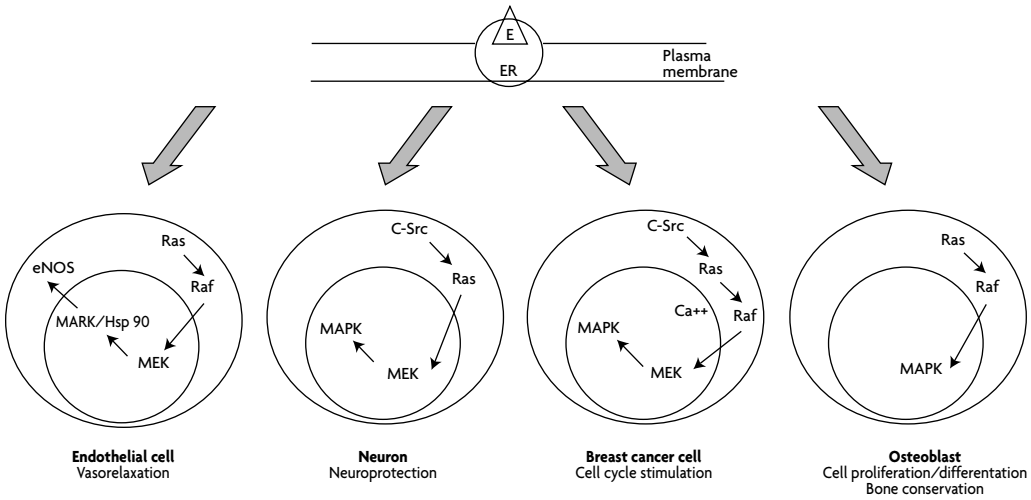
Ligand-independent activation of oestrogen receptors

6.25 The oestrogen receptor can also be activated in the absence of a ligand by phosphorylation. Epidermal growth factor, human epidermal growth factor receptor-2 (HER-2) and insulin are all capable of activating the ER through mechanisms involving the Ras signal transduction pathway. This pathway includes the mitogen-activated protein kinase (MAPK) family and is responsible for the phosphorylation of many proteins involved in cell signalling. Several studies have shown that phosphorylation by MAPK can bring about the activation of ER in the absence of ligand (Gillesby & Zacharewski, 1998). Thus, it is possible that interference with this pathway may produce oestrogen-like effects without direct ER-ligand interactions.

Cell surface oestrogen receptors

6.26 Investigations have suggested that cell-surface forms of ERα and ERß exist which are coupled to cytosolic signal transduction pathways (Collins & Webb, 1999). There is evidence to suggest that the nuclear and membrane ERs are derived from the same transcripts. Recent data have indicated a direct link between cell-surface ERs and the MAPK pathway. This suggests that ERs may be involved in signal transduction whereby extracellular messages are passed to the nucleus to stimulate gene expression with a subsequent rapid biological response. In this way, the effects of oestrogens may be similar to those of growth factors and may permit cross-talk. Thus, it will be important to establish to what extent similar changes can be induced by phytoestrogens (see Figure 6.7).

Figure 6.7 Proposed mechanisms of rapid actions of oestrogen in four cell types.
Oestrogen interacts with the plasma membrane ER of various cell types leading to the sequential activation of signal transduction pathways involving proteins such as c-Src, Ras, Raf and mitogen-activated protein kinase (MAPK). Androgen receptors may also interact with the messenger protein c-Src. Activation of various pathways leads to the types of downstream effects shown. Adapted from Collins & Webb (1999).



ER-independent mechanisms mediated through the ERE

6.27 Studies *in vitro* have shown that heterodimers of other receptors are capable of binding to the EREs. Possible ERE candidates are retinoic acid receptor-peroxisome proliferator activated receptor heterodimer (RXR/PPAR) and the arylhydrocarbon receptor-arylhydrocarbon receptor nuclear translocator heterodimer (AHR/ARNT) (Gillesby & Zacharewski, 1998; Klinge *et al*, 1999; McKenna *et al*, 1999; Nunez *et al*, 1995).

Oestrogen receptor and aromatase knockout mice

6.28 Knockout mice have been developed in which the ERα or ERß genes are inactive, these are known as αERKO and ßERKO mice. Double knockout mice (αßERKO) have also been developed in which both ERα and ERß are inactive. An aromatase knockout (ArKO) mouse has been developed, where the gene expressing the enzyme, aromatase (*Cyp19*) that converts testosterone to oestradiol has been inactivated. The development of these models has done much to increase understanding of oestrogen action and also helped to unmask unidentified oestrogen signalling systems, including those that are independent of either ERα or ERß (Das *et al,* 1997). The phenotypes of these genetically modified animals and their use as experimental models in the study of oestrogen action have been reviewed by Couse & Korach (1999), Gustafsson (1999) and Curtis & Korach (2000).

6.29 In summary, both sexes of the αERKO mouse are infertile. The female has cystic haemorrhagic follicles and no corpora lutea in the ovaries and the males exhibit testicular atrophy, decreased spermatogenesis and inactive sperm. There are also abnormalities in reproductive behaviour and breast development. Bone tissue is only slightly affected in females and the protective effects of oestrogen on the cardiovascular system are not overtly affected. Oestradiol levels are greatly elevated, which may explain the effects seen in the ovaries. Life-span is comparable to the wild type. The phenotypes observed in the αERKO animals are due to oestrogen insensitivity and confirm several functions previously believed to be dependent on ERα. These include:

- proliferative and differentiative actions required for the function of the adult female reproductive tract and mammary gland,
- obligatory involvement in growth factor signalling in the uterus and mammary gland,
- action as a component in the negative regulation of gonadotrophin gene transcription and LH levels in the hypothalamic-pituitary axis,
- actions in the positive regulation of prolactin synthesis and its secretion from the pituitary,
- progesterone receptor expression in several tissues,
- action as a promotional factor in oncogene-induced mammary neoplasia,
- involvement as a crucial component in the differentiation and activation of several behaviours in both the female and the male.

6.30 Certain oestrogen pathways in the αERKO female appear to remain intact such as the ability of the uterus to exhibit progesterone-induced decidualisation and maintenance of LH surge in the hypothalamus. Some αERKO phenotypes may be more pronounced by downstream effects of progesterone or prolactin or increased androgen sensitivity. It is also apparent that disruption of the ERα gene may have unmasked oestrogen-signalling systems that were not easily detectable in the wild-type such as those that are independent of both ERα and ERß.

6.31 The ERß-deficient (ßERKO) mouse, has been developed more recently and therefore is less well characterised than the αERKO mouse. Male ßERKO mice are, as far as is known, fully fertile, although the female mice exhibit reduced fertility due to a block in the last step of follicular development, which may be overcome when animals are treated with follicle stimulating and luteinising hormones. ßERKO animals also exhibit reduced abdominal fat. The use of ERß knockout mice has also established that ERα is instrumental in mediating sexual behaviour in male mice (Lubahn *et al*, 1993; Ogawa *et al*, 1999). Studies in αERKO and ßERKO mice suggest that the two receptors may compensate for each other to a certain extent since the phenotype in some cases is less pronounced than expected such as effects in the testes of ßERKO and in bone of both αERKO and ßERKO.

6.32 A study by Zhu *et al* (2002) reported that ßERKO male mice exhibit increased tail-cuff, systolic and diastolic blood pressure (9% increase) after 6-7 and 12 months of age respectively. Blood pressure remained elevated after 22 months in ßERKO male mice. Further to work showing that the predominant ER in the heart is ERß, the data demonstrate that ERß and oestrogen affect vascular tone by regulating ion channels, and play an important role in hypertension.

6.33 Aromatase knockout (ArKO) mice are characterised by female infertility as a result of a defect in ovulation that is accompanied by underdeveloped external genitalia, uterus and mammary glands. Oestradiol levels are undetectable while testosterone levels are elevated. In adult males, spermatogenesis and mating ability are impaired thus reducing fertility. Spermatogenesis, however, is not impaired in younger males although the reasons for this remain to be determined (O'Donnell *et al*, 2001; Robertson *et al*, 2001, 1999). Aromatase deficiency in humans is characterised by osteopenia and failure of epipheal closure (Carani *et al*, 1997; Morishima *et al*, 1995). Miyaura *et al* (2001) reported age- and sex-dependent bone loss in ArKO mice. At 4 weeks no defects were noted however, by 9 weeks there was marked loss of trabecular bone resulting from increased resorption. The degree of bone loss was similar for young adult animals of both sexes but was much more severe in older females. In addition, raised serum pyridinoline levels were noted in both sexes suggesting an increase in bone turnover. In contrast, a study by Oz *et al* (2000) reported differences in bone turnover between males and females with ArKO females exhibiting increased turnover whilst males showed decreased turnover with the suppression of bone formation.

Double Oestrogen receptor knockout (DERKO or αßERKO) mice

6.34 The generation of mice that do not express either ERα or ERß (double knockout mice or DERKO), has provided further information on the role of these receptors in regulating physiological and behavioural processes. Studies have shown that in male mice, ERα is instrumental in regulating bone growth and maturation (Vidal *et al*, 2000), thymus and spleen development (Erlandsson *et al*, 2001) and the development of obesity (Ohlsson *et al*, 2000).

6.35 The role of both ERα and ERß in the sexual behaviour and aggression of male mice has been studied in double knockout animals (Ogawa *et al*, 2000; Simpson & Davis, 2000). These experiments assessed the mating ability and aggressiveness of male mice. Mating ability was measured by recording mount, ejaculation and intromission frequency whilst aggressiveness was measured by recording behavioural responses after introduction of other male mice unable to respond to aggressive behaviour due to the absence of a functional olfactory system.

6.36 The results demonstrate that the absence of both ERα and ERß significantly reduces sexual and aggressive behaviour. However, the observation of reduced intromissions and ejaculations but normal sexual behaviour in ERα or ERß knockout mice suggests that one receptor may partially compensate for the other. The level of aggressiveness recorded in double knockout animals was very similar to that seen in ERα knockout mice. ERß knockout mice however, have been shown to have increased aggressive behaviour suggesting that ERß may have an inhibitory effect on ERα mediated functions (Ogawa *et al*, 2000).

6.37 At present it is unclear how these results may be interpreted in light of data on the localisation of ERα and ERß in the brain. Both ERs are present in the arcuate nucleus and the preoptic area of the hypothalamus. ERα is present in the ventromedial nucleus and ERß in the paraventricular nucleus. These regions of the hypothalamus are important in feeding behaviour, reproduction, sexual behaviour, and thermoregulation. Both receptors also appear to be present in the amygdala and hippocampus, areas involved in short term memory and emotion. ERß has also been identified in the cerebellum and in cortical regions (Kuiper *et al*, 1997; Shughrue *et al*, 1998).

6.38 The creation of these models has done much to increase our understanding of oestrogen action and also helped to identify new oestrogen-signalling systems, including those pathways independent of either ERα or ERß. Studies using DERKO mice exposed to phytoestrogens that will help to differentiate between responses by ERα and ERß have yet to be conducted.

Key points

- Oestrogens produce their effects by interacting with oestrogen receptors (ERs) predominantly located in nucleus of the cell. Two main isoforms of ER have been identified as ERα and ERß, although additional variants such as ERß2 have also been identified in the rat. The receptors are composed of five distinct functional domains, each of which has a different function. Experiments have shown there is some degree of amino acid homology between the receptors, with respect to the DNA-binding domain (greater than 94% homology) and to a lesser extent in the ligand-binding domain (55% homology).

- When an oestrogen molecule binds to an ER, it dimerises. The dimerised receptor binds to the oestrogen response element (ERE), a particular DNA sequence contained within specific genes. Binding to the ERE causes a conformational change in the receptor thus, initiating gene expression and resulting in a biological response for example increased gene expression.

- ERs may also interact with other DNA sequences such as AP1, which may widen the role these receptors play in mediating the effects of oestrogenic compounds. There is also evidence that oestrogenic responses may occur by non-genomic mechanisms that permit cross-talk between other nuclear receptors and cellular signalling systems.

- Phytoestrogens possess hydroxyl and phenolic groups spaced at a similar distance to those in the oestradiol structure. The chemical structure of phytoestrogens determines their affinity, selectivity and efficacy of their binding to oestrogen receptors.

- Genistein, coumestrol, and daidzein all show greater selectivity for binding to ERß relative to ERα.

- The generation of transgenic mice that do not posses ERα, ERß or aromatase enzymes will provide additional information on the role of these factors in development and reproduction and, what effect oestrogenic compounds may have on these animals.

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7. Additional effects mediated by phytoestrogens

Introduction

7.1 Phytoestrogens may produce oestrogenic effects either via direct binding to oestrogen receptors or by acting indirectly to modulate the concentrations of endogenous oestrogens. Phytoestrogens may also induce effects, which may not be mediated through oestrogen receptors. This chapter summarises the information on the oestrogenic and non-oestrogenic mechanisms of action of phytoestrogens.

Effects on oestrogen bioavailability

7.2 Circulating oestradiol is bound primarily to glycoproteins in blood, such as sex hormone binding globulin (SHBG) in humans or to α-fetoprotein (AFP) in rats and mice (Baker *et al*, 1998). In adult women, approximately 37% of circulating oestradiol is associated with SHBG and 61% to serum albumin. Only free or unbound oestradiol is available to be taken up into cells and thus, elicit biological effects. Consequently, the binding of hormone to serum proteins provides a mechanism by which cellular uptake and responses in target tissues can be limited (Nagel *et al*, 1998; 1999). The majority of testosterone (97%) present in plasma is bound to proteins, of this 40% is bound to SHBG. Thus, SHBG binding to both testosterone and oestrogen contributes to the total active steroid concentration *in vivo*.

7.3 It has been suggested that phytoestrogens may alter the concentrations of active sex hormone by:

- binding to SHBG to inhibit binding of oestrogens (and androgens) to increase their plasma concentrations i.e. an oestrogenic effect (or androgenic effect).

- stimulating the synthesis of SHBG thus, reducing the concentrations of free hormones i.e. an anti-oestrogenic effect.

While it is possible that phytoestrogens may displace oestrogens or androgens from SHBG or alter the concentration of SHBG available to bind oestrogens and androgens, there are no data to suggest phytoestrogens act by this mechanism to produce clinical effects (see paragraphs 7.6-7.13).

Influence of phytoestrogens on hormone binding to sex hormone binding globulin (SHBG)

7.4 Isoflavones and lignans bind to SHBG with a 1000-5000-fold lower affinity than oestradiol (Nagel *et al*, 1998; 1999). Dechaud *et al* (1999) investigated the binding of genistein and its glucoside (genistin) to human SHBG (hSHBG). Genistein, but not genistin, competitively inhibited binding of testosterone and oestradiol to hSHBG with IC_{50} values of 11.5 and 4.5 μM, respectively. Milligan *et al* (1998) reported that concentrations of >100 μM genistein, daidzein and coumestrol were required to competitively inhibit binding of oestradiol and dihydrotestosterone from steroid binding proteins. A study by Martin *et al* (1996) reported that phytoestrogen IC_{50} values of 10-50 μM inhibited testosterone and oestradiol binding to hSHBG in the rank order: enterolactone ⩾ equol > genistein >> enterodiol, daidzein. Thus, it is unlikely that such effects would occur *in vivo* as plasma concentrations of phytoestrogens are <5μM and would be present predominantly in conjugated form (see Chapter 5) and which are unable to bind to SHBG.

Influence of phytoestrogens on sex hormone binding globulin (SHBG)

In vitro studies

7.5 It has been shown that phytoestrogens can modulate the levels of hSHBG *in vitro* (Mousavi & Adlercreutz, 1993). Adlercreutz and co-workers demonstrated that lignans and isoflavones can increase both the synthesis and secretion of hSHBG in HepG2 liver cells (Adlercreutz *et al*, 1992; Loukovaara *et al*, 1995; Mousavi & Adlercreutz, 1993). They also reported that enterolactone (1-10 μM) stimulated the synthesis of hSHBG up to 50%, in a dose dependent manner but that higher concentrations of enterolactone (50 μM) inhibited synthesis (Adlercreutz *et al*, 1992). They also showed that genistein (5-30 μM) dose dependently induced hSHBG production up to 7-fold under similar culture conditions (Mousavi & Adlercreutz, 1993). Loukovaara *et al* (1995) found that 5 μM concentrations of daidzein, genistein, equol and oestradiol (250 nM) increased hSHBG concentrations without increasing hSHBG mRNA. The authors suggested that phytoestrogen regulation of hSHBG occurs by post-transcriptional regulation.

Studies in pre-menopausal women

7.6 Adlercreutz *et al* (1987; 1998) reported a positive correlation between SHBG concentrations, fibre intake and the urinary excretion of lignans and isoflavones in a study of 62 women.

7.7 In contrast, no effects on SHBG or sex hormone levels were observed in a randomised crossover trial (n= 14) when diets were supplemented by 64 and 128 mg isoflavones per day for 3 menstrual cycles (Duncan *et al*, 1999a). A randomised crossover trial (n= 18) investigating the effect of flaxseed ingestion, also failed to show any effect on SHBG concentrations although a 30-fold increase in urinary enterolactone excretion was observed (Phipps *et al*, 1993). In addition, a cross-sectional study by Nagata *et al* (1997) failed to show an association between soy intake (38 ± 26 g/day) and SHBG levels measured on days 11 and 22 of the menstrual cycle (n= 50). Studies by Cassidy *et al* (1994) and Martini *et al* (1999) did not observe any significant changes in SHBG concentrations following dietary supplementation with soy (n= 6 and 36, respectively).

7.8 A study by Duncan *et al* (2000) demonstrated that subjects (n= 50) able to metabolise daidzein to the more potent oestrogen equol had lower concentrations of testosterone (30%) and higher levels of SHBG (50%) than non-equol producers (n= 9). However, neither testosterone nor SHBG levels were correlated with urinary equol concentration.

Studies in post-menopausal women

7.9 A marginal effect of isoflavone consumption on SHBG or plasma sex hormone levels was observed following ingestion of 65 or 132 mg/day isoflavones for 93 days each in a randomised cross-over trial (n= 18) (Duncan *et al*, 1999b). No significant changes in SHBG, follicle stimulating hormone (FSH) or luteinising hormone (LH) levels were observed in a randomised study (n= 97) consisting of four weeks of consumption of a soybean-supplemented diet containing 165 mg/day isoflavones (Baird *et al*, 1995).

7.10 Adlercreutz *et al* (1992) observed a positive correlation between plasma SHBG and urinary excretion of lignans (r= 0.38) and total diphenols (including isoflavones) (r= 0.4) in three groups of post-menopausal women (11 omnivores, 10 vegetarians and 9 women with history of breast cancer).

7.11 An increase in SHBG levels was observed (r= 0.85) in a dietary intervention study when women received 30 g of soy milk (69 mg isoflavones/day) for 10 weeks. Plasma concentrations of isoflavones rose from 0.014 $\pm$ 0.01 μM at baseline to 0.53 $\pm$ 0.19 μM at the end of the study (Pino *et al*, 2000). Plasma levels of SHBG were also increased in women (n= 145) whose diet was supplemented with phytoestrogen rich foodstuffs for 12 weeks (Brzezinski *et al*, 1997). A mean 25% increase in plasma SHBG was also observed in women (n= 312) receiving a low saturated fat and carbohydrate, high mono and polyunsaturated fatty acid and phytoestrogen-rich diet (Berrino *et al*, 2001).

Studies in men

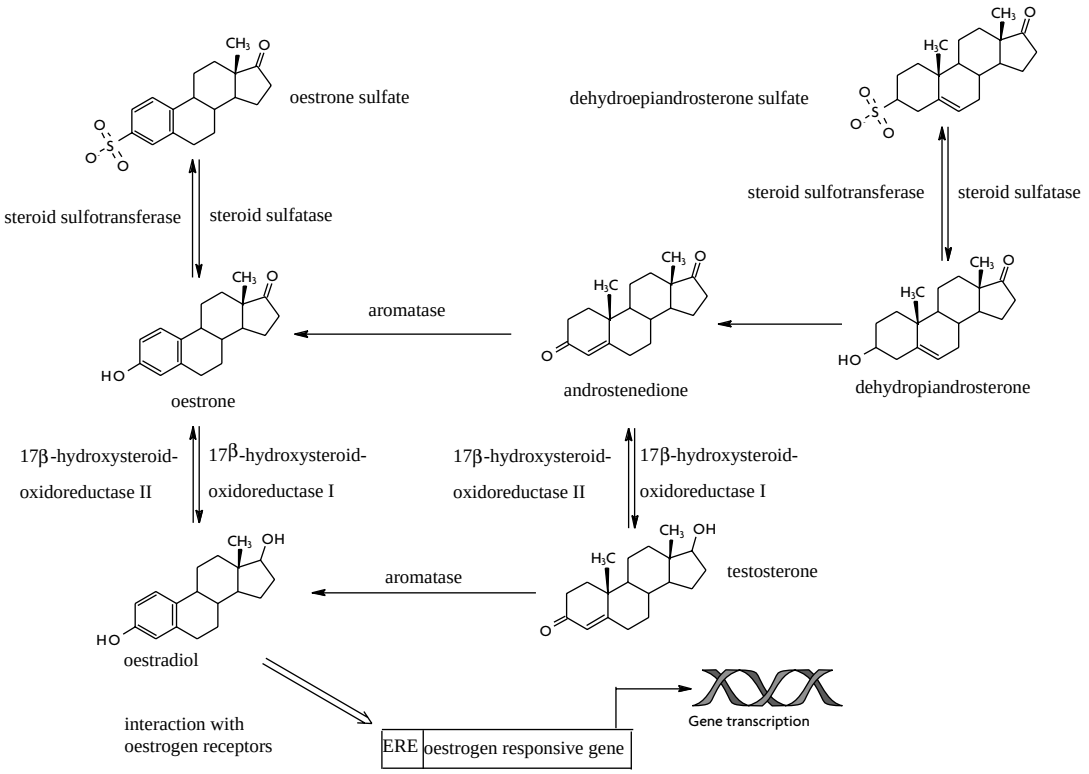
7.12 The effects on sex hormone and SHBG levels when replacing meat (150 g/day) with tofu (290 g/day) in isoenergetic diets was investigated in a randomised cross-over trial (n= 42). Urinary isoflavone levels increased 19-fold and SHBG levels increased by 9% on the tofu diet (Habito *et al*, 2000). In a cross-sectional study (n= 69, aged $\geqslant$ 35) reported soy intake was inversely associated with serum oestradiol concentration (r= -0.32). However, changes in SHBG were not statistically significant (Nagata *et al*, 2000).

7.13 A dietary intervention study (n= 7, aged 25-42 years) by Shultz *et al* (1991) investigated the effects of flaxseed consumption (57 μmol urinary lignans/day) for 2 weeks. Concentrations of free testosterone or SHBG did not significantly alter from baseline levels.

Effects on oestrogen biosynthesis and metabolism

7.14 A number of enzymes are involved in oestrogen synthesis and metabolism (see Figure 7.1). It is possible that phytoestrogens may modulate steroid concentrations by binding to these enzymes to inhibit their activity. However, there are limited data in this area and the Food Standards Agency is currently funding a research project examining the effect of phytoestrogens on steroid metabolising enzymes.

Figure 7.1 Enzymes involved in oestrogen biosynthesis and metabolism.
Adapted from Kirk *et al* (2001).

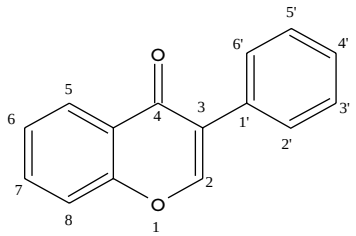


Inhibition of 17ß-hydroxysteroid oxidoreductases I and II

7.15 The 17ß-hydroxysteroid oxidoreductase (17ß-HSOR) enzymes, occur as two isoforms (I and II), involved in the inter-conversion of oestrone to the more active oestrogen, oestradiol. 17ß-HSOR I and II are key enzymes in oestrogen biosynthesis and metabolism and are expressed selectively in steroidogenic and some oestrogen sensitive tissues. The type I enzyme converts oestrone to oestradiol and type II catalyses the reverse reaction. Therefore, inhibition of 17ß-HSOR I reduces concentrations of oestradiol converting it to the less active oestrogen, oestrone (an anti-oestrogenic effect), whereas inhibition of the reverse reaction catalysed by 17ß-HSOR II would increase oestradiol, reducing oestrone concentrations (an oestrogenic effect).

7.16 Phytoestrogens have been found to inhibit both 17ß-HSOR enzymes *in vitro* and have shown a selectivity which is structure dependent (Makela *et al*, 1995; Santti *et al*, 1998; Krazeisen *et al*, 2001; 2002). The position and number of hydroxyl groups on the isoflavone structure appear to be the principal determinant of activity. Compounds with hydroxyl groups at the 5, 7 and 4' positions were the most potent inhibitors of 17ß-HSOR I and compounds with hydroxyl groups at positions 3, 5 and 7, inhibited 17ß-HSOR II (see Figure 7.2). In addition, hydroxylation at position 3, or methylation of the hydroxyl group at position 4' reduced 17ß-HSOR I activity while hydroxylation at position 3 reduced 17ß-HSOR II activity. The IC_{50} values of these compounds for inhibition of 17ß-HSOR I and II are in the range 0.1-1 μM (Makela *et al*, 1998). Thus, phytoestrogens may alter the activities 17ß-HSOR I and II to shift the equilibrium concentrations of oestrone and oestradiol. However, the direction of this equilibrium shift is dependent on phytoestrogen structure.

Figure 7.2 Potential hydroxylation position on the isoflavone structure.
The numbers correspond to the ring positions.



Aromatase inhibition

7.17 Aromatase is an enzyme involved in oestrogen synthesis, converting androgen to oestrogen. Lignans, flavones and, to a lesser extent, isoflavones, can inhibit cytochrome P_{450} aromatase (CYP19) in a competitive manner and therefore, may increase the ratio of endogenous androgen to oestrogen (Adlercreutz *et al*, 1993; Campbell & Kurzer, 1993; Chen *et al*, 1997; Kao *et al*, 1998; Kellis & Vickery, 1984; Pelissero *et al*, 1996). Isoflavones are less effective aromatase inhibitors compared with flavones e.g. naringenin (Kao *et al*, 1998) (see Table 7.1) and the high concentrations (≥ 100 μM) of isoflavones required to inhibit aromatase *in vitro* are unlikely to be achieved *in vivo* following dietary exposure to phytoestrogens.

Table 7.1 Aromatase inhibition by phytoestrogens.
Adapted from Kao *et al* (1998).

Compound	Ki (μM)
naringenin	5.1 ± 0.2
7,8-dihydroxyflavone	10 ± 1
biochanin A	12 ± 5
genistein	123 ± 8

Inhibition of glucuronidation

7.18 Zhu *et al* (1998) reported the inhibition of oestrone and oestradiol glucuronidation *in vitro* by polyphenols and flavonoids in rat liver microsomal fractions. The most potent compounds were found to inhibit with an IC_{50} of approximately 25 μM. Flavonoids have also been shown to induce phase I and II drug metabolising enzymes in rats including *p*-nitrophenol UDP-glucuronosyl transferase (Siess *et al*, 1996). Consequently, it is possible that inhibition of glucuronidation activity may be counteracted by increased synthesis of the enzyme in this species (e.g. rat liver). However, the effects of phytoestrogens have not been evaluated for either inhibition or induction of glucuronosyl transferase activity.

Inhibition of steroid sulfatase and sulfotransferase

7.19 Steroid sulfotransferase catalyses the addition of sulfate to steroid-like compounds and steroid sulfatase catalyses the reverse reaction. The equilibrium between these opposing reactions usually lies towards the sulfated compounds and their concentrations are 10-30-fold higher than the unconjugated forms (Harris *et al*, 2000). Steroid sulfatase activity is of particular importance to post-menopausal women as it produces biologically active oestrogens from the less active oestrone sulfate and dehydroepiandrosterone (DHEA) sulfate in breast tissue. In many breast cancer tumours, oestrogen sulfotransferase activity is lower than in normal tissue, and this may account for the increased sensitivity of breast tumours to oestrogen (Qian *et al*, 1998). A project in the Food Standards Agency phytoestrogen research programme is currently evaluating whether other phytoestrogens and their sulfated metabolites are effective inhibitors of these enzymes. Should phytoestrogens selectively inhibit one of these enzymes this would alter the respective concentrations of active and inactive oestrogens with potentially potent effects depending on the enzyme localisation in tissues. Results published from this study indicate that genistein and daidzein are potent inhibitors of oestradiol sulfation *in vitro*. In contrast, these compounds were poor inhibitors of steroid sulfatase (see Table 7.2). This suggests that they may act to either reduce the concentrations of sulfated steroids or, conversely, to increase the concentration of active steroids in peripheral tissues, depending on the location of the affected enzymes (Kirk *et al*, 2001).

7.20 Harris *et al* (2000) reported that 4-*p*-ethylphenol, a metabolite of genistein can act as a sulfotransferase substrate. However it remains to be determined if dietary phytoestrogens can generate sufficient 4-*p*-ethylphenol to reduce the activity of these enzymes in target tissues. Wong & Keung (1997) demonstrated that daidzein sulfoconjugates are potent inhibitors of steroid sulfatase and sulfotransferases (see Table 7.2).

Table 7.2 Steroid sulfatase and sulfotransferase inhibition by phytoestrogens.

Enzyme Inhibited	Phytoestrogen	IC_{50} (μM)
Steroid sulfatase	Genistein	>25[a]
	Daidzein	>50[a]
	Daidzein-4'-O-sulfate	6[b], 8.6[a]
	Daidzein-7,4'-di-O-sulfate	1.5[b]
Steroid sulfotransferase	Genistein	0.8[a]
	Daidzein	1.0[a]
	Daidzein-4'-O-sulfate	>100[b]
	Daidzein-7,4'-di-O-sulfate	>100[b]

[a]Data from Kirk *et al* (2001); [b]Data from Wong & Keung (1997).

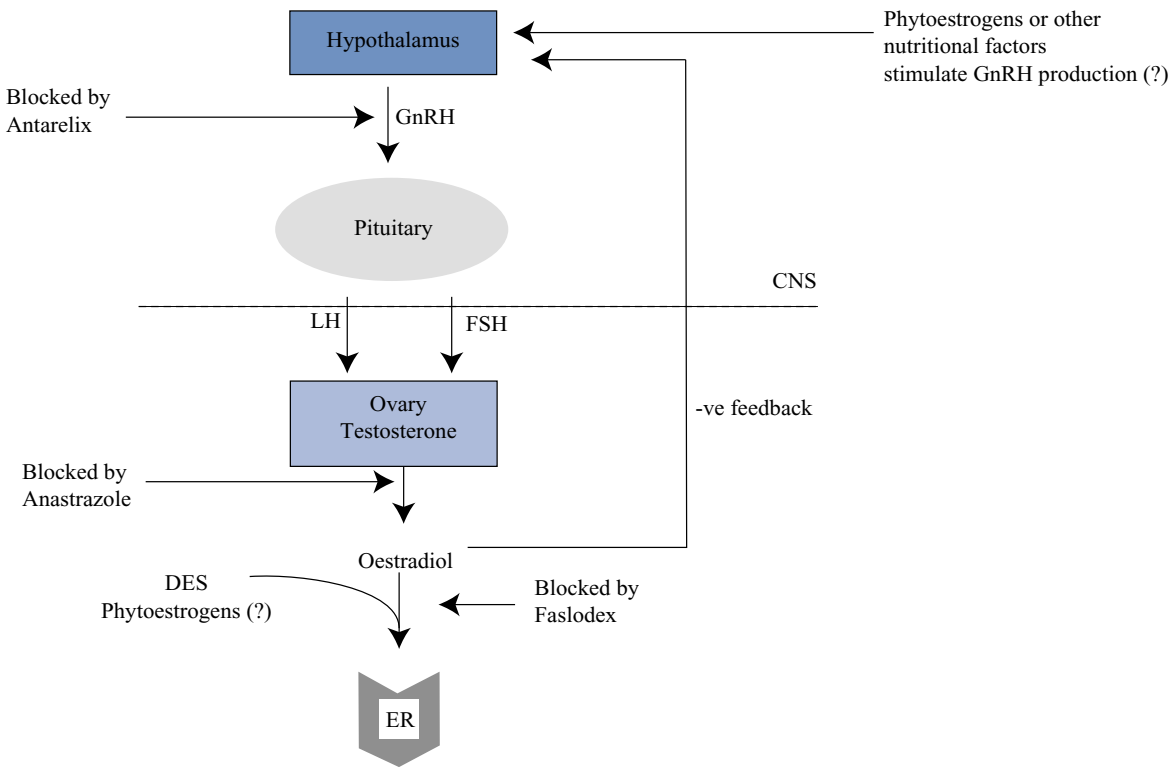
Effects on oestrogen production

7.21 The production of endogenous oestrogens is controlled in the central nervous system by the secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus. GnRH acts on the pituitary gland to increase secretion of the gonadotrophins, follicle stimulating hormone (FSH) and lutenising hormone (LH). Following their release, LH and FSH stimulate the maturation of the ova resulting in increased conversion of testosterone to oestradiol by the enzyme, aromatase. When sufficient levels of oestradiol have been reached, oestradiol exerts a negative feedback effect on the hypothalamus inhibiting further secretion of GnRH. The role of hormones in the production of sex hormones is explained in more detail in Chapter 9.

7.22 Experiments have shown that phytoestrogens may modulate the endogenous production of oestrogen. Ashby *et al* (2000) demonstrated that soy-based formulae elicited oestrogenic effects in an ovariectomised mouse model (see Chapter 8 for further details of this experimental system). The oestrogenic effects of soy-based formulae, diethylstilboestrol (DES) and oestradiol were abolished when the oestrogen receptor antagonist faslodex (FAS) was co-administered, implicating oestrogen receptors in the response (see Figure 7.3). Antarelix (ANT) blocked the action of GnRH as a result of exposure to soy-based formulae but not DES or oestradiol. Also the aromatase inhibitor anastrazole (ANAS) abolished the oestrogenic effects of soy formulae. Both GnRH and aromatase inhibition suggest that the oestrogenic effect of isoflavones in soy-based formulae are not mediated via direct interaction with

oestrogen receptors but via stimulation of the central nervous system by hypothalamic GnRH and subsequent production of endogenous oestrogens mediated by gonadotrophins released from the pituitary. However, similar oestrogenic effects were observed with cows' milk formulae and a phytoestrogen free rodent diet. The same inhibitors also abolished the oestrogenic effects. This suggests the oestrogenic effects observed may be mediated via dietary factors other than isoflavones. Thus, direct effects of isoflavones on oestrogen production by a centrally mediated mechanism remain to be established.

Figure 7.3 Investigation of centrally mediated oestrogenic effects of phytoestrogens.
The mechanisms responsible for the uterotrophic effects of soy-based formulae were investigated using selective pharmacological inhibitors of sex hormone production and action. The oestrogenic effects of the formulae were abolished by the ER antagonist faslodex, the aromatase inhibitor, anastrasole and the GnRH inhibitor, antarelix suggesting that the oestrogenic effects are mediated by stimulation of GnRH production resulting in increased production of endogenous oestrogens rather than by direct interaction with oestrogen receptors in the ovary.



Other biochemical effects of phytoestrogens

Induction of the steroid and xenobiotic pregnane X receptor

7.23 The pregnane X receptor (PXR) has also been described as the steroid X receptor and is highly expressed in the liver and intestine of mammalian species, including man. The receptor is activated by a diverse range of chemicals, including natural and synthetic steroids and induces the expression of CYP3A genes. In addition to modulating this xenobiotic metabolising capacity it is thought that PXR can also modulate CYP3A expression in response to endogenous hormones (Waxman, 1999; Savas *et al*, 1999). It has been suggested that PXR may act to monitor steroid and xenobiotic concentrations and upregulate genes that metabolise such compounds. Since PXR is expressed mainly in the gut and liver, exposure to dietary phytoestrogens could result in the increased metabolism of endogenous oestrogens.

7.24 There are differences in the structure of the PXR ligand-binding domain (LBD) between species. Rabbit, rat and human receptors share only ~ 80% amino acid homology in the ligand binding domain of the receptor (Jones *et al*, 2000). Mouse LBD PXR shares 95% sequence identity with rat but 79% with human (Zhang *et al*, 1999). Thus, the receptors between species can respond to chemicals differently (Blumberg *et al*, 1998; Lehmann *et al*, 1998; Jones *et al*, 2000). Blumberg *et al*, (1998) demonstrated that human PXR was activated by coumestrol whereas the mouse PXR was not. Therefore, the different compound specificities between human and rodent PXR may result in species differences in the metabolism and therefore, the potency of phytoestrogens in rodents and humans (Moore & Kliewer, 2000).

Thyroid peroxidase

7.25 Thyroid peroxidase (TPO) is an enzyme involved in the production of thyroid hormones. TPO catalyses the oxidation of iodide to iodine radical allowing iodination of tyrosines during synthesis of thyroid hormones, tri-iodothyronine (T_3) and thyroxine (T_4). *In vitro* studies suggest that some phytoestrogens may inhibit TPO activity to reduce the concentrations of thyroid hormones. Genistein, daidzein and biochanin A inhibited TPO catalysed thyroid hormone synthesis *in vitro* by acting as alternative substrates for iodination (see Table 7.3) (Divi *et al*, 1997; Divi & Doerge, 1996). Addition of iodine reduced this effect. If such inhibition were to occur *in vivo*, concentrations of thyroid hormones would be reduced and potentially stimulate the production of thyroid stimulating hormone (TSH). The implications of the interactions between phytoestrogens and thyroid peroxidase are described in Chapter 10.

Table 7.3 Inhibition of thyroid peroxidase (TPO) catalysed thyroid hormone synthesis by phytoestrogens.
Data from Divi *et al* (1997) and Divi & Doerge (1996).

Compound	Concentration ((M)	% Inhibition
Genistein	3.2	50
Daidzein	7.6	50
Biochanin A	6	15
Flavanone	>1500	2
Flavone	>2000	7

Inhibition of topoisomerase II *in vitro*

7.26 DNA topoisomerase II is a nuclear enzyme involved in replication, transcription, recombination, integration and transposition (Okura *et al*, 1988). Topoisomerase II catalyses the relaxation of supercoiled DNA, separating the two strands of DNA by breaking and joining DNA strands in ATP-dependent reactions (Bjergbaek *et al*, 2000; Wang *et al*, 2001). This allows the genes to be manipulated during replication and gene expression. Therefore, topoisomerase II plays an integral role in cell growth and death and is likely to mediate events that occur when cells are exposed to cytotoxic agents. Inhibition of topoisomerase II may disrupt progression through the cell cycle and initiate apoptosis.

7.27 A number of studies have shown that genistein can interact with topoisomerase II *in vitro* (Kaufmann *et al*, 1998; McCabe & Orrenius, 1993; Middleton *et al*, 2000; Polkowski & Mazurek, 2000). Okura *et al* (1988) demonstrated that genistein (3.7 μM) stabilises the topoisomerase II-DNA complex. Markovits *et al* (1989) and Yamashita *et al* (1990) showed that genistein induced stabilisation of this complex, stimulating topoisomerase II dependent DNA cleavage (in the ranges 80-370 μM and 12.5-250 μM, respectively). Strick *et al* (2000a) found genistein 50 μM) inhibited unwinding of DNA by topoisomerase II in primary hematopoietic cells and stimulated strand breakage. Robinson *et al* (1993) investigated the effect of genistein on the DNA cleavage and ATP hydrolysis steps of the topoisomerase II catalysed reactions. Genistein inhibited enzyme-catalysed ATP hydrolysis with an IC_{50} of 7 μM. However, genistein did not stimulate DNA cleavage (Robinson *et al*, 1993). Further studies by Strick *et al* (2000b) showed that genistein (50 μM) and daidzein (100 μM) induced cleavage of the *MLL* gene in primary hematopoietic cells. Further *in vitro* experiments suggested that inhibition of topoisomerase II catalysed re-ligation of DNA strand breaks as the mechanism of action.

Inhibition of topoisomerase II *in vivo*

7.28 There is no direct evidence for genistein interactions with topoisomerase II *in vivo*. However, Record *et al* (1997) and Zhou *et al* (1998) have suggested that genistein inhibition of topoisomerase II accounts for the chemo-protective effects observed in rodents treated with genistein. Record *et al* (1997) demonstrated

tumour growth in female mice implanted with B16 melanoma cells was inhibited by 50% when fed a diet containing 1 mg/day genistein.

7.29 Zhou *et al* (1998) showed similar effects in mice implanted with mouse bladder carcinoma cells. Genistein administered by intraperitoneal injection (50 mg/kg bw/day) or in feed (either 0.2-1% (w/w) soy concentrate or 20% (w/w) soy protein isolate) reduced tumour volumes by 40% and 1%, respectively. These treatments reduced cell growth, increased apoptosis and prevented angiogenesis, in the absence of toxicity in the normal bladder epithelia. Record *et al* (1997) and Zhou *et al* (1998) propose that genistein inhibition of topoisomerase II induced apoptosis with concomitant chemo-protective effects.

Inhibition of protein kinases

7.30 Protein kinases are essential enzymes for cellular growth and differentiation. They allow cells to respond to external stimuli through signal transduction pathways by catalysing the phosphorylation of serine, threonine and tyrosine residues in proteins (for review see Scott & Pawson, 2000). A number of studies have shown that genistein can inhibit tyrosine kinases *in vitro*.

7.31 Akiyama *et al* (1987) demonstrated that genistein (150 μM) inhibited tyrosine kinase activity by binding to the catalytic domain of the enzyme. Genistein (100 μM) suppressed growth of MCF-7 breast cancer cells in response to insulin by inhibiting insulin induced tyrosine kinase activity (Pagliacci *et al*, 1994). Spinozzi *et al* (1994) demonstrated that genistein dose-dependently inhibited the proliferation of a leukaemia cell line with a constitutively increased tyrosine phosphorylation pattern.

7.32 Genistein (in the range 75-112 μM) inhibited tyrosine kinase activity, prevented S-phase progression and inducing G2/M arrest and apoptosis. Dalu *et al* (1998) demonstrated that increasing concentrations of genistein in the diet (250-1000 mg/kg diet) inhibited tyrosine-phosphorylated proteins of 85 and 170 kD in rat prostate. Genistein (1000 mg/kg diet) repressed the expression of EGF (epidermal growth factor – a protein involved in cellular proliferation) and its phosphorylation product by 50% and inhibited expression of ERβ2. However, kinase inhibition may be cell dependent and modulated by the concentrations of oestradiol and other growth factors suggesting that genistein may not consistently inhibit tyrosine kinase (Panno *et al*, 1996; Peterson, 1995; Peterson & Barnes, 1993; Peterson & Barnes 1996; Peterson *et al*, 1996; Twaddle *et al,* 1999).

Effects on cellular growth and differentiation

7.33 The effects of genistein on cell growth and cell cycle progression have been investigated *in vitro*. Genistein (10-60 μM) inhibited the growth of a human gastric cancer cell line (HGC-27) in a dose-dependent manner with cell cycle arrest at the G2/M-phase (Matsukawa *et al*, 1993). G2/M arrest was also observed in multi-drug resistant human Jurkat and mouse leukaemia cells treated with genistein (40 μM) (Finlay *et al*, 1994). Spinozzi *et al* (1994) demonstrated genistein induced G_2/M- (18-37 μM) and S-phase arrest (74-110 μM) in Jurkat cells. Genistein (60-150 μM) arrested the cell cycle at G2/M-phase arrest in colon cancer cells inducing apoptosis (Salti *et al*, 2000). Similar results were found in human

myelogenous leukemia HL-60 and lyphocytic leukemia MOLT-4 cells at concentrations of genistein of 20 μM (Traganos *et al*, 1992). Zava & Duwe (1997) found that MCF-7 cells proliferated when exposed to 1 nM-10 μM genistein but inhibited cell growth at concentrations >10 μM. Hsieh *et al* (1998) also demonstrated that genistein (0.01-1 μM) increased cell growth in a dose-dependent manner but at concentrations >25-100 μM, cell growth was inhibited. Genistein (10 μM) increased the protein content, alkaline phosphatase activity and DNA content of metaphyseal tissues isolated from female rats (Yamaguchi & Gao, 1998) and in osteoblastic MC3T3-E1 cells (Sugimoto & Yamaguchi, 2000). Cell proliferation has also been observed Constantinou & Huberman (1995) studied the genistein-induced cell differentiation in several cell lines. Genistein (3.7-92 μM) inhibited cell proliferation in a dose-dependent manner. However, a number of cell lines were shown to differentiate (Constantinou & Huberman 1995). Peterson & Barnes (1991) demonstrated that genistein-inhibited growth in human breast cancer cell lines (IC_{50} ranging from 24-44 μM). Similar results in an oestrogen receptor negative cell line suggested inhibition was not receptor mediated. *In vitro* evidence that phytoestrogens inhibit cell growth and induce differentiation use concentrations that are unattainable through normal dietary consumption in humans and therefore these results are not applicable *in vivo*.

Genotoxic effects of phytoestrogens

7.34 The genotoxic and mutagenic properties of a number of phytoestrogens have been investigated in a number of *in vitro* systems. Kulling & Metzler (1997) demonstrated that genistein (> 25 μM) and coumestrol (50 μM) induced micronuclei in Chinese hamster V79 cells whereas daidzein did not. Coumestrol (25-100 μM) also induced DNA breaks in a dose-dependent manner. Daidzein did not induce strand breakage over this concentration range and genistein was not tested in this study.

7.35 Coumestrol (5-50 μM) induced dose-dependent mutations in the hypoxanthine guanine phosphoribosyl transferase gene. Genistein (25 μM) and daidzein (100 μM) also increased mutation frequency in this gene significantly above controls (Kulling & Metzler 1997). Boos & Stopper (2000) assessed the genotoxicity of genistein using the micronucleus, single cell electrophoresis and tk-locus mutation assays. Genistein induced DNA strand breaks (7-118 μM), mutations (10-80 μM) and micronuclei (6-100 μM), respectively in these assays. Similar results were reported by Morris *et al* (1998). Coumestrol (50-75 μM) and genistein (25 μM) induced chromatid breaks, gaps and interchanges in cultured lymphocytes (Kulling *et al*, 1999). Strand breakage induced by genistein and daidzein was investigated using the comet assay (Anderson *et al*, 1997). Exposure to genistein and daidzein resulted in weak and variable DNA damage. Misra *et al* (2002) assessed the *in vitro* mutagenicity of an isoflavone product (40-50% genistein, 18-25% daidzein and 1-4% glycitein). Isoflavones (genistein concentrations > 4 μM) were weakly mutagenic in 1/5 bacterial and 1/1 mammalian mutation assays after addition of metabolising systems.

7.36 The results of a study by Mitchell *et al* (2000) showed that genistein, daidzein, coumestrol and equol cause growth inhibition of PC-3 and LNCaP human prostate tumour cells at concentrations of <10 μM. Inhibition of LNCaP cells occurred at lower concentrations than PC-3 cells. Exposure to genistein caused DNA strand breakage at concentrations ⩽ 10 μM in both LNCaP and PC-3 cells. DNA damage was not

observed when cells were treated with daidzein. High concentrations of equol (> 50 μM) and coumestrol (> 250 μM) were required to cause DNA strand breakage in both cell lines. In addition, genistein (5 μM) was found to protect against H_2O_2-induced DNA strand breakage in PC-3 prostate cancer cells.

7.37 The *in vivo* mutagenicity of isoflavones has been investigated in only one study (Misra *et al*, 2002). Small non-dose dependent increases in micronuclei were reported at one time-point in male but not female mice fed genistein (0-1000 mg/kg diet) in the mouse micronucleus assay. The authors reported similar findings in historical control animals. In addition, there were no differences in the incidence, multiplicity or spectrum of tumours in either male or female p53 (-/-) mice fed genistein (50 mg/kg bw/day) compared with control animals. This study suggests that genistein at dietary levels is not mutagenic *in vivo*.

Anti-oxidant properties of phytoestrogens

7.38 Highly reactive oxygen species, such as hydroxyl radicals, have been shown to damage DNA, cellular proteins and lipids to play a role in the development of cancer (see Chapter 15). In addition, oxidation of lipids has been implicated in the development of cardiovascular disease (see Chapter 13). It has been suggested that isoflavones and lignans possess antioxidant properties Wei, *et al* (1993). A study by Harper *et al* (1999) investigated the antioxidant capability of genistein, equol, enterolactone and enterodiol using an *in vitro* system to measure the inhibition of free radical induced DNA damage. The rank order of inhibition of DNA damage was genistein > equol > enterolactone and enterodiol. However, the concentrations required were higher than those likely to occur *in vivo* from dietary exposure to phytoestrogens (i.e. IC_{50} for genistein was 9 μM).

7.39 Wei *et al* (1995) investigated the antioxidant properties of the phytoestrogen genistein. Superoxide and hydrogen peroxide free radical production was efficiently inhibited by genistein at concentrations of 5 and 50 μM, respectively *in vitro* assays.

7.40 Studies have suggested that phytoestrogens may protect against lipid peroxidation. Genistein was found to inhibit lipid peroxidation at concentrations >15 μM and low-density lipoprotein (LDL) oxidation at concentrations >20 μM but had no effect on lipid hydroperoxide concentrations (Patel *et al*, 2001). In an *in vitro* study by Wilson *et al* (2002), genistein and daidzein significantly inhibited LDL oxidation at 10 μM but not 1 μM. Similarly, Hodgson *et al* (1996) showed that the daidzein metabolites, equol and *O*-desmethylangolensin were more potent inhibitors of lipoprotein oxidation than daidzein or genistein at concentrations of 10 μM. Ascorbic acid has also been shown to enhance the inhibitory effects of genistein, daidzein and equol on LDL oxidation *in vitro* (Hwang *et al*, 2000).

7.41 A study by Arora *et al* (1998) compared the ability of genistein, genistin, daidzein, daidzin, biochanin A, formononetin and equol (all at 10 μM) to inhibit lipid peroxidation *in vitro*. The rank order of antioxidant activity was formononetin > biochanin A > daidzin > daidzein > genistin > genistein. Thus, the number and position of hydroxyl groups appear to be determining factors for antioxidant activity.

7.42 In a study in mice with increased susceptibility to skin tumours, dietary genistein (250 mg/kg diet) administration for 30 days increased the activities of superoxide dismutase and glutathione-*S*-transferase (enzymes involved in free radical scavenging) in the skin (Cai & Wei, 1996).

7.43 The antioxidant effects of phytoestrogens have been investigated in a number of human dietary trials. In a randomised cross-over trial, hyperlipidemic patients (19 men and 12 postmenopausal women) were fed soy (86 mg isoflavones/day) for 4 weeks. Decreases in oxidised LDL concentrations in blood ($p<0.001$) were reported after dietary soy supplementation (Jenkins *et al*, 2000).

7.44 The oxidation rate of LDL fractions from subjects (n= 6) was tested *ex vivo* before and after consumption of soy (12 mg genistein and 7 mg daidzein/day) for 14 days. Dietary soy reduced the rate of LDL oxidation ($p<0.02$) compared with baseline measurements (Tikkanen *et al*, 1998). Similarly, in a study by Kanazawa *et al* (1995), ingestion of soy (isoflavone content not determined) appeared to reduce lipoprotein peroxidation tested *ex vivo* in samples from subjects (n= 10) compared with controls ($p<0.05$).

7.45 In a randomised trial, men were fed soy milk (n= 4), rice milk (n= 3) or cows' milk (n= 3) for 4 weeks (Mitchell *et al*, 1999). Dietary soy supplementation reduced oxidative DNA base damage (but not peroxide induced DNA strand breaks) in lymphocytes from the soy fed group compared with the other groups ($p<0.05$), as assessed *ex vivo* using the Comet assay.

7.46 In a randomised cross-over trial, reductions in plasma 8-*epi*-prostaglandin $F_{2\alpha}$ (a biomarker for lipid peroxidation) concentrations (p= 0.028) and LDL oxidation (p= 0.017) tested *ex vivo* were seen in blood samples from subjects (n= 24) that had consumed soy (56 mg isoflavones) compared with alcohol extracted soy (2 mg isoflavones) for 17 days (Wiseman *et al*, 2000).

7.47 There was no difference in the oxidisability of LDL fractions tested *ex vivo* between test and control groups in a randomised cross-over trial in women (n= 14) consuming isoflavones (86 mg/day) for 2 menstrual cycles (Samman *et al*, 1999).

7.48 These human studies suggest that supplementation of the diet with soy may produce antioxidant effects such as inhibition of LDL oxidation. However, this property may not be attributable to the phytoestrogen content of soy.

Key points

- It is possible that phytoestrogens may produce oestrogenic effects, which occur by mechanisms other than direct interaction with oestrogen receptors.
- Phytoestrogens may stimulate secretion of hormones from the central nervous system to increase concentrations of oestrogens.
- Phytoestrogens may also modulate the concentrations of endogenous hormones by:
  - inhibition of hormone binding to sex hormone binding globulin (SHBG), which increases the concentrations of active oestradiol and testosterone.
  - stimulation of SHBG synthesis which can reduce concentrations of endogenous hormones.
  - inhibition of enzymes involved in oestrogen biosynthesis and metabolism to modulate concentrations of endogenous oestrogens.

However, much of the evidence for these effects comes from *in vitro* studies and require much higher concentrations of phytoestrogens than are likely to be achieved *in vivo* via dietary exposure. The studies carried out in humans have reported disparate effects. Therefore, at present it is not clear what such interactions contribute towards oestrogenic effects observed *in vivo*.

- The pregnane X receptor (PXR) may monitor steroid and xenobiotic concentrations and upregulate genes that metabolise such compounds. Since PXR is expressed mainly in the gut and liver, exposure to dietary phytoestrogens could result in the increased metabolism of endogenous oestrogens.
- *In vitro* experiments have shown that phytoestrogens can inhibit thyroid peroxidase, an enzyme involved in synthesis of thyroid hormones.
- *In vitro* experiments have demonstrated that genistein interacts with topoisomerase II and protein kinases which are enzymes involved in cellular proliferation and differentiation. However, the concentrations of phytoestrogens required to inhibit these enzymes are higher than concentrations of phytoestrogens likely to be achieved *in vivo* following dietary exposure. No studies have directly demonstrated such effects *in vivo*.
- There are limited data on the genotoxic potential of phytoestrogens. Studies have indicated that coumestrol and genistein may be genotoxic at concentrations very much higher than would be achieved *in vivo* from dietary exposure to phytoestrogens. The single study that has investigated the *in vivo* mutagenicity of isoflavones suggests that genistein at dietary levels is not mutagenic *in vivo*.

- Data suggests that phytoestrogens may possess antioxidant properties but at much higher concentrations than would be expected to be achieved *in vivo* following dietary exposure. Human studies suggest that dietary soy may have antioxidant effects such as inhibition of LDL oxidation. However, these effects may not be attributable to the phytoestrogen content of soy.

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8. Estimation of phytoestrogen oestrogenic potency

Introduction

8.1 The oestrogenic potential of phytoestrogens is the most thoroughly documented property of these compounds. As outlined in Chapters 6 and 7, phytoestrogens may elicit oestrogenic effects either by direct interaction with oestrogen receptors or indirectly by modulation of endogenous oestrogen levels. Oestrogenic potency is expressed as the ratio of the activity of a test compound relative to that of oestradiol, the major human oestrogen in the same assay. This comparison offers a perspective on the potential biological effects of phytoestrogens. In general, most phytoestrogens are relatively weak oestrogens, requiring much higher concentrations than oestradiol to produce an equivalent biological response.

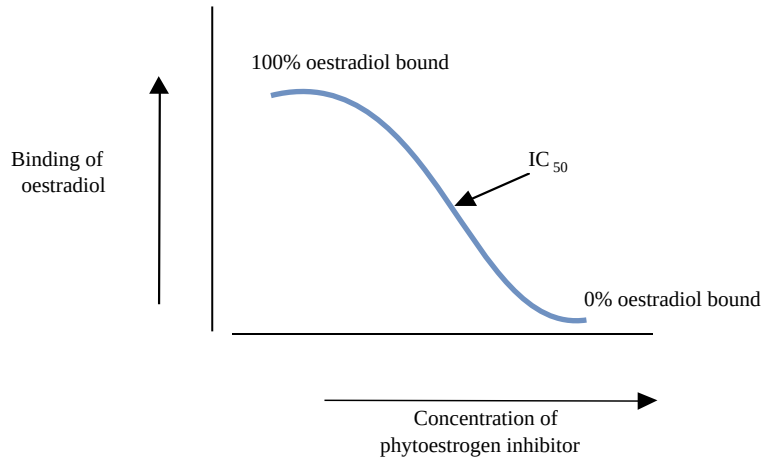
8.2 A variety of experimental methods are used to assess the oestrogenicity of phytoestrogens, and the estimate of oestrogenic potency can vary significantly between methods. This chapter reviews the range of *in vitro* and *in vivo* experimental methods in use.

8.3 In general, *in vivo* assays provide a more holistic view of oestrogenic activity. These assays incorporate many different biological processes, including absorption and metabolism, which influence oestrogenic activity. Many of the *in vitro* methods are a simple measurement of direct interaction with the oestrogen receptor and are not necessarily predictive of oestrogenic activity *in vivo*.

Oestrogen receptor binding assays

8.4 Oestrogen receptor binding assays provide a quantitative indication of the oestrogenic activity of a phytoestrogen relative to that of oestradiol at the receptor level. They are conducted *in vitro* and examine the binding affinity of phytoestrogens to oestrogen receptors, which is termed the relative binding affinity (RBA). RBAs are usually expressed as the concentration of a compound required to inhibit oestradiol binding by 50% or IC_{50} (Blair *et al*, 2000) (see Figure 8.1). Thus, RBAs provide a simple measurement of the affinity of a compound to the receptor but do not indicate whether a compound will behave as an agonist, antagonist or partial agonist (see paragraphs 6.20-6.24).

Figure 8.1 Determination of IC_{50} from a competitive ligand-binding assay.
The binding of oestradiol to oestrogen receptors is measured in the presence of increasing concentrations of a phytoestrogen. The IC_{50} value for a phytoestrogen is the concentration of compound required to inhibit oestradiol binding by 50%.



8.5 Many studies have investigated the binding affinity of isoflavones to oestrogen receptors. However, there is a paucity of data for lignans. The RBA of the same phytoestrogen may differ markedly between studies. The range of RBAs reported for phytoestrogens are shown in Table 8.1.

Table 8.1 Ranges of relative binding affinities (RBA) for phytoestrogens to oestrogen receptors.
RBAs are relative to oestradiol, which has been assigned an arbitrary binding affinity of 1. Ranges of RBA are represented as individual studies report different values of binding affinity using this assay method.

Phytoestrogen	Range of RBA[a]		
Coumestrol	0.002	–	1.0
Equol	0.001	–	0.003
Genistein	0.0001	–	0.0088
Biochanin A	0.0015	–	0.00005
Daidzein	0.0001	–	0.0008
Formononetin	0.000046	–	0.000067

[a]Data from Breithofer *et al* (1998; Collins *et al* (1997; Gehm *et al* (1997; Graumann *et al* (1999); Hopert *et al* (1998); Hunter *et al* (1999); Kitaoka *et al* (1998); Kuiper *et al* (1997; 1998); Miksicek (1995); Petit *et al* (1997); Sathyamoorthy & Wang (1997); Stahl *et al* (1998); Wang *et al* (1995); Zava & Duwe (1997); Zhang *et al* (1999a and b).

8.6 It is probable that experimental protocols vary between laboratories, and this may contribute to inter-laboratory differences in the results. Additionally, variations in the oestrogen receptor subtype and variants used in the assays may account for the range of values obtained for a single phytoestrogen. As described in 6.22-6.23, phytoestrogens can bind preferentially to the ERβ subtype. There is a number of variants of oestrogen receptors and phytoestrogens that can also bind (with different affinities) to these receptor variants. For example, binding studies to ERβ variants have demonstrated that coumestrol and genistein bind to ERβ2 with a weaker affinity than to ERβ1 (see Table 8.2; Petersen *et al*, 1998).

Table 8.2 Binding affinities of oestradiol, coumestrol and genistein to the β1 and β2 variants of the oestrogen receptor.

Compound	ERβ1 binding affinity/nM	ERβ2 bonding affinity/nM
Oestradiol	0.17	3.02
Coumestrol	0.11	7.76
Genistein	0.26	41.5

Data taken from Petersen *et al* (1998).

8.7 When studies are taken together, a rank order of RBA can be derived for phytoestrogens as follows: oestradiol $\geqslant$ coumestrol $>$ 8-prenylnaringenin $>$ genistein and equol $>$ daidzein $>$ glycitein $>$ biochanin A, formononetin, 6-prenylnarinigenin, xanthohumol and isoxanthohumol (Breithofer *et al*, 1998; Collins *et al*, 1997; Gehm *et al*, 1997; Graumann *et al*, 1999; Hopert *et al*, 1998; Hunter *et al*, 1999; Kitaoka *et al*, 1998; Kuiper *et al*, 1997; 1998; Miksicek, 1995; Milligan *et al*, 1999; Petit *et al*, 1997; Sathyamoorthy & Wang, 1997; Song *et al*, 1999; Stahl *et al*, 1998; Wang *et al*, 1995; Zava & Duwe, 1997; Zhang *et al*, 1999a; 1999b).

8.8 Metabolism of phytoestrogens can result in structural changes for example through glucuronidation or sulfation of hydroxyl groups. These changes can significantly hinder the compound binding to the receptor which lowers the affinity of the metabolite relative to the parent compound (Breinholt & Larsen, 1998; Cheek *et al*, 1998). For example, the glucuronides of genistein and daidzein bind with lower affinity to the oestrogen receptors when compared with their unconjugated forms (Zhang *et al*, 1999a; 1999b).

Cell proliferation assays

8.9 Oestrogen dependent cell proliferation is used as a screening assay for oestrogenic activity. Phytoestrogens have been shown to both stimulate and inhibit cell proliferation in oestrogen dependent cell lines. These effects appear to be concentration dependent, as phytoestrogens stimulate proliferation in the 0.1-10 μM concentration range but inhibit proliferation at higher concentrations ($>$ 10 μM). It has been suggested that the proliferative effects of phytoestrogens may reflect direct receptor mediated responses, whereas the inhibitory effect are not directly mediated via the oestrogen receptors. This proposal is supported by evidence that phytoestrogens do not stimulate proliferation of

ER-negative cells, but are still inhibitory to their growth at high concentrations (Pagliacci *et al*, 1994; Wang *et al*, 1996; Record *et al*, 1997; Wang & Kurzer MS, 1997; Zava & Duwe, 1997; Barnes, 1998; Miodini *et al*, 1999).

8.10 The potency of many phytoestrogens has been evaluated using these assays and a range of parameters is used to estimate oestrogenicity (see Table 8.3). However, not all of these parameters are measured in each study which makes both inter-study comparisons and ranking of oestrogenic potency difficult (Breinholt & Larsen, 1998; Hsu *et al*, 1999; Kitaoka *et al*, 1998; Mousavi & Adlercreutz, 1992; Sathyamoorthy & Wang, 1997; Wang & Kurzer, 1997; Zava & Duwe, 1997).

Table 8.3 Parameters used to evaluate oestrogenic activity in cell proliferation assays.

Parameter	What is measured
EC_{min}	Lowest concentration of compound which promotes cell proliferation.
C_{max}	Concentration of compound that promotes maximal cell proliferation.
EC_{50}	Concentration that gives 50% of the maximum response.
Max_I	Concentration of compound inducing maximal expression level of an oestrogen responsive gene. Not a measure of cell proliferation *per se* but can be used as supporting evidence of oestrogenic activity.

8.11 On the basis of C_{max} data, coumestrol, genistein and enterolactone are estimated as three orders of magnitude less potent than oestradiol. Other phytoestrogens are estimated as at least another order of magnitude less potent (Mousavi & Adlercreutz (1992); Wang & Kurzer (1997); Zava & Duwe (1997)).

8.12 The EC_{50} is probably the most useful parameter when making intra- and inter-study comparisons, although is difficult to compare the values for agonists and partial agonists (see paragraph 6.20-6.24). Very few studies use the EC_{50} parameter with the exception of reports by Breinholt & Larsen (1998) and Kitaoka *et al* (1998). In the study carried out by Breinholt and Larsen (1998), 23 potential phytoestrogens were tested in a breast cell proliferation assay. These data indicate that all the phytoestrogens were at least 3-4 orders of magnitude less potent than oestradiol, with isoflavones having the highest potency. No single phytoestrogen elicited a maximal response greater than 80% that of oestradiol. A study by Hunter *et al* (1999) evaluated oestrogenic potency of phytoestrogens based on EC_{25} rather than EC_{50} data. In this study, EC_{25} values of 1 and 0.1 nM were estimated for coumestrol and genistein, respectively, whereas oestradiol was 1 pM. Kitaoka *et al* (1998) demonstrated that 8-prenylnaringenin was approximately 3 orders of magnitude less potent than oestradiol.

Recombinant mammalian and yeast cell-based transcription assays

8.13 The oestrogenic potency of phytoestrogens has been assessed using mammalian cells engineered to express a reporter gene under the control of an oestrogen response element (ERE). In these assays, the reporter gene product is usually an enzyme and is measured either indirectly (by measurement of enzymatic activity of the reporter gene product), or directly by quantification of the transcript or protein (Mayr *et al*, 1992; Sathyamoorthy & Wang, 1997; Zava & Duwe, 1997). Similar systems have been developed in yeast, which have been engineered to express mammalian forms of oestrogen receptors (Coldham *et al*, 1997).

8.14 Most of these studies have assessed the oestrogenicity of phytoestrogens by comparison with oestradiol. However, the data are not always amenable to inter-study comparison as different test systems are employed or investigators have not studied the same compounds. The range of potencies of various phytoestrogens reported from reporter gene assays is shown in Table 8.4. The wide range of values obtained with different cell types and reporter assays may be due to contrasting kinetics in cellular metabolism, uptake and membrane transport (Nagel *et al*, 1998; 1999). For example, yeast cells have the ability to transport specific steroid compounds out of the cell (Kralli *et al*, 1995). Consequently, differences in the efflux of test compounds will affect the estimation of oestrogenic potency. The design of the recombinant system will also determine the sensitivity of the system. Ashby *et al* (1999) demonstrated that the source of ERE used in a cell-based assay can influence the extent of oestrogenic activity.

Table 8.4 Range of oestrogenic potencies obtained for phytoestrogens determined by cell-based transcription assays.

Potencies are relative to oestradiol, which has been assigned an arbitrary binding affinity of 1. Ranges of potency are represented as individual studies report different values using this type of assay.

Phytoestrogen	Range of potency[a]		
Coumestrol	0.0003	–	0.09
Genistein	0.000001	–	0.002
Equol	0.00023	–	0.001
Daidzein	0.0000024	–	0.00014
Biochanin A	0.000000 1	–	0.0045

[a]Data from Arnold *et al* (1996); Ashby *et al* (1999); Baker *et al* (1999); Breinholt & Larsen (1998); Breithofer *et al* (1998); Coldham *et al* (1997); Collins *et al* (1997); Gaido *et al* (1997); Hopert *et al* (1998); Hunter *et al* (1999); Petit *et al* (1997); Sathyamoorthy & Wang (1997); Willard & Frawley (1998); Zava & Duwe (1997).

8.15 Relative potencies can be determined using EC_{50} values relative to the EC_{50} for oestradiol. However, many compounds are partial agonists and therefore, do not give as great a response as oestradiol no matter what concentration of test compound is used. Thus, comparisons of EC_{50} values, derived from cell-based transcription assays may not be accurately made (Kuiper *et al*, 1998).

8.16 Coumestrol is often shown to be the most potent phytoestrogen in these test systems (100-1000-fold less potent than oestradiol), followed by genistein, 8-prenylnaringenin, equol, daidzein, biochanin A and formononetin ($\geqslant$ 1000-fold less potent than oestradiol) (Arnold *et al*, 1996; Ashby *et al*, 1999; Baker *et al*, 1999; Breinholt & Larsen, 1998; Breithofer *et al*, 1998; Coldham *et al*, 1997; Collins *et al*, 1997; Gaido *et al*, 1997; Hopert *et al*, 1998; Hunter *et al*, 1999; Milligan *et al*, 1999; Petit *et al*, 1997; Sathyamoorthy & Wang, 1997; Willard & Frawley, 1998; Zava & Duwe, 1997).

8.17 The potencies of individual phytoestrogens have been assessed in a yeast cell system expressing a human ERα and an ERE reporter gene, relative to oestradiol (1) as: coumestrol (0.0067), 8-prenylnaringenin (0.004), genistein (0.0005), daidzein (0.00001), biochanin A (0.00009), formononetin (0.00006) and glycitein (0.0000002) (Coldham *et al*, 1999; Coldham & Sauer, 2000).

8.18 Arnold *et al* (1996) showed that the oestrogenic activities of both oestradiol and coumestrol were reduced by 75% in a reporter gene assay when physiological levels of serum, SHBG or α-fetoprotein were included. These proteins lowered the concentrations of the free forms of these compounds. However, isoflavones bind to SHBG with a 1000-5000-fold lower affinity than oestradiol (Nagel *et al*, 1998; 1999). Thus, in the presence of serum proteins, the potencies of phytoestrogens relative to oestradiol may be expected to increase when compared to potencies evaluated under serum-free conditions.

Potency of phytoestrogen mixtures

8.19 Data on the oestrogenic potency of mixtures of phytoestrogens are limited. Willard & Frawley (1998) demonstrated that co-treatment with equimolar concentrations of genistein, daidzein, formononetin and equol gave an additive oestrogenic response in a reporter gene assay than that observed for any individual phytoestrogen. However, Collins *et al* (1997) demonstrated that biochanin A and flavone inhibited the activity of oestradiol in a reporter gene assay, which is predictable for these weak partial agonists.

Interpretation of *in vitro* potency data

8.20 An estimation of oestrogenic potency, which is derived from a receptor binding assay provides an indication of whether a compound can bind to an oestrogen receptor and the strength of that binding. However, it reveals little about the ability of the compound to activate the receptor. Cell-based assays which measure proliferative responses or reporter gene expression indicate whether a compound can act as an oestrogen receptor agonist, partial agonist or antagonist. These assays can provide an approximate assessment of biological activity and can also be used to provide a rank order of potency.

8.21 Receptor binding or cell-based assays are useful tools for screening foods or plants in order to identify novel phytoestrogens. However, *in vitro* assay systems have a number of limitations in terms of predicting biological activity. They do not reflect the specific tissue distributions of phytoestrogens. In addition, these assays do not incorporate any measure of the bioavailability, metabolism or the extent of binding to serum proteins, pharmacokinetics and metabolism of phytoestrogens. All of these factors can modify the *in vivo* biological activity of phytoestrogens. Thus, *in vitro* systems will fail, by and large, to be predictive of the true potency of phytoestrogens *in vivo*, but can indicate their relative potential.

In vivo assessment of oestrogenic effects

8.22 The *in vivo* oestrogenic activity of phytoestrogens has been assessed in a number of experimental models (reviewed in Rudel, 1997). In contrast to *in vitro* methods, *in vivo* assays take account of factors such as bioavailability, pharmacokinetics, metabolism, distribution and interactions with binding and transport proteins. Therefore, *in vivo* assays offer a more holistic way to assess the oestrogenicity of a compound. In addition, whereas most *in vitro* assays assess phytoestrogen interactions with individual oestrogen receptors, *in vivo* assays measure the composite response from both direct and indirect mechanisms of phytoestrogen action (outlined in Chapters 6 and 7).

8.23 A range of different end points can be used as indicators of oestrogenic activity (see Table 8.5). The oestrogenic responses induced in an *in vivo* model are dependent on a number of experimental factors outlined below.

Species

8.24 The majority of research into the oestrogenic properties of phytoestrogens has been carried out in rodents. Other species such as rabbits and primates have been used, but to a much lesser extent. Inter-species differences in the ADME of phytoestrogens have a strong influence on the ultimate oestrogenic effect. Additionally, inter-species differences in fetal, neonatal and pubertal development, endogenous oestrogen concentrations as well as endocrine physiology and function will also determine the magnitude and type of biological response following phytoestrogen exposure (Jobling, 1998). Inter-strain differences in susceptibility to oestrogenic effects have been reported in mice (Spearow *et al*, 1999; 2001). Therefore, it is important that the influence of species and strain differences are recognised and taken into account when extrapolating experimental findings to humans.

Route of administration

8.25 The route of administration has a major influence on the oestrogenic potency of phytoestrogens. In terms of human exposure to phytoestrogens, oral administration is the most relevant. However, many studies have used intraperitoneal or subcutaneous administration, which allows compounds to enter the systemic circulation, by-passing metabolism in the gut and/or liver. Thus, if phytoestrogens are administered by these routes, a more potent oestrogenic response is induced as much of the deactivating effect of metabolism on phytoestrogens has been by-passed. Although not relevant to dietary exposures, use of these routes of administration establishes whether a compound can in principle produce oestrogenic effects and helps identify target tissues.

Effect of timing and duration of exposure

8.26 The sensitivity of the endocrine system to the effects of phytoestrogens is dependent upon the developmental stage and the age when exposure occurs i.e. *in utero*, perinatal, neonatal, pre-pubertal or adult exposure. Thus, there is an age-related sensitivity to oestrogens and the timing of exposure is a critical determinant of effect. Multiple doses of a compound can exert greater and possibly longer-term oestrogenic effects than a single exposure. The effect of multiple exposures and their timing will be discussed in detail in Chapter 9.

Table 8.5 Endpoints used to assess the oestrogenic activity of phytoestrogens *in vivo*.
Most methods use rodents as the test model and effects are assessed by comparison with untreated animals.

End point	Oestrogenic response assessed	Example of study reporting oestrogenic effects to phytoestrogens
Female		
Development of oestrogen sensitive tissues	Increase in weight of oestrogen-sensitive tissue e.g. uterine growth (uterotrophic assay), uterine vascular permeability.	Ashby *et al* (2000); Milligan *et al* (1998)
Hormone concentrations	Alterations of hormone concentrations e.g. LH and FSH.	Register *et al* (1995); Hobson *et al* (1977)
Age of puberty	Advancement of day of vaginal opening and/or first estrus.	Ashby et al (2000)
Estrus cycle length	Alterations in length of phases of the estrus cycle.	Whitten et al (1995)
Cell differentiation	Stimulation of growth e.g. early development of mammary tissue.	Tou & Thompson (1999), Hilakivi-Clarke *et al* (1999)
Histopathological changes	Proliferation or differentiation of oestrogen sensitive cells e.g. epithelial cornification of the endometrium.	Galey *et al* (1993)
Gene expression	Stimulation of expression of an oestrogen-responsive gene e.g. uterine peroxidase, oestrogen receptor expression.	FSA project report T05005
Cell proliferation	Stimulation of cell proliferation in oestrogen-sensitive tissues e.g. mammary gland.	Arts *et al* (1992), Wang *et al* (1995)
Male		
Gene expression	Stimulation of expression of an oestrogen-responsive gene e.g. prostatic c-fos, oestrogen receptor expression.	Strauss *et al* (1998)
Hormone concentrations	Alteration of hormone concentrations e.g. testosterone.	FSA project report T05005
Age of puberty	Alteration in the day of prepuputial separation.	Nagao *et al* (2001)
Development of oestrogen sensitive tissue	Decreased organ growth e.g. testis and prostate weight.	Atanassova *et al* (2000), Strauss *et al* (1998)

Effect of diet

8.27 Standard rodent diets can be supplemented with ingredients such as soybeans, flax, wheat, barley, corn, alfalfa and oats, which contain phytoestrogens (Thigpen e*t al*, (1999a and 1999b). Thigpen *et al* (1999a) analysed twelve standard animal diets and reported that daidzein and genistein were present in ten out of the twelve diets. The concentrations of daidzein and genistein in these diets ranged from 53-277 mg/kg and 69-491 mg/kg, respectively. Odum *et al* (2001) showed that such rodent diets were oestrogenic when compared with phytoestrogen-free diets. Therefore, standard rodent diets can provide a significant source of phytoestrogens and this background exposure may reduce the sensitivity of animals to phytoestrogens. Thus, phytoestrogen-free diets are the diets of choice when assessing the oestrogenicity of phytoestrogens *in vivo*.

8.28 The oestrogenic effects of phytoestrogens in animal models and how they are extrapolated to and interpreted in the context of human exposures is discussed in Chapter 9.

Assessment of oestrogenic potency (the uterotrophic assay)

8.29 As discussed in the previous section a wide range of biological end points has been used to assess oestrogenic activity *in vivo*. Most methods involve analysis of female reproductive tissue development and function. However, other than the uterotrophic assay, few endpoints are suitable for making quantitative measurements of oestrogenic potency.

8.30 The rodent uterotrophic assay measures uterine growth in animals treated with a test compound compared with controls treated with an oestrogen such as oestradiol or diethylstilboestrol (DES). The potency value is expressed as the dose of phytoestrogen required to produce an equivalent change in uterine weight (Bickoff, 1962).

8.31 The uterotrophic assay is performed in immature or ovariectomised animals. Such animals have lower concentrations of endogenous oestrogens which increases the sensitivity of the assay to exogenous oestrogens. Inter-species differences in the uterotrophic response to genistein have been reported as well as differences in sensitivity to route of exposure. Also, considerable inter-laboratory variations are reported for specific compounds (Whitten & Patisaul, 2001). This makes inter-study comparisons difficult and the results must be placed in the context of the experimental methodology. For example, potency values of coumestrol from uterotrophic assays ranged from 100-2500 times lower than that of oestradiol (Baker *et al*, 1999; Bickoff, 1962; Galey *et al*, 1993; Markaverich *et al*, 1995; Whitten *et al*, 1992).

8.32 The absence of any standardised protocol for the uterotrophic assay has been recognised by the Organisation for Economic Co-operation and Development (OECD) and an international programme is in progress to validate the rodent uterotrophic assay to allow compounds that are suspected agonists and antagonists of oestrogen to be identified (Kanno *et al*, 2001). The programme aims to validate the 3 day uterotrophic assay in immature intact and adult ovariectomised rat models. Compounds are administered by oral gavage or subcutaneous injection and dose-response relationships derived. Although the uterotrophic response is considered an oestrogenic effect it has yet to be confirmed as an adverse health effect.

8.33 The oestrogenic response is dependent on the dose administered, and generally a dose-dependent response is produced. However, there are exceptions to this. The uterine response can be divided into two phases: an early phase, which occurs ~2-4 hours after the initial exposure. This phase is characterised by increased vascular permeability, oedema and eosinophilia in the uterus. The second phase response includes changes in cell division, differentiation and uterine growth. The first phase is dependent on the oestrogenic potency of the compound whereas, the second phase is dependent on both the oestrogenic potency and the dosing schedule. Thus, a single administration of a compound, which is a weak oestrogen, can induce the first phase, but thereafter, the response declines and may not induce second phase uterine activity. Whereas, a potent oestrogen may induce phase one and two uterine activity after a single administration. Large or repeated doses of a weak oestrogen may also induce the second phase uterine activity shown by strong oestrogens. For example, subcutaneous administration of either oestradiol or oestriol (a weaker oestrogen) results in uterine activity within a few hours. However, 24 hours after administration, oestriol is unable to mimic the phase two effects of oestradiol. If oestriol is administered repeatedly it can produce a range of oestrogenic responses similar to oestradiol (Milligan *et al*, 1998).

8.34 Although many studies have demonstrated the uterotrophic activity of phytoestrogens, there have been very few systematic and large-scale assessments of their relative oestrogenic potency. Bickoff (1962) defined the oestrogenic potency of phytoestrogens in an immature mouse model as the dose required to induce the development of a uterus weighing at least 25 mg. The potency of several phytoestrogens was assessed in this early assay and coumestrol was found to be the most potent phytoestrogen, although it was 200 and 3000-fold less potent than oestrone and diethylstilboestrol, respectively. Genistein, daidzein, biochanin A and formononetin were found to be 30-100 fold less active than coumestrol. Song *et al*, (1999) demonstrated that glycitein and genistein were 33,000, and 100,000-fold less potent than DES in a mouse uterotrophic assay. 8-Prenylnaringenin was 3000-fold less potent than oestradiol in a rat uterotrophic assay (Miyamoto *et al*, 1998). The relative oestrogenic potency of the lignans has not been assessed using this assay.

8.35 There are male equivalents to the uterotrophic assay which detect androgenic activity. The castrated male rat assay, described by Hershberger *et al* (1953), has been used to detect androgenic and anti-androgenic chemicals. However, this assay is limited by the need for surgical castration. Several alternative approaches have been proposed including an assay which uses peri-pubertal male rats (PND 22-35) and monitors changes in testes, epididymides, seminal vesicle and prostate weights (Ashby & Lefevre, 2000). Phytoestrogens have not been tested in these assays.

Phytoestrogen potency determined by *in vitro* and *in vivo* methods

8.36 The methods currently used to measure the oestrogenic potency of phytoestrogens are summarised in table 8.6

8.37 Constructing a single rank order of oestrogenic potency of phytoestrogens from combined *in vitro* and *in vivo* data is difficult. Different compounds have been tested and no single compound has been tested in all test models. There is significant variation in the potency of phytoestrogens, which differ according

to the test model, methodology and end-point(s) employed. Figure 8.3 demonstrates the range of potencies reported in the literature.

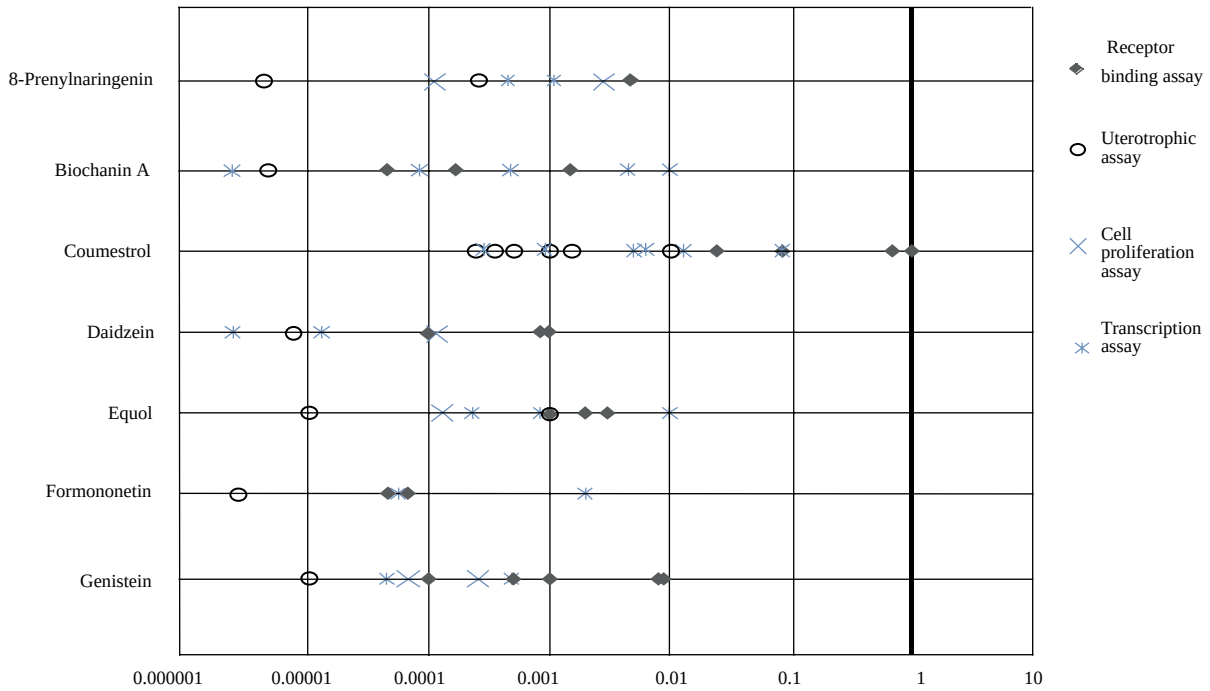
8.38 The oestrogenic potency measured in these assays may provide an indication of the biological potency of a phytoestrogen. For many compounds *in vitro* activity is correlated with oestrogenicity *in vivo*. However, this is not consistently the case, for example, 8-prenylnarigenin is relatively potent in *in vitro* assays but relatively inactive *in vivo* (Coldham & Sauer, 2001). Conversely glycitein is a relatively weak oestrogen in *in vitro* assays but a relatively potent oestrogen *in vivo* (Song *et al*, 1999). True comparisons of the oestrogenic potencies of phytoestrogens requires standardisation of the test methods. Furthermore, assessments of potency by any one method are likely to be inadequate and full characterisation of a phytoestrogen may require a battery of tests, including both *in vitro* and *in vivo* methods and yet still yield results that have different relevance for effects in humans.

Table 8.6 *In vitro* **and** *in vivo* **methods used for the assessment of oestrogenic activity and potency of phytoestrogens.**

Method	Comments
In vitro	
Receptor binding assay	
Measures the affinity between oestrogens and oestrogen receptors.	Assay is easy to perform. Offers a choice of receptor source e.g. possibility to select subtype and isoform. Assay measures affinity to oestrogen receptors. Not possible to distinguish agonists from antagonists.
Cell proliferation assay	
Indirect measure of oestrogenic activity via ability of a compound to stimulate proliferation in an oestrogen-responsive cell line (e.g. MCF-7 cells).	Assay is easy to perform. Potency estimates can be derived. Can distinguish agonists from antagonists. Cell proliferation may occur independent of oestrogen receptors. Cell lines can differ in their response to an oestrogen.
Reporter gene assay	
Indirect measurement of oestrogenic activity via expression of a reporter gene engineered into a cell line.	Provides estimation of potency. Can distinguish agonists from antagonists. Artificial system and dependent on cell line, response element and reporter gene used.
Analysis of changes in gene expression	
Estimation of oestrogen induced gene expression.	Provides estimation of potency. Can be used to investigate tissue specific effects. Does not inform about functional response.
In vivo	
Uterotrophic assay	
Measurement of uterine growth in response to phytoestrogens in a rodent model low in endogenous oestrogens.	Provides estimation of potency. Absorption, metabolism and excretion are incorporated in the assessment. Only measures oestrogenic effects in one tissue.

Figure 8.3 Range of oestrogenic potencies of phytoestrogens as determined by different assay systems.

Potency is relative to oestradiol set at 1 (bold line). Each data point represents a measurement of potency derived from independent studies[a].



[a] Data from Arnold *et al* (1996); Ashby *et al* (1999); Baker *et al* (1999); Bickoff (1962); Boettger-Tong *et al* (1998); Breinholt & Larsen (1998); Breithofer *et al* (1998); Coldham *et al* (1997); Coldham & Sauer (2001); Collins *et al* (1997); Gaido *et al* (1997); Galey *et al* (1993); Gehm *et al* (1997); Graumann *et al* (1999); Hobson (1977); Hopert *et al* (1998); Hsieh *et al* (1998); Hsu *et al* (1999); Hunter *et al* (1999); Kitaoka *et al* (1998); Kuiper *et al* (1997; 1998); Lamartiniere *et al* (1998); Markaverich *et al* (1995); Medlock *et al* (1995a; 1995b); Mellanen *et al* (1996); Miksicek (1995); Milligan *et al* (1999); Miyamoto *et al* (1998); Mousavi & Adlercreutz (1992); Petit *et al* (1997); Rosenblum *et al* (1993); Ruh *et al* (1995); Santell *et al* (1997); Sathyamoorthy & Wang (1997); Stahl *et al* (1998); Tang & Adams (1980); Turnbull *et al* (1999); Turner *et al* (1999); Wang & Kurzer (1997); Wang *et al* (1995); Whitten *et al* (1992); Willard & Frawley (1998); Zava & Duwe (1997); Zhang *et al* (1999a; 1999b).

Key points

- The oestrogenic activity of phytoestrogens can be assessed by *in vitro* methods such as receptor binding, cellular proliferation and reporter gene assays and *in vivo* using the uterotrophic assay.
- Receptor binding assays measure the simple interaction of phytoestrogens with oestrogen receptors but do not provide an indication of receptor activation.
- Relative binding affinities derived from receptor binding assays are dependent on the subtype and variant of the receptor used (see chapter 6).
- Potency values from *in vitro* assays are dependent on the test system e.g. cell line and reporter construct used.
- *In vivo* assays provide a more holistic assessment of oestrogenic potency. These assays incorporate oestrogenic effects of phytoestrogens such as modulation of endogenous oestrogen concentrations as well as direct interactions with oestrogen receptors. In addition, they include biological process such as absorption, distribution metabolism and excretion, and plasma protein binding that influence oestrogenic activity.
- Potency values from the uterotrophic assay are dependent on a number of experimental parameters such as: animal species and strain, route of administration, hormonal status, stage of development and diet.
- As potency values are dependent on the assay used, and vary considerably, the relative absolute oestrogenic potency of phytoestrogens is difficult to determine. However, taking the results of both *in vitro* and *in vivo* studies of phytoestrogen potency together, a rank order of oestrogenic potency may be estimated: oestradiol $\geqslant$ coumestrol $>$ genistein and equol $>$ glycitein $>$ 8-prenylnaringenin $>$ daidzein $>$ formononetin, biochanin A, 6-prenylnarinigenin, xanthohumol and isoxanthohumol. The oestrogenic potencies of the conjugated metabolites are much lower than those of the parent phytoestrogens.

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9. Effects of phytoestrogens on fertility and development

Introduction

Historical evidence for adverse effects of exposure to oestrogenic chemicals

9.1 Phytoestrogens were first associated with adverse effects on mammalian development and fertility from observations of animals consuming phytoestrogen-rich plants. Ewes feeding on Australian clover developed abnormal plasma concentrations of endogenous hormones with subsequent loss of fertility (Bennett *et al*, 1946; Moersch *et al*, 1967; Obst & Seamark, 1975). The syndrome was termed "Clover Disease".

9.2 Subsequent investigations showed that when pregnant ewes were fed on yarloop clover, plasma progesterone and oestrogen concentrations were lowered. This resulted in a substantial reduction (27%) of the mated ewes achieving successful conception, compared with ewes (95%) fed on grass. Similar, but more extreme, effects on fertility were observed in ewes administered large quantities of oestradiol (300 mg) for periods of up to 26 months (Adams & Sanders, 1988).

9.3 These effects on fertility by a known oestrogen led to the hypothesis that oestrogenic compounds in the clover were responsible for the adverse effects on fertility. Compounds of similar structure to oestradiol were identified in several types of clover: formononetin, biochanin A, and genistein in White clover (*Trifolium repens*), Subterranean clover (*Trifolium subterraneum*) and Yarloop (*Trifolium yanninicum*) and coumestrol in *Medicago sativa* (Shutt, 1976). It is thought that sheep may be particularly susceptible to the oestrogenic effects of these phytoestrogens due to the efficient conversion of formononetin to the more oestrogenic compound, equol and the limited deactivation of the potent oestrogen, coumestrol by metabolism (Shutt, 1976). Improved farming practices have since prevented further incidences of "Clover Disease" (Little, 1996).

9.4 Additional evidence that dietary exposure to oestrogenic compounds could adversely affect reproduction in mammals was shown in research on captive female cheetahs. High concentrations of genistein and daidzein in commercial feed, together with the limited ability of this species to deactivate these compounds metabolically, resulted in veno-occlusive disease and reproductive failure (Setchell *et al*, 1987). Fertility problems in the females of mammalian species such as the cow, sheep, rabbit, guinea pig and mouse have also been reported (Adams, 1995; Batterham *et al*, 1965; Cheng *et al*, 1954; Humfrey, 1998; Kallela *et al*, 1984). These effects have raised concerns that dietary phytoestrogens may have similar effects on development and fertility in other species including the human.

9.5 It is established that exposure to potent oestrogens *in utero* can have long-term adverse effects on human development and fertility in males and, more severely, in female offspring. The synthetic oestrogen, diethylstilboestrol (DES) of comparable potency to oestradiol, initially developed as a drug to prevent miscarriage, was used widely from the 1940s. The subsequent discovery that *in utero* exposure to DES induced abnormal development of the reproductive system during puberty in male and female

offspring and vaginal adenocarcinoma resulted in a ban by the US Food and Drug Administration (FDA) in 1971 (Giusti *et al*, 1995; Swan, 2000). These findings have raised concerns that exposure to phytoestrogens *in utero* may also give rise to similar adverse effects later in life.

9.6 This chapter outlines the role of hormones in human sexual development and reproductive function and reviews the experimental evidence for effects of phytoestrogens on sexual development and reproductive function. Human data are extremely limited and the majority of information is derived from experimental studies in laboratory animals, some of which may not be relevant to the human. The published research is reviewed and the health implications are discussed in the context of both the experimental and human data.

Role of hormones in human sexual differentiation

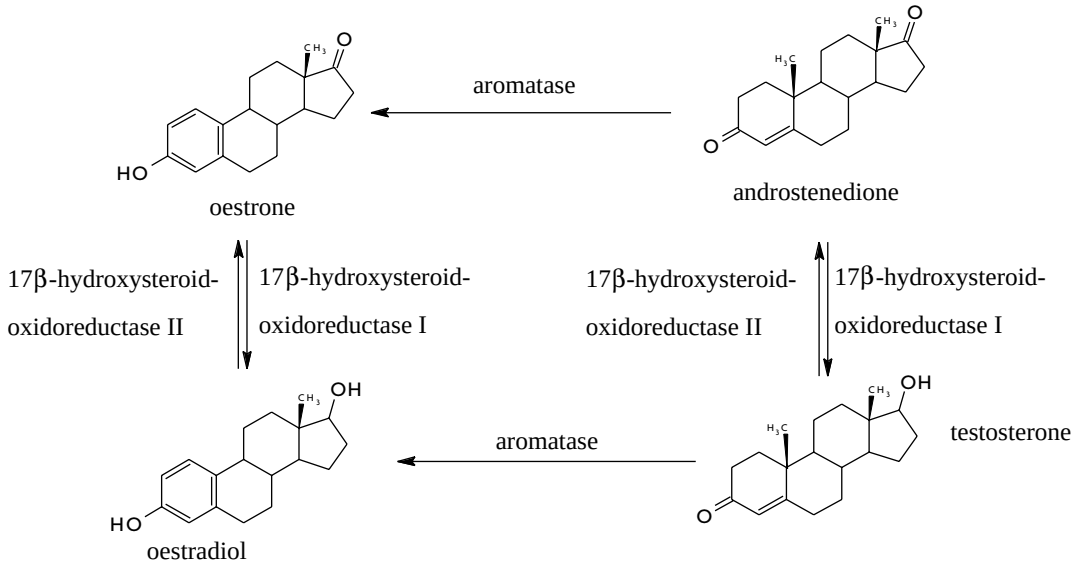
9.7 A fetus is either genetically male or female (Graves, 2001). However, all mammalian fetuses will automatically develop into phenotypic females unless male hormones (androgens) are produced. These hormones, primarily testosterone, change the phenotype of the fetus from female to male and play critical roles in masculinisation. This requirement for androgens illustrates the vulnerability of the male fetus to factors that can affect the production/action of androgens during this short, critical time period of fetal development.

Male development

9.8 The precise role that oestrogens play in male reproductive development is unclear, but in general, oestrogens tend to have 'demasculinising' or anti-androgenic effects. In fetal and neonatal life, this probably results from suppression of testosterone production (Haavisto *et al*, 2001; Williams *et al*, 2001a), or loss of androgen receptors (McKinnell *et al*, 2001). Oestrogens are synthesised from androgens *via* the action of a single enzyme (aromatase), and there is a close relationship between the actions of these two hormones (see Figure 9.1).

9.9 In terms of steroid hormone production, the ovary is quiescent and remains so until puberty over a decade later. Hormones produced by the fetal testes, particularly androgens, are essential for masculinisation of the male fetus during the first trimester of human pregnancy. Furthermore, in the first 12 months postnatally, the male infant produces androgens, in amounts equivalent to those of an adult man. This has been termed the 'neonatal testosterone surge' but, as yet, the functional purpose of this hormonal surge is unclear (Mann & Fraser, 1996). Suppression of the neonatal surge in primates results in long-term effects such as altered thymus weight and an increase in the number of Leydig cells in the testes (Mann *et al*, 1998). It can also retard the pubertal rise in testosterone levels and elongation of the penis, though these both eventually normalise (Brown *et al*, 1999). However, it is reported that suppression of the neonatal testosterone surge has little or no effect on sexual behaviour and fertility (Lunn *et al*, 1997).

Figure 9.1 The conversion of androgens to oestrogens mediated by aromatase.



9.10 Because oestrogens can exert demasculinising effects, the concern that dietary phytoestrogens might affect the sexual differentiation and development process is understandable. Also as infants are breast- or bottle-fed throughout the neonatal period, exposure to chemical compounds in milk is of particular significance at this stage of life. In this regard, the high phytoestrogen content of soy-based milk formula is a cause of concern.

Effects of alterations of hormone levels during fetal/neonatal life

9.11 Interference with, or inadequate production of, androgens and disruption of the balance between androgen and oestrogen levels will impact on the degree of masculinisation of the male fetus (Williams *et al*, 2001a). The more severe the interference, the more severe the manifestation, with complete lack of masculinisation (i.e. genotypic male with female genitalia) representing the most extreme consequence, whereas hypospadias and cryptorchidism represent progressively milder consequences. Such conditions are usually recognised at birth. However, when less extreme interference of androgen action occurs, or there is exposure to relatively low levels of oestrogens, no obvious consequences may be evident at birth and they may only manifest in adulthood.

9.12 These manifestations may be separated from the 'exposure event' by several decades, which makes it extremely difficult to establish causal relationships. Nevertheless, there are examples of such a causal link. For instance, the occurrence of testicular germ cell cancer in young adult men probably results from the development of aberrant germ cells in the testes in fetal life (Rajpert-De Meyts *et al*, 1998). Another example relates to sperm production and fertility in adulthood. Considering that sperm are not produced until late puberty, it may seem surprising that the number and/or quality can be influenced by

events occurring in the fetal or neonatal testis. However, the germ cells (from which sperm are produced) and the Sertoli cells (which control and organise the process of sperm production) are both present in the testis from early in pregnancy. The Sertoli cells proliferate during fetal life and especially in neonatal life, the latter coinciding with the neonatal testosterone surge and further Sertoli cell proliferation occurs at some time close to puberty (Sharpe *et al*, 1999).

9.13 The increase in Sertoli cell numbers during testicular development is very important in terms of male fertility. Each Sertoli cell supports a fixed number of germ cells, therefore in normal individuals there is a linear relationship between the number of Sertoli cells and sperm production (Johnson *et al*, 1984), and the latter is the main determinant of sperm counts in men. However, other factors such as ejaculatory frequency, infections/disease may also affect sperm counts in the ejaculate. The increase in Sertoli cell number that occurs during fetal and neonatal life may therefore be an important determinant of sperm counts in adult men. As proliferation of Sertoli cells at these times is partly dependent on stimulation by hormones secreted from the pituitary gland, any interference with this hormonal stimulation may result in lower Sertoli cell numbers. This has been shown to be the case in experimental studies in marmoset monkeys, in which Sertoli cell proliferation occurs at similar time periods as in the human (Sharpe *et al*, 2000). However, this same study also showed that even when Sertoli cell number was reduced by ~30% by the end of the neonatal period, compensation for reduced Sertoli cell number was able to occur after the neonatal period, such that normal numbers were present by adulthood. Similar compensation may therefore occur in the human if subnormal numbers of Sertoli cells are present neonatally. It is also known that other hormones, such as thyroid hormone and growth hormone, can affect Sertoli cell proliferation. Therefore, exposure to any factor that alters production of these hormones may affect the reproductive system *via* their effects on Sertoli cells.

9.14 In rats, Sertoli cell proliferation continues until postnatal day (PND) 15 and after this time no further proliferation occurs. Thus, by measuring Sertoli cell proliferation, it is possible to determine the effect of placental or lactational chemical exposure during the post-natal period or the entire length of Sertoli cell development (Sharpe *et al*, 1999). However, in humans and other primates, Sertoli cell proliferation can still occur after the post-natal period, probably up to just prior to puberty (Sharpe *et al*, 2000).

9.15 Normal male development involves major, dynamic changes to the testes and reproductive system in fetal and neonatal life. Together, these changes ensure that in adulthood, two decades later, a fully functional reproductive system is capable of producing very large numbers of sperm each day to ensure fertility. All of the changes are hormone-mediated to a greater or lesser degree. Furthermore, the hormones produced to 'shape' the reproductive system also have 'masculinising' effects throughout the body, including the shaping of male sexual behaviour and drive, and again these effects probably occur mainly in early fetal life in the human.

Female development

9.16 In contrast, the development of the female reproductive system is relatively passive. It does not depend on hormonal stimulation and does not require hormonal activity in the neonatal period as in the male. These fundamental differences mean that males are inherently more susceptible than females to disruption of hormone-dependent sexual differentiation. However, there is still a risk that inappropriate exposure of the developing female fetus to exogenous oestrogens might induce permanent changes to the brain or reproductive system, as illustrated in some offspring of women treated with DES during pregnancy.

Hormones and 'endocrine disruption': critical windows of vulnerability

9.17 In fetal and neonatal life, when the reproductive system is developing, androgenic and oestrogenic hormones can exert 'organisational' effects that permanently shape the reproductive system and its function. In contrast, during puberty and adulthood the same hormones exert 'activational' and 'functional' effects, respectively, on a 'pre-formed' reproductive system. Inappropriate hormonal exposures during either of these periods, especially in adulthood, are less likely to cause permanent changes as most of the organisational changes have already taken place. The primary reason for this age related difference is that hormonal systems are homeostatic in adult males and females (see Figures 9.2 and 9.3). In other words, they are designed to compensate if hormone levels rise or fall above a particular level (threshold) in order that a constant or appropriate level of hormone is maintained. However, in early life the set point for this threshold is being established and therefore, the hormonal systems are not yet under homeostatic control.

9.18 Consequently, all endocrine systems are balanced in adulthood, but not in early life, by a negative feedback loop in which the hormone being stimulated reaches a set concentration and then automatically inhibits its own production. In the case of androgens in the male, the 'stimulator' is lutenising hormone (LH; see Figure. 9.2) while for oestrogen in the female the 'stimulator' is both follicle stimulating hormone (FSH) and LH, depending on the stage of follicle development (see Figure 9.3).

9.19 Therefore, if an adult woman is exposed to an exogenous oestrogen such as a phytoestrogen, the level of endogenous oestrogen production will normally be lowered to ensure relatively constant 'oestrogen' levels. In contrast, exposure of a fetus or neonate to phytoestrogens has the potential to induce permanent effects because of disruption of the organisational changes occurring in the fetus or neonate at this time.

The importance of exposure levels

9.20 The purpose of the negative feedback system illustrated in Figures 9.2 and 9.3 is to ensure that hormone levels are maintained within a range that guarantees normal function. Therefore, even if humans are exposed during adulthood to oestrogenic compounds (phytoestrogens), it does not necessarily follow that the exposure will have a biological consequence. The exposure must be of sufficient duration

and/or magnitude that it activates the homeostatic systems (see Figures 9.2 and 9.3). In theory, the hormonal systems should re-adjust. However, potential health consequences are conceivable if exposure to the phytoestrogen is of such magnitude and/or duration that it 'swamps' the system or results in 'side-effects' because of the homeostatic adjustments. For example, suppression of LH by additional negative feedback results in failure of ovulation.

9.21 This applies also to exposures during fetal and/or neonatal life, but with two important differences. First, adjustments to the levels of fetal and/or neonatal hormones to compensate for the exposure to exogenous hormones cannot be made and therefore, the exposure may lead to target-organ effects. Secondly, it is during this period that the threshold level for the regulatory feedback systems is established. Inappropriate exposure during this critical period may permanently alter sensitivity to hormonal signals such that the endocrine system may never function properly. For example, inappropriate exposure of newborn female rodents to oestrogens alters the 'setting up' of their hypothalamic-pituitary-ovarian hormonal axis (see Figure 9.3), such that in adulthood they are unable to exhibit normal ovarian cycles[11].

Effects of phytoestrogens on non-reproductive tissues

9.22 The previous sections have specifically outlined the development of the reproductive system. However, it is clear from the distribution of oestrogen receptors (see Chapter 6), that oestrogens (and androgens) act on many different tissues and cells in the body. These range from the brain, to bone, fat, immune system and cardiovascular system (Sharpe, 1998). Recent studies of transgenic mice in which the oestrogen receptors or the aromatase enzyme have been inactivated, show changes in fat cells/fat accumulation, lipid metabolism, insulin resistance, vascular repair and bone mineralisation among other processes (Couse & Korach, 1999, see chapter 6). It is not yet clear which of these changes may be reversible, but at least in aromatase deficient mice and humans, many of the effects are reversible following restoration of normal oestrogen activity (Grumbach & Auchus, 1999).

9.23 A number of mammalian developmental stages are sensitive to disruption by exogenous oestrogens and androgens. Therefore, the timing of exposure is a major determinant on the subsequent effects on development. There are differences in the timing and sequence of specific developmental events between humans and rodents (see Figures 9.4 and 9.5). For example, sexual differentiation occurs from weeks 5-19 of pregnancy in humans, but in rodents occurs during a relatively short period from gestational days 12-20, with some aspects being completed during the neonatal period. Furthermore, sexual differentiation in the brain (which is dependent on the action of androgens and oestrogens) occurs *in utero* in humans, but during the neonatal period in rodents (Becu-Villalobos *et al*, 1997). Thus, administration of a phytoestrogen at a particular stage of rodent development may not be equivalent to the same period in humans. In addition, there are differences in the time span of sexual maturation between species. In comparison with humans and other primate neonates, newborn rodents are relatively underdeveloped and their development may be more sensitive than primates and humans to the action of phytoestrogens during the neonatal period.

[11] It has been suggested that exposures during the fetal or neonatal period, which alter the programming of hormonal homeostasis may not necessarily result in adverse health effects later in life (for example see Desai & Hales (1997)).

Figure 9.2 Hormonal regulation of the male reproductive system.
In the male, gonadotrophin releasing hormone (GnRH) secreted from the hypothalamus stimulates secretion of lutenising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. LH and FSH regulate testicular activity. LH stimulates Leydig cells to produce testosterone. In the neonate, testosterone is aromatised to oestradiol, which is thought to masculinise the CNS. FSH acts on Sertoli cells to stimulate germ cell spermatogenesis. FSH also stimulates secretion of inhibin, which together with testosterone (and oestradiol) are involved in regulating GnRH secretion from the hypothalamus.

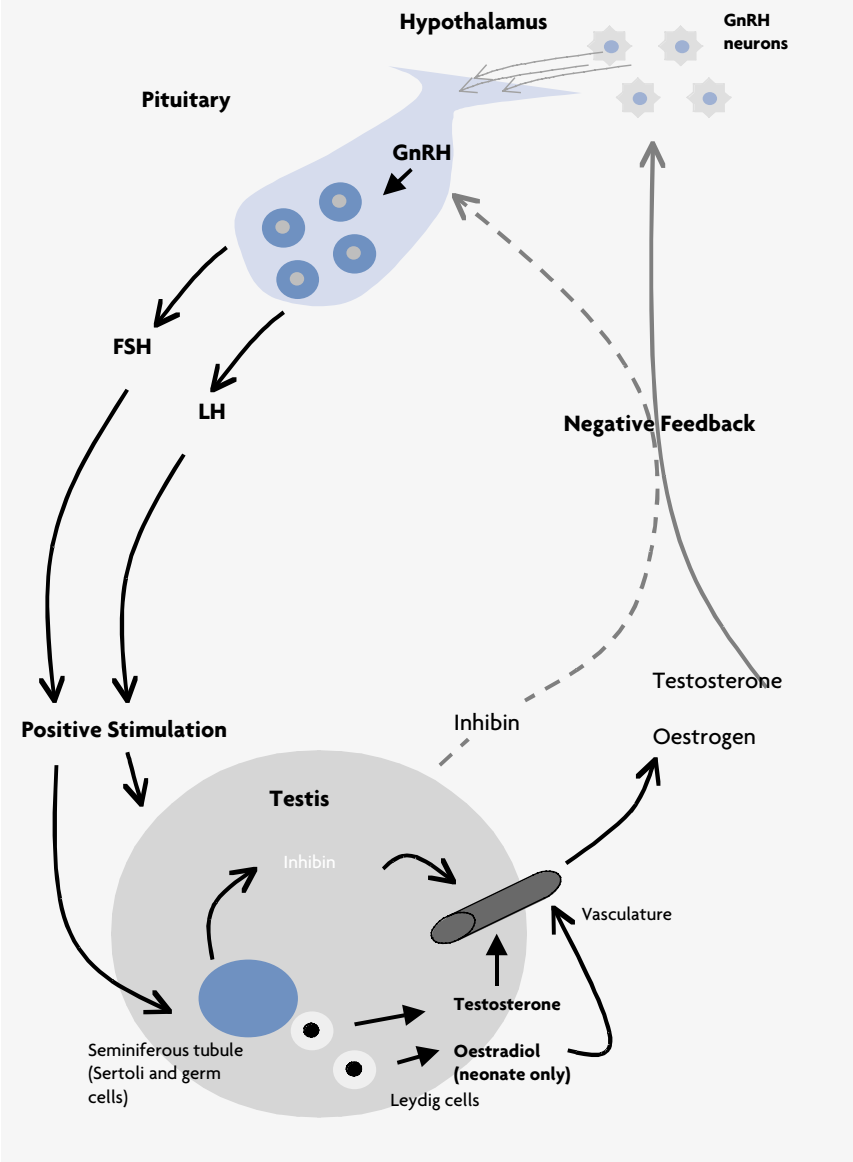
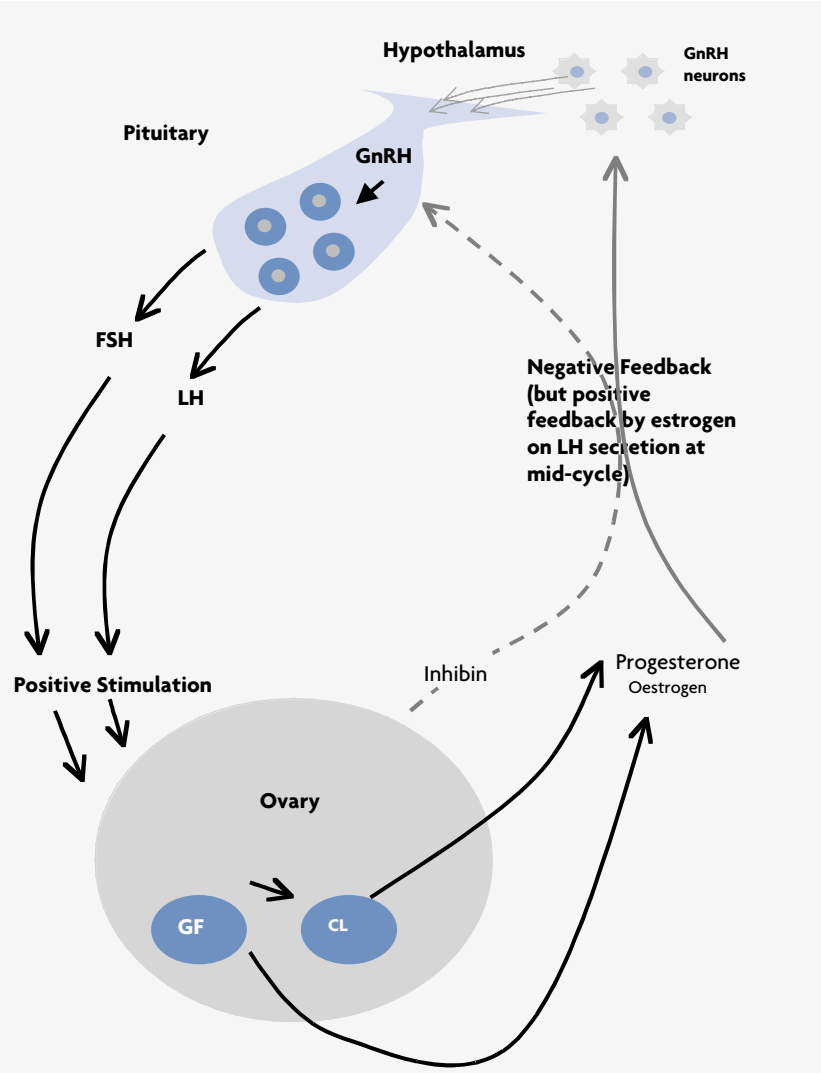


Figure 9.3 Hormonal regulation of the female reproductive system.
In the female, gonadotrophin releasing hormone (GnRH) secreted from the hypothalamus stimulates secretion of lutenising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. FSH and LH are secreted at different rates throughout the menstrual cycle. At the beginning of the cycle (follicular phase), FSH promotes the development of follicles, one of which develops more rapidly into a graffian follicle (GF) (the other follicles degenerate). The GF secretes oestradiol, progesterone and inhibin. At mid-cycle LH causes the GF to rupture resulting in ovulation and formation of the corpus luteum (CL). The CL secretes progesterone (the luteal phase of the cycle). Oestradiol, inhibin and progesterone regulate GnRH secretion from the hypothalamus. At the end of the cycle progesterone secretion stops, leading to menstruation. The menstrual cycle continues, unless interrupted by pregnancy, until menopause.



Organogenesis

9.24 Organogenesis (organ development) is a critical period during development when the fetus is susceptible to toxic agents (Pryor *et al*, 2000). In humans, this process occurs between weeks 3-8 of gestation, but between days 7-17 of gestation in the rat (Dencker & Eriksson, 1998). A further sensitive period in the development of many mammals occurs during the rapid acceleration of brain growth. In humans, this corresponds to the third trimester of pregnancy and continues throughout the first years of life, whereas in rodents this corresponds to the first 3-4 weeks of postnatal life (Dencker & Eriksson, 1998).

Spermatogenesis

9.25 Spermatogenesis (the production of spermatozoa from germ cells) is similar in rodents, primates and humans and can be disrupted by chemicals that interact directly with the testis or by chemicals that modulate plasma gonadotrophin or sex hormone concentrations. Effects on spermatogenesis can be evaluated by measuring sperm number, motility, morphology, fertilisation ability and by histopathological examination of the testis and epididymis (Working *et al*, 1988).

9.26 Daily production of sperm is different between species and is more efficient in rodents than in humans (Sharpe, 1994). Substantial reductions in sperm count can be induced in rodents before fertility is impaired. In contrast, in humans, relatively low numbers of sperm are produced and small effects may affect fertility or time taken to achieve a pregnancy in some individuals. Therefore, rodents may not be a suitable model for effects on human sperm production and fertility (Spielmann, 1998).

Mammary Gland Development

9.27 Rodent and human mammary gland development is very similar (Russo *et al*, 1990). In humans, mammary gland development takes place in two stages, formation (during organogenesis) and gland stimulation during puberty.

Reproductive cycles

9.28 In female mammals, ovulatory cycles occur with regular frequency. These involve growth of a follicle containing the oocyte (egg), ovulation of the mature oocyte and conversion of the ovulated follicles to a corpus luteum. A rise in oestrogen production characterises the period of follicular growth and a rise in progesterone production signals that a corpus luteum has formed (and that ovulation of an egg has occurred). The corpus luteum will continue to produce progesterone for a set period (approximately 14 days in the human) and will then degenerate unless pregnancy intervenes and 'rescues' the corpus luteum from degenerating. If pregnancy does not occur, new follicular growth commences and a new ovulatory cycle ensues.

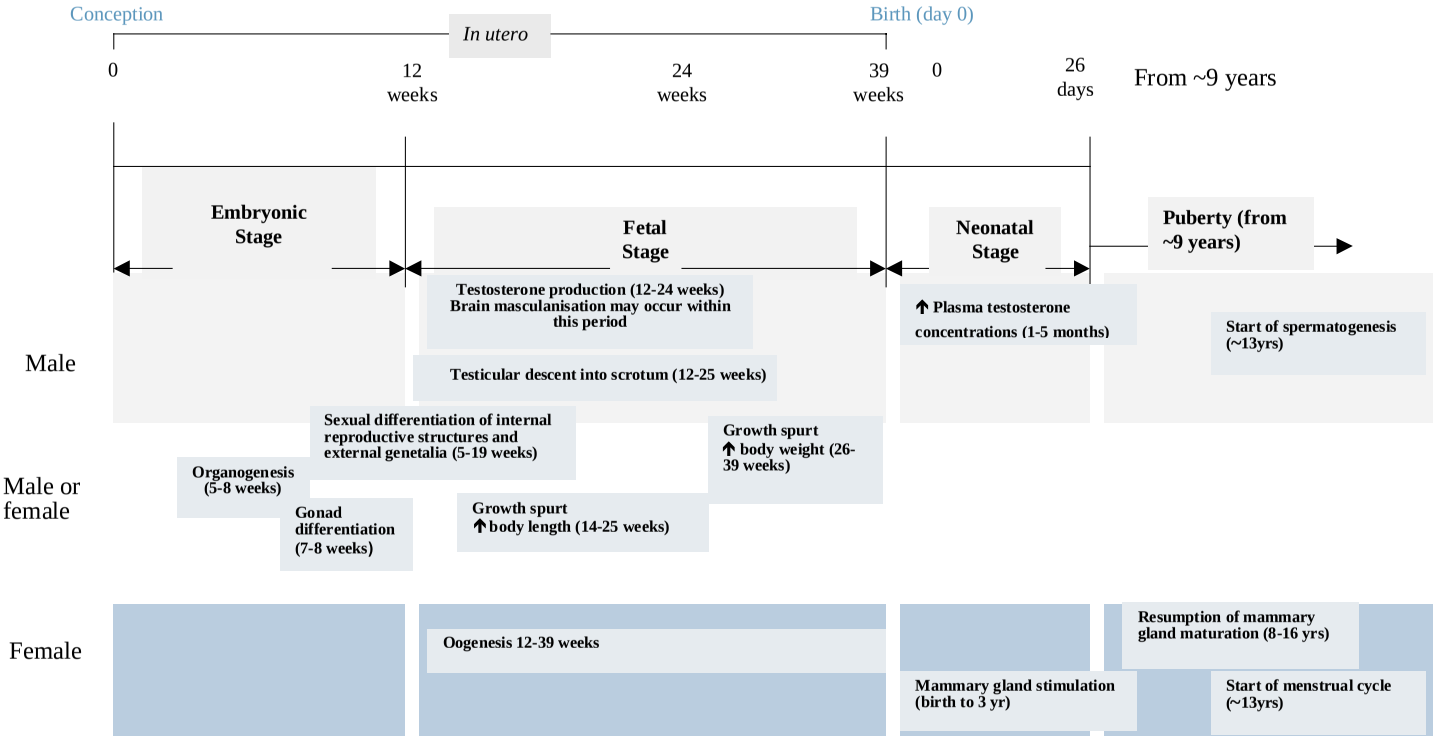
9.29 Most mammals have similar ovulatory cycles but there are some differences. For example, rats and mice have very short (4-5 days) ovulatory cycles whereas primates have much longer ovulatory cycles (e.g. approximately 28 days in humans). A further difference is that many mammals, including rodents, ovulate numerous eggs per cycle whereas primates usually ovulate only 1 or 2 eggs per cycle. Finally, in some primates, but not in most rodents, the ovulatory cycle is 'menstrual', which refers to shedding of the lining of the womb as a result of loss of progesterone support for the womb lining with degeneration of the corpus luteum.

Reproductive life-span

9.30 Healthy adult males continue to make sperm throughout the life cycle whereas women lose the capacity to reproduce at the menopause (approximately 45-50 years of age). The latter is a consequence of the depletion of oocytes from the ovary. The number of oocytes in the ovary is determined during fetal development such that by around birth there are approximately 1-2 million oocytes in the human ovary. Fewer than 500 of these are destined to ovulate and the remainder will partially develop and then undergo atresia (degeneration).

Figure 9.4 Critical stages in human development.

Some of the major events that occur in male and female human development from conception to puberty are shown. It is unclear when mascualisation of the brain and other body organs/tissues occurs in the male but it is likely to be within the period when testosterone production by the fetal testis is highest *in utero* (12-19 weeks).



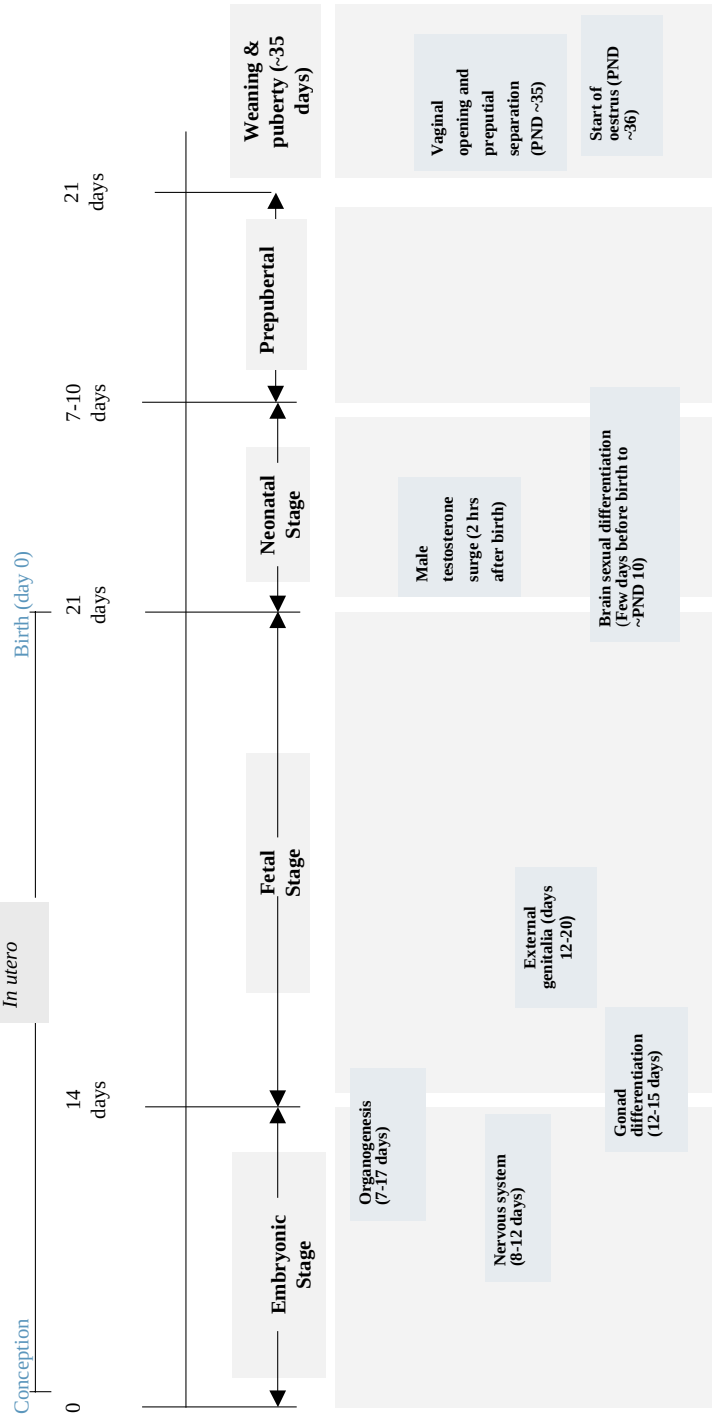


Figure 9.5 Critical stages in rat development.
Some of the major events that occur in rat development from conception to weaning and puberty are shown.

In vivo studies of the effects of phytoestrogens on sexual development and reproduction

Introduction

9.31 An accurate assessment of the human health implications needs to examine physiological levels of phytoestrogens and routes of administration that are relevant to human health.

9.32 Experiments to examine the effects of phytoestrogens on human reproduction or sexual development are extremely difficult to conduct for both practical and ethical reasons. Most of the published work has been performed using laboratory animals, mainly rodent species. The extrapolation and interpretation of this research to humans is complicated by a number of species differences, most notably in sexual development and reproductive function. A small number of studies has been conducted in non-human primates, which are of more relevance in terms of human risk assessment. However, there are ethical considerations that limit the use of these experimental models.

9.33 Factors such as species, age, gender, diet, dose, route of administration and metabolism, strongly influence the ultimate biological response to phytoestrogen exposure (see Chapter 8). A major limitation to the interpretation of experimental research on phytoestrogens is that many studies do not report doses on a body weight basis (mg of compound/kg bw/day). Therefore, comparisons between studies cannot always be made. Also, many studies have used the subcutaneous route of administration. This route by-passes gut microflora and hepatic first pass metabolism, which has a major impact on the biological potency of phytoestrogens. Additionally, many studies have not administered purified phytoestrogens but used rodent diets supplemented with soy or flaxseed as a source of isoflavones or lignans, respectively. This complicates interpretation as the phytoestrogen content is often undetermined and other active constituents in soy or flaxseed may modify the biological response.

Studies on the effects of phytoestrogens on rodents

Isoflavones

9.34 The biological effects of isoflavones or soy have been examined during the following periods in the rodent life-cycle:

- *In utero* exposure
- Perinatal exposure
- Exposure during the neonatal, prepubertal and/or pubertal periods
- Continued exposure through the perinatal, prepubertal and/or pubertal periods
- Multigeneration studies

In utero exposure

9.35 As yet, only two studies have restricted exposure to isoflavones to the *in utero* period. In the first study, pregnant rats received a subcutaneous injection of various concentrations of genistein (0, 5 or 25 mg/day), on gestational days 16-20 (Levy *et al*, 1995). No differences in litter size were observed between treatment groups, although birth weights were lower in the offspring exposed to 25 mg genistein/day. Delayed sexual maturation of the female offspring was reported although the effect was not dose-dependent. Female offspring were ovariectomised to allow assessment of pituitary function. There was no difference in pituitary responsiveness to GnRH stimulation between treatment groups.

9.36 The second study examined the effect of *in utero* exposure on mammary gland development in female mice (Hilakivi-Clarke *et al*, 1998). Pregnant mice were administered genistein (20 μg/day) by subcutaneous injection from days 15-20 of gestation. In the female offspring, advanced mammary gland proliferation and differentiation as well as the time of vaginal opening was evident.

Perinatal exposure

9.37 A number of studies have been published on the effects of isoflavone exposure during the perinatal period (gestation and lactation).

9.38 Pregnant rats received dietary genistein (5 mg/kg diet estimated to approximate 0.2 mg/kg bw/day) from gestational day 17 (Awoniyi *et al*, 1998). After weaning, female offspring continued on the genistein diet or were switched to an isoflavone-free diet until PND 70. A significant reduction in absolute ovary and uterus weights as well as plasma oestradiol and progesterone concentrations was observed in both treatment groups at PND 21. However, these differences were not apparent at PND 70. The time of vaginal opening was unaffected, however irregular oestrus cyclicity was noted in 25% of animals (treated up to PND 21) compared with controls (12%). Irregular oestrus was also found in similar numbers of animals (27%) treated until PND 70 suggesting that exposure to genistein in the pre-weaning period was responsible (see paragraph 9.21). Histological examination of the ovary and uterus revealed morphological abnormalities and fewer functional corpora lutea in animals from both treatment groups (Awoniyi *et al*, 1998). Effects in male offspring were not reported in this study.

9.39 In contrast, a study by Kang *et al* (2002) showed that when genistein was administered at doses higher than in the previous study no long-term effects on sexual development were apparent. Pregnant rats received genistein by oral gavage during gestation and lactation (0, 0.4 or 4 mg/kg bw/day). No significant differences in offspring weights, gender, litter size or male and female anogenital distance were observed between treatment groups. No effects on time of vaginal opening were evident in female offspring. No effects on sperm count or sperm motility were noted in male offspring. There were no differences in reproductive organ weights or histology in either sex at PND 100.

9.40 In a study by Casanova *et al* (1999), pregnant rats were fed genistein supplemented diets (0, 160, 200 or 1000 mg genistein/kg of feed) during gestation and the offspring were maintained on these diets until puberty. No significant differences in the litter size or birth weights were observed between the different treatment groups. In female offspring in the highest treatment group, the time of vaginal opening was accelerated (>2 days) and relative uterine weights (at PND 21) were significantly increased compared with controls. In male offspring, no effect on the age of preputial separation or testes and prostate weights were apparent between treatment groups.

9.41 A study by Fritz *et al* (1998) suggests that exposure to dietary genistein can advance mammary gland development. In this study, dietary genistein (0, 25 or 250 mg/kg diet) administered to pregnant rats during the gestational and lactational periods did not alter time of vaginal opening, uterine weight, mammary gland size or vaginal, uterine and ovarian histopathology in the female offspring. However, a dose-dependent advance in the differentiation of the primary mammary ductile structures was reported. In male offspring, the time of testes descent was not altered in the treated groups (the histopathology of the male reproductive organs was not examined). Serum concentrations of total and free genistein were approximately 1.8 and 0.1 μM measured at PND 21 after administration of the highest dose (Fritz *et al*, 1998).

9.42 Exposure to isoflavones, given as soy in the diet, affected the sexual maturation of female rats but did not impact on subsequent mating and fertility. Female mice received a diet supplemented with soy extract (0, 0.7, 1.2 or 2.4% (w/w), equivalent to 0, 840, 1440 and 2880 mg isoflavones/kg, respectively) from weaning, through adulthood until 7 days after delivery of their first litter (Gallo *et al*, 1999). Vaginal opening occurred significantly earlier in all treatment groups. In animals in the highest dose group, oestrus cycle lengths were significantly longer. In addition, relative uterine weight was increased. Vaginal inflammation, hyperkeratosis and dyskeratosis and ovarian oedema, endothelial hyperplasia and leucocytic infiltration were noted on histological examination. No significant differences in the number of pregnancies, gestational time, number of offspring produced or the characteristics of the litters between treatment groups were reported. Although dietary soy resulted in advanced puberty, lengthened oestrus and induced abnormalities in the reproductive tissues, this did not affect significantly the fertility of female rodents. The effects of soy on sexual maturation and fertility in the male were not evaluated in this study.

9.43 In a study by Cotroneo *et al* (2001), no effects on plasma concentrations of sex hormones or expression of uterine sex hormone receptors were apparent in the offspring of female rats treated with genistein (250 mg/kg diet) throughout pregnancy and lactation.

Exposure during the neonatal, prepubertal and/or pubertal periods

9.44 The effects of isoflavones on pre-weaning development and subsequent sexual development and function have been examined in rodents. The neonatal (PND 1-10) and prepubertal (PND 11-21) stages are a particularly sensitive stage of rodent development as maturation of the reproductive organs takes place during this time.

9.45 Subcutaneous treatment of female rats (males were not examined) with genistein (500 mg/kg bw/day) either during the neonatal or prepubertal stage had differing effects (Lamartiniere *et al*, 1998). Treatment during the neonatal stage (PND 2, 4 and 6) resulted in significantly decreased uterine weights at PND 21 and 50, as well as a reduction in plasma progesterone and the number of corpora lutea, and an increase in the numbers of atretic antral and growing follicles at PND 50. In contrast, treatment during the prepubertal stage (PND 16, 18 and 20) caused a significant but transient increase in uterine weight at PND 22, however the uterine weight had normalised by PND 50 and no effects on plasma progesterone level or on ovarian follicular development were reported. However, prepubertal treatment resulted in early differentiation of mammary tissue.

9.46 Genistein when administered subcutaneously at relatively high concentrations (500 mg/kg bw/day) can induce oestrogenic effects in both intact and ovariectomised animals. The comparative sensitivity of intact and ovariectomised prepubertal female rats (males were not examined in this study) to genistein was examined by Cotroneo *et al* (2001). In this study, animals received a subcutaneous injection of genistein (500 mg/kg bw/day) on PND 16, 18 and 20. After treatment, the plasma concentration of genistein was approximately 5.6 μM (2 μM unconjugated genistein). Relative uterine weights were significantly increased at PND 21 in both ovariectomised and intact animals with hypertrophy of the luminal and glandular epithelia of the uterus. An increase in progesterone receptor concentration but decreased ERα and androgen receptor concentrations was evident in the uterus of both intact and ovariectomised animals. ERα expression was still lower in treated animals at PND 50, but not PND 100 (ERβ was not analysed). Sex hormone concentrations were measured in intact animals. Genistein treatment increased oestradiol, reduced progesterone and had no effect on testosterone plasma concentrations.

9.47 Equivalent increases in uterine weight were observed after subcutaneous administration of genistein (50 mg/kg bw/day) or DES (0.001 mg/kg bw/day) to female mice on PND 1-5 (Newbold *et al*, 2001). After 18 months, animals in both treatment groups developed abnormalities of the reproductive tract including cystic ovaries, absence of corpora lutea, abnormal oviducts, squamous metaplasia, atypical hyperplasia of the uterus and an increased incidence of uterine adenocarcinoma (35% and 31% for genistein and DES, respectively). A parallel study in male animals was not conducted.

9.48 In a comparative study of male and female neonatal rats, animals were dosed subcutaneously with genistein (0, 0.2 or 4 mg/kg bw/day) from PND 1-6 and orally (0, 4 or 40 mg genistein/kg bw/day) from PND 7-21 (Lewis *et al*, 2002). Dose equivalence studies showed that the subcutaneous doses would provide plasma concentrations of genistein equivalent to oral doses of 4 or 40 mg genistein/kg bw/day, respectively, although the subcutaneous route does not allow for conjugation by first pass metabolism. In females, increased uterine weights at PND 22, advancement of the time of vaginal opening, permanent oestrus and reduced plasma progesterone concentrations were evident in animals dosed with 40 mg genistein/kg bw/day. No effects were evident in females dosed with 4 mg genistein/kg bw/day. In males, no consistent effects with treatment were reported.

9.49 In a series of studies, Hughes (1988) and Hughes *et al* (1991a and b) studied GnRH-induced LH release in female ovariectomised rats administered with genistein by intravenous injection (single dose of 0.01, 0.1, 1 or 10 μg/kg bw), oral gavage (single dose of 0.1, 1 or 10 mg/kg bw) or subcutaneous injection (3 doses of 0.8 or 8 mg/kg bw/day). Intravenous treatment with genistein inhibited LH release at doses of 0.01 and 10 μg/kg bw but LH release was enhanced at the 0.1 μg/kg bw dose and did not differ from controls at the 1μg/kg bw dose (Hughes *et al*, 1988). In contrast, oral administration of genistein had no effect on LH release (Hughes *et al*, 1991a) and subcutaneous administration of genistein inhibited LH release (Hughes *et al*, 1991b).

9.50 When female rats (effects in the male were not examined) were fed soy- or cows' milk-based infant formula from PND 21/22 to 24/25 both formulae induced uterotrophic responses (Ashby *et al*, 2000). The most marked response was seen in animals fed soy infant formula, however, the effects could not be causally associated to the isoflavone content as the isoflavone-free cows' milk formula had similar activity. Vaginal opening and first oestrus were advanced when soy formula feeding was continued from PND 21-55 (the effect of cows' milk formula feeding on these parameters was not tested). In contrast, oestrogenic effects were not observed when ovariectomised animals were fed soy formula suggesting modulation of endogenous oestrogens rather than direct action on reproductive tissues as the mechanism of action. Co-administration of inhibitors of sex hormone production with the soy formula lent support to this mechanism (see paragraphs 7.22-7.23). Assessment of the daily intake of the soy formula indicated that rodents consumed three times as much as human infants. When the formula was diluted to provide the animals with the equivalent intake on a body weight basis to human infants, no effects on sexual development were reported.

9.51 Genistein in the diet can induce oestrogenic effects on the uterus and mammary glands as well as hypothalamic-pituitary axis in ovariectomised female rats (Santell *et al*, 1997). In this study, dietary genistein (750 mg/kg diet) administered from PND 70 for 5-14 days inhibited ovariectomy-induced mammary gland regression and increased uterine weights. Prolactin secretion was also stimulated indicating that genistein can act on the hypothalamic-pituitary axis.

9.52 In contrast, no oestrogenic effects were reported in a study of ovariectomised PND 40 rats fed either a soy diet or a soy diet with reduced isoflavone content for a period of two months (Tansey *et al*, 1998). No effects on vaginal cornification or uterus weight were reported for either diet suggesting that soy-containing diets may not be able to compensate for the loss of endogenous oestrogens. The isoflavone content of the soy was not reported in this study, but it is possible the intake of genistein from the soy in the diets was not comparable with that used in the study by Santell *et al* (1997).

9.53 Subcutaneous administration of genistein (4 mg/kg bw/day) to male rats on alternate days between PND 2-18 significantly increased germ cell apoptosis, retarded seminiferous tubule lumen formation and reduced testis weight, plasma FSH and spermatocyte/Sertoli cell volume ratio by PND 18 (Atanassova *et al*, 1999). No effects on inhibin B concentrations and Sertoli cell nuclear volume were observed. Other than the reduction of spermatocyte/Sertoli cell volume ratio, the differences between genistein treated and control animals were undetectable at PND 25 and differences in testis weight were no longer evident

in adulthood. When the reproductive function of these animals was assessed at adulthood, a trend towards a reduction in the number of mating events and litter size was reported but it was not statistically significant.

9.54 However, lifetime exposure to soy did delay male reproductive development with manifestations detected in adulthood in rats. A lifetime exposure to a soy-containing diet (15.5% soy flour) resulted in increased FSH concentrations and reduced body and testis weight compared with animals fed a soy-free diet. All of the observed changes were significant but of small magnitude (Atanassova *et al*, 2000).

9.55 In a study by Weber *et al* (2001), reduced body and prostate weight and plasma testosterone and androstenedione levels were evident in male rats fed a soy-containing diet (600 mg isoflavones/kg) from PND 50 for 5 weeks compared with animals fed an isoflavone-free diet. However, no significant differences in plasma LH and oestradiol levels or 5α-reductase activity were reported. A parallel study was not conducted in female animals.

9.56 A comparative study on the effects of subcutaneous administration of genistein (4 mg/kg bw/day) to male rats on PND 2-12 showed that in contrast to DES, genistein treatment had no effect on gross morphology or sex hormone receptor expression in the seminal vesicles (Williams *et al*, 2001b).

9.57 When genistein was administered by subcutaneous injection to neonatal male mice (effects in female mice were not examined) on PND 1-3 at a dose level of 1 mg/animal/day (which equates to ~500 mg/kg bw/day) a persistent reduction in ventral lobe and relative prostate weights was observed in adulthood (Strauss *et al*, 1998). In a further experiment in adult male mice, genistein (2.5 mg/kg bw/day) administered by subcutaneous injection for 9 days reduced testicular and serum testosterone and pituitary LH concentrations and prostate weight (Strauss *et al*, 1998).

9.58 In a study of spontaneous vulvar carcinoma incidence, female weaning mice were fed various isoflavone diets (0-228 mg isoflavones/kg diet) (Thigpen *et al*, 2001). After 3 months the incidence of vulvar carcinoma in the highest treatment group was significantly greater compared with the control group (6/16 versus 12/16 animals for the control and high treatment groups, respectively).

9.59 The effect of exogenous oestrogens on sexual development and fertility was examined in the male offspring of mice (female offspring were not examined) administered with either DES (0-50 μg/day) or genistein (0-1000 μg/day) by subcutaneous injection from PND 1-5 (Shibayama *et al*, 2001). A dose-dependent reduction in relative testis weight, sperm count and sperm motility was observed in DES treated mice but not in the genistein treated animals. However, ERα and androgen receptor expression in the testes decreased dose-dependently with genistein and DES treatment.

9.60 Equol, a metabolite of daidzein has been shown to have oestrogenic activity. Subcutaneous administration of equol (10, 100 and 1000 μg/day) to female rats on PND 1-5 resulted in reduced relative uterine weights only in the highest treatment group at PND 25 (Medlock *et al*, 1995). However, this effect was transient, as no significant differences in uterine weight were evident at PND 60.

9.61 A study by Wang *et al* (1995) demonstrated that subcutaneous administration of formononetin (40 mg/kg bw/day) for five days to ovariectomised female mice increased mammary gland proliferation 3 fold. An increase in mammary gland oestrogen receptor expression and prolactin was also observed. The authors conclude that formononetin can act as an oestrogen receptor agonist in mammary tissue and may stimulate proliferation directly *via* the oestrogen receptor or indirectly, by inducing prolactin secretion *via* the pituitary gland.

9.62 It is clear that isoflavones can produce effects on the sexual development of male and female rodents when administered at relatively high doses by subcutaneous injection. One study has examined the effects of oral administration of genistein over a range of doses. Male and female neonatal rats were administered genistein (0, 12.5, 25, 50 or 100 mg/kg bw/day) by oral gavage on PND 1-5 (Nagao *et al*, 2001). In females, no effects on the time of vaginal opening or oestrus cycle length were noted in any of the treatment groups. No significant effects on female mating were observed, but fertility was significantly reduced in all of the treatment groups. Polyovular follicles were detected in treated but not in control females. Atrophic ovaries with no corpora lutea were observed in 1/5 rats in the 50 mg/kg bw/day group and 5/10 in the 100 mg/kg bw/day group in 18 week old females that had failed to become pregnant.

9.63 In males, the time of preputial separation, serum testosterone concentrations, sperm counts as well as testes, seminal vesicle and ventral prostate weights were comparable to controls. No effects on male mating or fertility were observed. The testes, seminal vesicle and ventral prostate were histopathologically normal. These results indicate that neonatal dosing of genistein can cause dysfunction of postpubertal reproductive performance at doses $\geqslant$ 12.5 mg/kg bw/day as well as abnormal development of reproductive organs in female rats at doses $\geqslant$ 50 mg/kg bw/day. However, dietary genistein has little detectable effect on male reproductive development and fertility in doses up to 100 mg/kg bw/day (Nagao *et al*, 2001).

Continued exposure through the perinatal, neonatal, prepubertal and/or pubertal periods

9.64 A number of studies have been published on the effects of continued exposure to isoflavones through the perinatal and subsequent periods. In this study, pregnant rats received dietary genistein (5 mg/kg diet estimated to approximate 0.2 mg/kg bw/day for an adult rat) from gestational day 17. After weaning, male offspring (female offspring were not examined) either continued on this diet or were switched to an isoflavone-free diet until PND 70 (Roberts *et al*, 2000). Epididymal weights were significantly reduced in both treatment groups at PND 130 but no effects on sperm counts were evident. LH concentrations were significantly reduced at PND 21 and 130 in both treatment groups, but this reduction was not consistently maintained, as LH concentrations were comparable to controls at PND 70. No effects on plasma testosterone or FSH concentrations were observed. These findings suggest that in rats, the critical period of exposure to genistein is the perinatal period.

9.65 In a study by Lamartiniere *et al* (2002), female rats received dietary daidzein (0, 250 or 1000 mg/kg diet) prior to mating and throughout pregnancy and lactation. The offspring received the same diets up until PND 50. There were no significant effects on the number of, or the ano-genital distances of, the offspring. The plasma concentrations of daidzein and equol were similar in both the pregnant females and the fetuses. In the female offspring, plasma progesterone concentrations were significantly reduced in the highest dose group and oestradiol concentrations showed a dose dependent (non-significant) decrease. Mammary gland, uterine and ovarian weights were not significantly different between groups at PND 50 and no histopathological changes were reported. Thus, in this study the only detectable effect on female sexual development was a reduction in plasma progesterone concentrations (a parallel study in male offspring was not conducted).

9.66 The effects of genistein on the reproductive function and sexual development were examined following exposure of pregnant rats and their offspring. Animals were exposed to genistein (0, 300 or 800 mg/kg diet) throughout gestation and lactation. The offspring continued on the same diet until PND 100 (You *et al*, 2002). The exposure to the offspring was estimated at 44 and 125 mg/kg bw/day (at PND 28-31) and 16 and 43 mg/kg/day (at PND 97-100), for the intermediate and high doses, respectively.

9.67 Genistein had no effect on preputial separation in male offspring in this study. However, a dose-dependent advancement in the time of vaginal opening was observed in females. Genistein treatment increased the oestrus portion of the cycle and decreased metoestrus and dioestrus but the length of the oestrus cycle in rats was not affected. Treatment had no effect on sex-dependent locomotor activity. The histology of the male and female reproductive organs was normal (You *et al*, 2002).

9.68 The effect of genistein on androgen and ER receptor expression in the prostate was examined in male rats exposed to genistein throughout gestation to PND 70 (0, 25 or 250 mg/kg diet). Treatment with genistein reduced androgen receptor, ERα and ERβ expression in the prostate in a dose-dependent manner. No differences in the histopathology or weight of the reproductive tract were evident between treatment groups (Fritz *et al*, 2002).

9.69 The oestrogenic effects of soy on the uterus and prostate of mice was examined following exposure to pregnant mice and their offspring (Makela *et al*, 1995). Pregnant mice were fed a 0 or 7% (w/w) soy diet (the isoflavone content was not determined) during gestation and lactation and the offspring were continued on these diets after weaning. DES was also administered to some animals on PND 1-3. In the female offspring, dietary soy and DES increased uterine weights in the immature animals. However, in animals co-administered with soy and DES, exposure to soy reduced the uterotrophic effect of DES. Similar effects were observed in the prostate of male offspring. Prostate weights were increased at 9 months in animals exposed to either soy or DES. However, soy exposure inhibited the prostatic growth induced by DES treatment.

Multigeneration studies

9.70 The most rigorous assessment of fertility and development effects is through multigeneration studies. Typically these studies examine a number of aspects of male and female reproductive and developmental toxicity (libido, fertility, pregnancy and lactation) as well as the effects on survival, growth, development and reproductive capacity of the offspring. These effects can be assessed over a number of generations (Barlow *et al*, 2002).

9.71 Two studies have been conducted in rodents (Flynn *et al*, 2000; Badger *et al*, 2001). The scope of these studies was somewhat limited, as the full range of end-points was not assessed.

9.72 No effects on the rate or success of breeding, litter size, body and organ weight or birth length were observed following a multigenerational soy feeding study. Animals received a diet supplemented with soy protein isolate over three generations (Badger *et al*, 2001). The age of onset of puberty did not differ in F_1 males (as assessed by preputial gland separation) but vaginal opening was advanced (2 days) in F_1 females. In this study exposure to soy advanced puberty in females, but exposure to soy had no detectable effect on the reproductive capacity of male or female rats. However, the intake and isoflavone content of the soy diet was not reported which makes the findings difficult to extrapolate to likely human exposures.

9.73 A study by Flynn *et al* (2000) showed that genistein had no effect on maternal nursing behaviour observed across and between generations when rats were fed genistein (500 mg/kg diet) over four generations.

9.74 The data from the experimental studies of the effects of isoflavones and soy on rodents are summarised in Table 9.1.

Table 9.1: Effects of isoflavones on rodent development and fertility.

Timing of exposure/Species	Route of administration (duration)	Dose	Results	Reference
In utero				
Rat	s.c. (GD 16-20)	25 mg genistein/day	⇩ Birth weights, delayed vaginal opening	Levy *et al* (1995)
Mouse	s.c. (GD 15-20)	20 μg genistein/day	Advanced time of vaginal opening and mammary gland growth	Hilakivi-Clarke *et al* (1998)
Perinatal				
Rat	Dietary (GD 10-PND 70)	5 mg genistein/kg diet	⇩ Uterus & ovary weight, ⇩ oestradiol and progesterone, irregular oestrus, abnormal uterine and ovarian histology	Awoniyi *et al* (1998)
Rat	Dietary (GD 10-PND 21)	4 mg genistein/kg bw/day	No long term effects on sexual development	Kang *et al* (2002)
Rat	Dietary (GD 0-PND 21)	250 mg genistein/kg diet	No effect on sex hormone concentrations or receptor levels	Cotroneo *et al* (2001)
Rat	Dietary (GD 0-PND 21)	250 mg genistein/kg diet	Advancement of mammary gland differentiation	Fritz *et al* (1998)
Rat	Dietary (GD 0-PND 21)	1000 mg genistein/kg diet	⇧ Relative uterine weight, accelerated female puberty	Casanova *et al* (1999)
Rat	Dietary (GD 0-PND 7)	2.4% soy extract in diet	⇧ Oestrus cycle length, ⇧ uterus weight, abnormal vaginal, uterine and ovarian histology	Gallo *et al* (1999)
Neonatal, prepubertal and/or pubertal				
Mouse	s.c. (PND 1-3) s.c. (9 days in adults)	1mg genistein/kg bw/day 2.5 mg genistein/kg bw/day	⇩ Ventral lobe and prostate weight ⇩ Testicular & serum testosterone and pituitary LH concentrations, prostate weight	Strauss *et al* (1998)
Mouse	s.c. (PND 1-5)	1 mg genistein/day	No effect on testis weight or spermatogenesis	Shibayama *et al* (2001)

Table 9.1: Effects of isoflavones on rodent development and fertility. *(continued)*

Timing of exposure/Species	Route of administration (duration)	Dose	Results	Reference
Rat	s.c. (PND 2-18) Dietary (continuous)	4 mg genistein/kg bw/day Soy diet	⇩ testes weight (reversible), ⇧ germ cell apoptosis, ⇩ spermatocyte/Sertoli cell volume, ⇩ FSH ⇩ testes weight (irreversible)	Atanassova *et al* (2000)
Rat	s.c. (PND 2-12)	4mg genistein/kg bw/day	No effect on seminal vesicles	Williams *et al* (2001)
Mouse	s.c. (PND 1-5)	50 mg genistein/kg bw/day	⇧ Uterine weight, absence of corpora lutea, abnormalities to oviduct and uterine adenocarcinoma.	Newbold *et al* (2001)
Mouse	Dietary (3 months)	228 mg isoflavones/kg diet	⇧ Vulvar carcinoma	Thigpen *et al* (2001)
Rat	Oral gavage (PND 1-5)	50 mg genistein/kg bw/day	Disrupted fertility in females, histological changes in uterus and ovaries	Nagao *et al* (2001)
Rat	s.c. (PND 2-6) s.c. (PND 16-20)	500 mg genistein/kg bw/day	⇩ Uterine weight & corpora luteum. ⇩ plasma progesterone. ⇧ Uterine weight. Advancement of mammary gland differentiation. No effect on sex hormone concentrations, ovarian development or menstrual cycle	Lamartiniere *et al* (1998)
Rat (ovariectomised)	i.v. (single dose)	0.01-10 μg genistein/kg bw	0.01 and 10 μg genistein/kg bw ⇩ GnRH-induced LH release 0.1 μg genistein/kg bw ⇧ GnRH-induced LH release	Hughes *et al* (1988)
Rat (ovariectomised)	Oral gavage (single dose)	0.1-10 mg genistein/kg bw	No effect on GnRH-induced LH release	Hughes *et al* (1991a)
Rat (ovariectomised)	s.c. (3 daily doses)	0.8 or 8 mg genistein/kg bw	⇩ GnRH-induced LH release	Hughes *et al* (1991b)
Rat (ovariectomised & intact)	s.c. (PND 16-20)	500 mg genistein/kg bw/day	⇧ Uterine weights, ⇧ PR, ⇩ ERα, ⇩ AR expression	Cotroneo *et al* (2001)

Table 9.1: Effects of isoflavones on rodent development and fertility. *(continued)*

Timing of exposure/Species	Route of administration (duration)	Dose	Results	Reference
Rat (ovariectomised & intact)	Dietary (PND 70-75)	750 mg genistein/kg diet	⇧ Uterine weights, inhibition of mammary gland regression (no effect on intact immature animals)	Santell *et al* (1997)
Rat	Dietary (PND 50-99)	600 mg isoflavones/kg diet	⇩ Prostate and body weight ⇩ Plasma testosterone and androstenedione	Weber *et al* (2001)
Rat (ovariectomised)	Dietary (PND 40-100)	Soy	No effect on uterine weight or vaginal histology	Tansey *et al* (1998)
Mouse	s.c. (PND 1-5)	1 mg equol/day	⇩ Uterine weight	Medlock *et al* (1995a and b)
Mouse (ovariectomised)	s.c. (5 days)	40 mg formononetin/kg bw/day	⇧ Mammary gland proliferation	Wang *et al* (1995)
Rat	Dietary (PND 21/22-24/25) Dietary (PND 21-55)	Soy infant formula	⇧ Uterine weight ⇧ female anogenital distance and advanced first oestrus	Ashby *et al* (2000)
Rat	s.c. (PND 1-6) then oral gavage (PND 7-21)	4 mg then 40 mg genistein/kg bw/day	⇧ Uterine weight, advanced time of vaginal opening, permanent oestrus, ⇩ Plasma progesterone in females. No effects in males.	Lewis *et al* (2002)

Table 9.1: Effects of isoflavones on rodent development and fertility. *(continued)*

Timing of exposure/Species	Route of administration (duration)	Dose	Results	Reference
Continued perinatal and/or neonatal to pubertal				
Rat	Dietary (GD 17-PND 70)	50 μg genistein/day	⇩ Serum testosterone and LH, ⇩ Epididymal weights	Roberts *et al* (2000)
Rat	Dietary (GD 0-PND 100)	800 mg genistein/kg diet	Advanced vaginal opening, altered oestrus cycling	You *et al* (2002)
Rat	Dietary (GD 0-PND 100)	1000 mg daidzein/kg diet	No reproductive parameters altered in males or females	Lamartiniere *et al* (2002)
Rat	Dietary (GD 0-PND 70)	25 mg genistein/kg diet	⇩ AR, ERα and ERβ expression in the prostate	Fritz *et al* (2002)
Mouse	Dietary (GD 0-PND 270)	7% (w/w) soy	⇧ Uterine weight, ⇧ prostate weight	Makele *et al* (1995)
Multigenerational				
Rat	Dietary	500 mg genistein/kg diet	No effect on maternal nursing behaviour	Flynn *et al* (2000)
Rat	Dietary	Soy protein isolate (dose not determined)	No effect on birth weight, length or relative organ weight, advanced vaginal opening in F_1 females	Badger *et al* (2001)

GD- gestational day, PND- postnatal day, s.c.- subcutaneous injection, i.v.- intravenous injection; FSH- follicle stimulating hormone, LH- lutenising hormone, ER- oestrogen receptor, PR- progesterone receptor, AR- androgen receptor.

Coumestrol

9.75 *In vitro* and *in vivo* assays of oestrogenicity suggest that coumestrol may be the most potent phytoestrogen identified to date (see Chapter 8). Exposure to coumestrol has been shown to induce a range of oestrogenic effects.

9.76 Increases in absolute uterine weight were reported after coumestrol was administered by subcutaneous injection (single injection of 50-200 μg) or orally (50-100 μg for 3 days) to immature female ovariectomised rats (Markaverich *et al*, 1995).

9.77 Similar findings were reported in studies by Ashby *et al* (1999) and Tinwell *et al* (2000). Coumestrol (60 mg/kg bw/day) was administered by oral gavage to intact immature (Ashby *et al*, 1999), ovariectomised immature or ovariectomised mature female rats (Tinwell *et al*, 2000) on 3 consecutive days. Increases in uterine weight were evident from all treatments and were accompanied by uterine hyperplasia.

9.78 Subcutaneous administration of coumestrol (range 0.001-100 μg/day) during PND 1-5 advanced vaginal opening in mice at all doses (Burroughs *et al*, 1990a). At 20-22 months of age, abnormalities of the reproductive tract were observed such as cervicovaginal pegs and downgrowths at all doses except 25 and 50 μg, cervical adenosis (≥ 0.08 μg), uterine squamous metaplasia (≥ 50 μg) haemorrhagic follicles (0.1, 5 and 100 μg/day), ovarian ceroid deposition (≥ 5 μg/day) and absent corpora lutea (100 μg/day). These effects were also observed in a similar study of ovariectomised mice (Burroughs *et al*, 1990b). The effects of equivalent exposures in the male were not studied.

9.79 Two studies by Medlock *et al* (1995a and b) showed that coumestrol can produce effects similar to that of potent oestrogens such as diethylstilboestrol (DES) and oestradiol in female rat neonates. Subcutaneous administration of oestradiol, DES and coumestrol (100 μg/day) on PND 1-5 or 1-10 increased uterine weight by PND 5, but subsequently reduced uterine growth and suppressed uterine ERα levels by PND 25 compared to controls. When coumestrol was administered on PND 10-14 coumestrol inhibited uterine gland genesis.

9.80 No adverse effects on spermatogenesis or neuroendocrine function were reported in male mice (females mice were not examined) following neonatal exposure to coumestrol (100 μg/day) administered by subcutaneous injection on PND 1-5 (Awoniyi *et al*, 1997). Coumestrol had no effect on the weights of testes and sex accessory organs, or sperm count measured at PND 60. Similarly, there were no significant changes in serum testosterone, LH and FSH concentrations.

9.81 In a study of GnRH-induced LH release, Hughes (1988) administered coumestrol (single dose in the range 0.01-10 μg/kg bw) by intravenous injection to female ovariectomised rats. Treatment with coumestrol inhibited LH release at all doses.

9.82 Suppressed pituitary responsiveness to GnRH stimulation was reported when coumestrol (20 mg) was intravenously infused over 8.5 hours to adult female ovariectomised rats (McGarvey *et al*, 2001).

9.83 Dietary studies suggest that neonatal or prepubertal exposure to dietary coumestrol in relatively large doses (100 mg/kg diet) can produce long-term effects on reproductive parameters in both females and males.

9.84 The effect of neonatal dietary exposure was examined in rats in two studies by Whitten *et al* (1993; 1995). Rats were exposed either during PND 1-10 or 1-21 *via* breast milk from mothers fed coumestrol (100 mg/kg diet). After weaning, the offspring were switched to a coumestrol-free diet. In females, no effect on the oestrus cycle was seen in animals treated for 10 days, however, the animals treated throughout lactation showed irregular oestrus cycling at PND 99-108 and by PND 132-143, 83% of animals exhibited persistent oestrus. A reduction in latency of mounting behaviour and ejaculation frequency was observed in male offspring after 10 or 21 days of treatment. However, testis weights and plasma testosterone levels were unaffected.

9.85 When immature female rats received dietary coumestrol (0, 50 or 100 mg/kg of diet) for 90 hours, a significant increase in progesterone receptor concentrations and uterine weight was observed at a dose of 100 mg/kg diet. When the treatment was extended to 180 hours, similar effects were seen in both treatment groups (Whitten *et al*, 1992).

9.86 In a further study, increased uterine and pituitary expression of progesterone receptors was observed in prepubertal female rats treated from PND 21-24 with 100 mg/kg coumestrol in the diet (Whitten & Naftolin, 1992). A longer term treatment was included in this experiment (PND 22-60) which resulted in significant reductions in the time of vaginal opening and first oestrus. No differences in oestrus cyclicity were found at first oestrus. However, by PND 116-131, irregular cycling was observed in approximately half the treated animals.

9.87 The data from the experimental studies of the effects of coumestrol on rodents are summarised in Table 9.2.

Table 9.2: Effects of coumestrol on rodent development and fertility.

Species	Route of administration (duration)	Dose	Results	Reference
Rat (ovariectomised)	s.c. (single dose to immature animals) oral (3 days to immature animals)	50-200 μg 50-100μg/day	⇧ Uterine weight	Markaverich *et al* (1995)
Rat	Oral gavage (3 days to immature animals)	60 mg/kg bw/day	⇧ Uterine weight and uterine hyperplasia	Ashby *et al* (1999)
Rat (ovariectomised)	Oral gavage (3 days to immature animals) Oral gavage (3 days to mature animals)	60 mg/kg bw/day	⇧ Uterine weight and uterine hyperplasia	Tinwell *et al* (2000)
Mouse	s.c. (PND 1-5)	0.001-100 μg/day	Advanced vaginal opening, abnormalities of the reproductive tract (all doses). No corpus luteum (100 μg/day)	Burroughs *et al* (1990a)
Mouse (ovariectomised)	s.c. (PND 1-5)	0.001-100 μg/day	Advanced vaginal opening, abnormalities of the reproductive tract (all doses). No corpus luteum (100 μg/day)	Burroughs *et al* (1990b)
Rat	s.c. (PND 10-14)	100 μg/day	Altered uterine growth	Medlock *et al* (1995a and b)
Rat (ovariectomised)	i.v. (single dose)	0.01-10 μg/kg bw	⇩ GnRH-induced LH release	Hughes (1988)
Rat	i.v. infusion (8.5 hours)	20 mg	⇩ pituitary responsiveness	McGarvey *et al* (2001)
Rat	Dietary (PND 25-28)	50 mg/kg diet	⇧ Uterine weight and progesterone receptors	Whitten *et al* (1992)

Table 9.2: Effects of coumestrol on rodent development and fertility. *(continued)*

Species	Route of administration (duration)	Dose	Results	Reference
Rat	Dietary (PND 21-24) Dietary (PND 21-60)	100 mg/kg diet	⇧ Uterine weight and progesterone receptors Early vaginal opening and menstrual cycle irregularity	Whitten & Naftolin (1992)
Rat	Dietary (PND 1-21)	100 mg/kg in maternal diet	Irregular menstrual cyclicity, persistent oestrus state	Whitten *et al* (1993)
Rat	Dietary (PND 1-10) Dietary (PND 1-21)	100 mg/kg in maternal diet	Females: no effect. Males: ⇩ sexual behaviour/function Females: permanent oestrus. Males: ⇩ sexual behaviour/function	Whitten *et al* (1995)
Rat	s.c. (PND 1-5)	100 μg/day	No effect on spermatogenesis or sex hormone concentrations.	Awoniyi *et al* (1997)

PND – post natal day, s.c. – subcutaneous injection, i.v. – intravenous.

Lignans

9.88 Published studies have predominantly used flaxseed (linseed) as a source of lignans. Flaxseed is rich in the lignan secoisolariciresinol, but may also contain other active compounds, which makes a causative association with biological effects difficult. However, comparative studies on the effects of flaxseed and purified secoisolariciresinol do support the association.

9.89 Pregnant rats received secoisolariciresinol diglucoside (1.5 mg/day) either by oral gavage or *via* diets supplemented with 0%, 5% or 10% (w/w) flaxseed throughout gestation, birth and weaning (Tou *et al*, 1998). The offspring received the flaxseed-free diet up until PND 132. No significant differences in gestation length, parturition, litter size, offspring survival or the ratio of males to females were observed in any treatment group.

9.90 Female animals in the 10% (w/w) flaxseed treatment group demonstrated early onset of puberty and lengthened oestrus cycles. In contrast, puberty was delayed in the secoisolariciresinol and 5% (w/w) flaxseed-exposed groups. In males, no effects were reported on testes, seminal vesicle or prostate weights in any treatment group. However, accessory sex gland and prostate weights were significantly greater in the 10% (w/w) flaxseed group. These results suggest that perinatal exposure to compounds in flaxseed can produce hormonal effects in male and female rodents. Oestrogenic effects were observed in female rodents at relatively high doses although the delay of puberty in females suggests that flaxseed may have anti-oestrogenic effects at lower doses (Tou *et al*, 1998).

9.91 In a study by Tou *et al* (1999), the offspring from mothers fed control, 5% or 10% (w/w) flaxseed diets throughout gestation and lactation were maintained on these diets until PND 132. Some animals fed the control diet were switched to a diet containing 5% or 10% (w/w) flaxseed after weaning. In the females fed 10% (w/w) flaxseed, vaginal opening was advanced, oestrus prolonged and serum oestradiol concentrations elevated at PND 50 and 132 compared with the other groups. In contrast, vaginal opening was delayed with no effect on oestrus cyclicity in the 5% (w/w) flaxseed treatment group. Exposure to flaxseed limited to during the post-weaning period had no effect on age of vaginal opening or oestrus cyclicity (Tou *et al*, 1999).

9.92 In males, elevated testosterone concentrations (at PND 132) and increased sex accessory gland, seminal vesicle, prostate and testes weights were reported only in the 10% (w/w) flaxseed group. When exposure to flaxseed was restricted to the post-weaning period there were no effects on male sex organs. The results suggest that exposure during the perinatal period is the critical time for induction of the observed effects (Tou *et al*, 1999).

9.93 None of the effects noted in the previous studies (Tou *et al*, 1998; 1999) were observed in a study by Ward *et al* (2001). In this study, male and female rat offspring were exposed from birth *via* the milk of mothers fed 10% (w/w) flaxseed or secoisolariciresinol (177 mg/kg diet equivalent to the concentration of lignan present in the 10% (w/w) flaxseed diet) and continued on these diets after weaning. This suggests *in utero* exposure is the critical time for induction of the observed effects.

9.94 A study by Tou & Thompson (1999) suggests that perinatal exposure to lignans may promote differentiation of specific cells in mammary glands. When pregnant rats were fed a 10% (w/w) flaxseed diet during gestation and lactation, early differentiation of mammary gland tissue was stimulated in the female offspring. Similar effects were reported from continuous exposure from the perinatal to the post-weaning period. When exposure was limited to the post-weaning period, no effects were evident. Similar but weaker effects were observed when rats were fed a diet incorporating 5% (w/w) flaxseed or secoisolariciresinol diglucoside (88 mg/kg diet, estimated as equivalent to the concentration of lignan in the 5% (w/w) flaxseed diet). These results suggest that exposure to dietary lignans during the *in utero* and neonatal periods can accelerate breast maturation.

9.95 Male reproductive development and function were unaffected but alterations in sex hormone concentrations were observed following continuous exposure to flaxseed from gestation to adulthood. No significant differences in testis, seminal vesicle and epididymal weights, spermatid numbers, testosterone concentrations or sperm morphology were noted in the male offspring of rats on a diet supplemented with 0%, 20% or 40% (w/w) flaxseed from gestation to weaning (Sprando *et al*, 2000a). There was a dose dependent increase in the concentrations of plasma LH (but not FSH) and seminiferous tubule fluid testosterone in animals fed 20% and 40% (w/w) flaxseed (Sprando *et al*, 2000a). No effects on testis structure and spermatogenesis were evident in animals in the treatment groups. An increase in plasma testosterone was noted only in the 40% flaxseed treatment group in a similar study by Sprando *et al* (2000b). Parallel experiments were not conducted in females.

9.96 The data from the experimental studies of the effects of lignans or flaxseed on rodents are summarised in Table 9.3

Table 9.3: Effects of lignans on rodent development and fertility.

Species	Route of administration (duration)	Dose	Results	Reference
Rat	Seco: oral gavage (GD 0- PND 21) Flaxseed: dietary (GD 0- PND 21)	1.5 mg/day seco diglucoside 5% and 10% (w/w) flaxseed in diet	Females: advanced puberty (10% (w/w) flaxseed), delayed puberty (5% (w/w) flaxseed or seco) Males: ⇧ accessory sex gland, prostate weights (10% (w/w) flaxseed)	Tou *et al*. 1998
Rat	Dietary (GD 0- PND 132)	5% and 10% (w/w) flaxseed diet	Females: delayed age of puberty (5% (w/w) flaxseed), advanced puberty and prolonged oestrus, ⇧ oestradiol concentrations (10% (w/w) flaxseed) Males: ⇧ accessory sex gland, seminal vesicle, testes, prostate weights (10% (w/w) flaxseed)	Tou *et al*. 1999
Rat	Dietary (GD 0- PND 21)	10% (w/w) flaxseed diet	Promotion of mammary gland differentiation in neonates	Tou & Thompson (1999)
Rat	Dietary (PND1- PND 132)	10% (w/w) flaxseed (177 mg seco/kg) diet	No effects	Ward *et al* (2001)
Rat	Dietary (GD 0- PND 70)	20% or 40% (w/w) flaxseed diet	⇧ Serum testosterone concentrations (40% (w/w) flaxseed) ⇩ Prostate weight (20% (w/w) flaxseed)	Sprando *et al*. (2000a and b)

GD – gestational day, PND- post natal day, seco- secoisolariciresinol.

Studies on the effects of phytoestrogens in primates

9.97 It is generally accepted that primates are more relevant than rodents in terms of evaluating adverse health effects in humans. Few studies evaluating the effects of phytoestrogens on primates have been published and often the number of animals used is small, making it difficult to evaluate the statistical significance of the results.

9.98 The effects of equine oestrogens (used in hormone replacement therapy) and soy extract (~26.6 mg of genistein/day for 6 months) on the vaginal cytology of ovariectomised cynomolgus macaques were compared in a study by Cline *et al* (1996). Treatment with equine oestrogens induced marked vaginal maturation, whereas the soybean extract had no effect.

9.99 In a similar primate model, ovariectomised adult female macaques were treated with oestradiol, an isoflavone-rich soy protein isolate or were co-administered with both treatments daily for 6 months (Foth & Cline, 1998). Although the precise doses administered were not reported, they were described as being equivalent on an energy basis to doses of 1 mg oestradiol/day and 148 mg soy protein isolate/day in the female adult human. No effects of soy protein on endometrial or mammary tissue were evident after 6 months of treatment. In contrast, uterine weights were significantly increased in the oestradiol treated group, which was accompanied by increased endometrial hyperplasia and proliferation of mammary gland tissue. In this study, co-administration with soy protein isolate appeared to partially antagonise the effects of oestradiol on the mammary gland.

9.100 The effect of dietary soy on the plasma hormone concentrations was assessed in prepubertal female and male rhesus monkeys in a randomised cross-over study (Anthony *et al*, 1996). Animals were fed a diet supplemented with soy (containing either 1 or 9 mg isoflavones/kg diet) for 24 weeks of each arm. No significant effects on plasma oestradiol, testosterone, dehydroepiandrosterone sulfate, free thyroxine and sex hormone binding globulin concentrations or in uterine, prostatic and testicular weights between the dietary groups. No significant differences in testicular weights or testicular histology were reported in a longer term study in which prepubertal male cynomolgus monkeys were fed either a casein-, soy- or an isoflavone free soy-based diet for 14 months (Anthony *et al*, 1997). The isoflavone intake was not reported in this study.

9.101 Pregnant rhesus monkeys were fed genistein (8 mg/kg bw/day) for 7 weeks in addition to a soy-containing diet (the isoflavone concentrations of the feed were not determined) (Harrison *et al*, 1999). No significant differences in maternal, fetal or placental weights were evident at delivery. A non-significant trend towards an increase in maternal plasma concentrations of oestrone and dehydroepiandrosterone sulfate and maternal and fetal progesterone concentrations was noted in the genistein treated group. Serum oestradiol concentrations were 58% greater in maternal and 78% greater in fetal genistein treated groups. No gross changes in placental villous morphology were evident as a result of genistein treatment.

9.102 The effects of soy-based infant formulae on sexual development and fertility were investigated in male marmosets. Co-twin male marmosets were fed either cows' milk or soy-based infant formula from PND 4/5 to 35/45 (Sharpe *et al*, 2002). Isoflavone intakes from soy formula were estimated at 1.6-3.5 mg/kg bw/day. No differences in the intake of formula were evident between the twins. Blood samples collected at PND 18 or 19 and PND 35-45 indicated that the testosterone surge was suppressed in the soy formula-fed animals. Mean testosterone concentrations ranged from 2.8-31 ng/mL in cows' milk formula fed animals compared with 1.2-3.1 ng/mL in soy formula fed animals. A paired comparison between twin-sets showed a 53-70% reduction in plasma testosterone levels in 11/13 of soy formula fed animals.

9.103 No differences in testis weights were evident between the feeding groups (at PND 35/45). Leydig cell numbers increased by an average of 74% in soy-formula fed animals, but no consistent differences in Sertoli or germ cell numbers were evident. It might be expected that the decrease in testosterone produced could be due to decreased Leydig cell numbers, however, large increases in Leydig cell numbers were found.

9.104 These results suggest that isoflavones (or other components in soy formula) can modulate testosterone concentrations in neonates and suppress the neonatal testosterone surge. In addition, soy formula can stimulate Leydig cell proliferation but has no effect on Sertoli or germ cell numbers. The impact or long-term consequences of these effects on the male reproductive system remain to be investigated. An equivalent study in female primates has not been conducted.

9.105 The data from the experimental studies of the effects of genistein or soy on primates are summarised in Table 9.4.

Table 9.4: Effects of isoflavones or soya on primate development and fertility.

Species	Route of administration (duration)	Dose	Results	Reference
Rhesus monkeys (pregnant)	Dietary (7 weeks)	8 mg genistein/kg bw/day	⇧ maternal & fetal oestradiol	Harrison *et al* (1999)
Cynomolgus monkeys (ovariectomised)	Dietary (6 months)	26.6 mg genistein/day	No effects	Cline *et al* (1996)
Macaques (ovariectomised)	Dietary (6 months)	Soy protein isolate/day	Oestradiol induced mammary gland proliferation due to oestradiol antagonised by soy	Foth & Cline (1998)
Rhesus monkeys (prepubertal)	Dietary (24 weeks)	9 mg isoflavones/kg bw/day	No change in serum oestradiol and testosterone concentrations or prostate, testicle or uterine weight	Anthony *et al* (1996)
Marmosets	Dietary (PND 4/5-35/45)	soy infant formula (1.6-3.5 mg/kg bw/day)	⇩ testosterone, ⇧ Leydig cells	Sharpe *et al* (2002)

PND – post natal day.

Studies on the effects of phytoestrogens in humans

9.106 Information on the effects of phytoestrogens on fertility and development in humans is limited. Anecdotal reports have suggested that plant-based compounds can produce oestrogenic effects in humans. Abnormalities in the menstrual cycle of women working with hops and in Dutch women consuming tulip bulbs were associated with exposure to phytoestrogens (Milligan *et al*, 1999; Labov, 1977).

9.107 The effect of a maternal diet on the incidence of hypospadias (a disorder of male sexual differentiation) was investigated in boys (n=7928) born to mothers taking part in the Avon longitudinal study of pregnancy and childhood (North & Golding, 2000). Hypospadias were identified in 51 individuals. There were significant differences in the proportion of hypospadia cases with vegetarian diet or iron supplemented diets in the first half of pregnancy. Vegetarian mothers had an adjusted odds ratio of 4.99 (95% CI 2.10-11.88) of giving birth to a boy with hypospadias compared with omnivores who did not supplement their diet with iron (the odds ratio associated with dietary iron supplementation was 2.07 (95% CI 1.00-4.32)). Hypospadias were also associated with influenza in the first 3 months of pregnancy (adjusted odds ratio 3.19 (95% CI 1.50-6.78)). The authors suggest the greater exposure to phytoestrogens from a vegetarian compared with the omnivorous diet may be a factor in the development of hypospadias. However, the definition of vegetarian used in this study did not differentiate between vegetarian diets as defined by this report and other diets that included meat and no direct measurement of phytoestrogen exposure was conducted.

9.108 A matched pairs case control study of girls in Puerto Rico (n=120 pairs) with premature thelarche (breast development before 8 years of age), investigated the possible role of different factors including exposure to exogenous oestrogens in the aetiology of this condition (Freni-Titulaer *et al*, 1986). Details of dietary and environmental oestrogen exposure as well as maternal history of premature sexual development were examined in a retrospective interview of the mothers. No conclusive associations were found in subjects aged $\geqslant$ 2 years at the onset of thelarche. However, significant associations were found between subjects experiencing thelarche before 2 years of age (n=85 pairs) and consumption of soy infant formula (univariant analysis: OR 2.2, (95% CI 1.0-5.2); multivariant analysis: OR 2.7, (95% CI 1.1-6.8)). Associations in this group of individuals were also found with maternal ovarian cysts (multivariant analysis: OR 6.8, (95% CI 1.4-33.0)) and consumption of fresh chicken (multivariant analysis: OR 4.9, (95% CI 1.1-21.9)). Advancement of puberty has been reported in populations in the United States, however, the underlying causes of this are unclear (Herman-Giddens *et al*, 1997; Kaplowitz *et al*, 2001). There have been no published reports of populations in which boys have experienced pubertal advancement.

9.109 The effects of isoflavones on sex hormone concentrations and semen quality have been investigated in a short-term intervention trial. A group of non-vegetarian men aged 18-35 years (n=15) were given a standardised soy extract (40 mg total isoflavone/day) over a 2 month period (Mitchell *et al*, 2001). Mean plasma concentrations of total genistein and daidzein were 1 and 0.5 μM, respectively. Dietary supplementation with isoflavones did not affect oestradiol, testosterone, FSH and LH concentrations or

sperm volume, count, motility, morphology or testicular volume. Although it was a small study, the results indicate that short-term exposure to isoflavones (40 mg/day) during adulthood does not appear to affect male sex hormone concentrations or semen quality.

9.110 A retrospective cohort study of male and female adults (20-34 years old) fed either soy (n=248) or cows' milk (n=563) formula as infants was reported by Strom *et al* (2001). The participants were identified from infant feeding studies and a survey of reproductive parameters (e.g. menstrual history, pubertal maturation and pregnancy outcome) together with demographic and health characteristics, were assessed by telephone questionnaires. However, the dietary intake of phytoestrogens was not estimated.

9.111 Over 30 different clinically relevant and potentially oestrogenic outcomes were assessed in the two groups but no statistically significant differences were observed in most outcomes. The soy-based formula group did report longer duration of menstrual bleeding (+0.37 days (95% CI 0.06-0.68)) and greater menstrual discomfort (RR 1.77 (95% CI 1.04-3.00)). However, the clinical significance of these findings to females is unclear and the authors suggest that given the large number of comparisons evaluated these associations may be due to chance. Although many of the outcomes examined in this study are dependent upon participant recall, the results do not suggest obvious associations between consumption of soy-based infant formula and gross effects on male and female development and fertility (Strom *et al*, 2001).

Interpretation of data in relation to human exposure

9.112 The data on rodents indicate that exposure to isoflavones, coumestrol and lignans can produce oestrogenic effects in males but may be more pronounced in females. Perinatal, neonatal or prepubertal exposures appears to produce the most marked effects. In studies of isoflavones, advancement of puberty and mammary gland tissue differentiation, irregular oestrus cycling and abnormal histology of the reproductive tract are the most common effects reported in females. However, alterations in sex hormone concentrations have also been reported in male and female animals. Data on coumestrol suggest that this phytoestrogen is more potent, producing similar effects at lower doses, however few studies have been published. Published data suggest dietary secoisolariciresinol also produces oestrogenic effects in rodents.

9.113 Extrapolation of these data to humans are difficult for two reasons. Firstly, although the experimental data clearly demonstrate that phytoestrogens can produce oestrogenic effects in rodents, the human health implications of these effects are unclear due to species differences in sexual development and experimental considerations such as route of administration.

9.114 Secondly, data from human studies of the ADME of isoflavones (see Chapter 5) allow correlations between isoflavone intake and plasma concentrations to be made. However, there are few data to allow similar correlations between intake and plasma concentrations of isoflavones in rodents. Thus, it is difficult to assess the oestrogenic effects observed in rodents in the context of likely human exposures. There is a paucity of data on human intake and ADME of coumestrol and the lignans.

9.115 Data from primate studies are more useful in terms of risk assessment but are limited in number and scope. The studies suggest that *in utero* and neonatal exposures to isoflavones can alter concentrations of endogenous sex hormones. The data from a comparative study of twinned male marmosets fed soy- or cows' milk-based infant formula at equivalent human intakes show suppression of the neonatal testosterone surge and an increase in Leydig cells in the testes. However, Sertoli or germ cell numbers were unaffected (Sharpe *et al*, 2002). Although, the human health implications of these results are unclear they show that soy infant formula can alter parameters of sexual development in the male marmoset.

9.116 Human data on the effects of soy-based infant formula on sexual development are limited to two studies. One study specifically looked at the effects of soy infant formula but did not show associations between soy-based infant formula and obvious adverse health effects, with the exception of small increases in the duration and discomfort of menstruation (Strom *et al,* 2001). The other study examining the possible associations between a number of dietary and environmental factors with premature breast development in girls, reported a weak association with soy formula feeding (Freni-Titulaer *et al*, 1986). However, both studies were based on recall and did not involve any direct measurements of hormone levels or other parameters in the subjects. Human studies specifically examining the potential effects of *in utero* exposure to phytoestrogens have not been conducted.

Key Points

- Testosterone produced by the fetal testes is essential for proper sexual development of the male. Development of the female is not hormone dependent. However, exposure of the male and female fetus to oestrogens or androgens can disturb normal sexual differentiation, the potential effects being different in the male and female. For example, menstrual disturbances in females, or low sperm counts in males. The timing of exposure is a critical determinant of effect.

- Studies on the effects of phytoestrogens on human development and fertility are limited in number and scope. There are no published human studies examining the potential effects of *in utero* exposure to phytoestrogens.

- It is extremely difficult to examine the effects of phytoestrogens on human development and reproduction for both practical and ethical reasons. Hence most of the published research has been conducted in laboratory animals such as rodents and to a lesser extent (for ethical reasons) in primates.

- Significant species differences in sexual development between rodents, non-human primates and humans make the extrapolation of the data from *in vivo* experimental studies to humans extremely difficult.

- The rodent data are of limited use in human risk assessment as the human equivalents of oestrogenic responses in rodents are unclear. In addition, rodent experiments often administer much higher doses than those observed for dietary exposures in humans and use the subcutaneous route of administration, which excludes gastrointestinal and hepatic metabolism.

- Experiments in rodents suggest that coumestrol, and to a lesser extent genistein and secoisolariciresinol, produce oestrogenic effects in both male and female rodents but effects may be more pronounced in the female rodent. Exposure during the perinatal, neonatal or prepubertal stages of development produce the most marked effects. However, the significance of these effects such as alterations in sex hormone concentrations, advancement of vaginal opening, mammary gland development, irregular oestrus cyclicity and abnormal histology of the reproductive tract to humans is unclear.

- Dietary consumption of soy-based infant formula reduces the neonatal surge in testosterone and increases Leydig cell number in the testes of male marmosets. However, Sertoli or germ cell numbers were unaffected. The human health implications of these results are unclear.

- One human study published to date has specifically examined the effect of soy-based formula feeding on sexual development and fertility. The data do not provide evidence for obvious adverse clinical effects on sexual development or reproductive health with the exception of small increases in the duration and discomfort of menstruation. However, the study was based on recall and did not involve any direct measurements of hormone levels or other parameters in the subjects.

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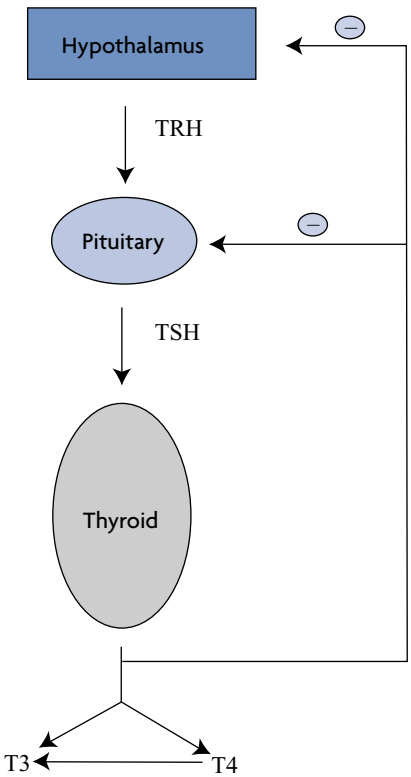
10. Effect of phytoestrogens on the thyroid gland and thyroid function

Introduction

10.1 The thyroid gland is responsible for the production of hormones involved in regulating metabolism, bodyweight and oxygen requirements, as well as normal growth and development during childhood. The thyroid hormones, known as tri-iodothyronine (T_3) and thyroxine (T_4), are synthesised in the gland from iodine and the amino acid tyrosine. The amount of T_3 and T_4 produced by the thyroid gland is controlled by thyroid stimulating hormone (TSH). TSH is secreted from the pituitary gland and is regulated by the central nervous system (CNS). A schematic representation of thyroid hormone synthesis and secretion is given in Figure 10.1.

Figure 10.1 Control of thyroid hormone synthesis and secretion.
The thyroid gland is part of the hypothalamic-pituitary thyroid axis and thyroid hormone secretion is controlled by negative feedback (–). Thyroid-releasing hormone (TRH) from the hypothalamus stimulates thyroid stimulating hormone (TSH) from the pituitary, which stimulates thyroid hormone release.



10.2 Any inhibition of thyroid hormone synthesis will result in an increased secretion of TSH by the pituitary as the body attempts to compensate for the reduced thyroxine concentration. However, greatly elevated concentrations of TSH can result in enlargement of the thyroid gland, a condition that is known as goitre.

A number of compounds, including some that are naturally present in food, have been shown to cause goitre; these compounds being known collectively as goitrogens.

10.3 The development of the thyroid and the pituitary glands in the fetus occurs during weeks 10-12 of gestation. At this stage of development, both glands are relatively inactive and the fetus is dependent on placental transfer of T_4 from the mother. From 18-24 weeks of gestation, the fetal pituitary begins to secrete TSH and fetal serum TSH concentrations rise, remaining relatively high between mid-gestation and term. The concentration of T_4 also increases during this period. The concentration of T_3 in fetal serum is low throughout gestation, associated with low concentrations of deiodinase, an enzyme that converts T_4 to T_3. The levels of fetal T_3 increase modestly towards term and a sharp increase is observed within 2 hours following delivery. During fetal development there may be an increased sensitivity to goitrogens and studies suggest abnormalities arise when normal thyroid physiology is already disrupted (Morreale de Escobar *et al*, 1988; Thorpe-Beeston *et al*, 1992).

10.4 The hypothalamic-pituitary-thyroid axis operates as a feedback regulatory system. The decrease in the circulating concentration of thyroxine is met by an increase in TSH secretion from the pituitary, which results in a return of circulating thyroxine concentrations to a "set" point. In disease states where there is excessive thyroxine production, pituitary TSH secretion is switched off. Where thyroxine production is compromised (as in autoimmune hypothyroidism), the pituitary attempts to overcome the deficiency in thyroxine by increased TSH secretion. This may be partly successful initially with normalisation of thyroxine concentrations in the circulation at the expense of increased TSH secretion from the pituitary. As the disease process advances and more thyroid gland is destroyed, thyroxine concentrations fall and TSH concentrations rise.

10.5 The term goitre simply refers to an enlargement of the thyroid gland (see Table 10.1). This may occur in both hyper- and hypothyroidism. In hyperthyroidism, this is due to the presence of thyroid stimulating antibodies, which have a trophic effect on the gland. In hypothyroidism, autoimmune destruction of the gland takes place so that the enlargement of the gland in this situation results from the increased production of TSH along with infiltration of the gland by lymphocytes involved in the autoimmune destructive process. Hypothyroidism may also occur in areas of iodine deficiency. Iodine is an essential precursor of thyroid hormone synthesis and therefore insufficient iodine in the diet reduces thyroid hormone levels, resulting in a compensatory increase in TSH and goitre. However, very high dietary iodine concentrations (30 times the daily requirement of iodine, see paragraphs 10.23-10.24) can also prevent thyroid hormone production by inhibiting thyroperoxidase (TPO) an enzyme involved in hormone biosynthesis (Rang & Dale, 1987).

Table 10.1 Thyroid function tests in thyroid disease

Condition	Serum TSH concentration	Serum free thyroxine concentration
Hyperthyroidism (overactivity of the thyroid gland)	Suppressed below normal range	Elevated above normal range
Euthyroid (normal function of the thyroid gland)	Within normal range	Within normal range
Hypothyroidism (underactivity of the thyroid gland)	Elevated above normal range	Suppressed below normal range

10.6 Certain plants contain goitrogenic compounds that can interfere with the activity of the thyroid gland. For example, vegetables of the *Brassicaceae* family, particularly cabbage and turnip, contain a precursor of the anti-thyroid agent, goitrin. Goitrin prevents iodine uptake by the thyroid and the production of T_3 and T_4. This results in an increase in TSH levels, ultimately resulting in goitre. In both the Sudan and the Republic of Guinea, iodine deficiency together with consumption of large quantities of millet, which contains the goitrogenic compounds apigenin and luteolin, has resulted in endemic goitre (Gaitan *et al*, 1989; Sartelet *et al*, 1996).

Soy and isoflavones: effects on the thyroid

Animal studies

10.7 The goitrogenic activity of soybeans was first reported in the 1930s when studies showed that soy-fed rats developed goitre (McCarrison, 1933). Since then other studies have shown dietary soy or isoflavones can affect the thyroid function of rodents.

10.8 A study by Filisetti & Lajolo (1981) demonstrated that feeding a defatted, non-autoclaved soy bean extract to rodents for 16 days caused an increase in thyroid weights and increased iodine uptake by the thyroid, whereas feeding a raw soybean extract increased thyroid weights but reduced iodine uptake. No effects on thyroid weight were observed in rodents fed a defatted, autoclaved soybean extract, although iodine uptake was slightly higher than controls. A subsequent feeding study for 29 days showed that a non-autoclaved soybean extract increased iodine uptake whereas an autoclaved extract inhibited iodine uptake. Measurement of thyroid hormones suggests this inhibition resulted in increased synthesis of T_3 and T_4. The authors conclude that while heat treatment inactivated the component of soy responsible for effects on thyroid weight it did not inactivate the component responsible for altering iodine uptake.

10.9 The effect of dietary soy on serum thyroid hormone concentrations was assessed in male rats and hamsters (Balmir *et al*, 1996). Animals were fed one of four diets for 4 weeks. The diets were similar in all respects with the exception of the source of protein added: isolated soy protein, isolated soy protein extracted with organic solvent, casein or casein with 0.36 mg of the soy extract/g protein. The isoflavone concentrations of the diets were not reported. In rats, serum T_4 concentrations were significantly higher in animals that consumed the soy extract supplemented casein diet compared with the other dietary groups. In hamsters, serum T_4 concentrations were significantly higher in animals fed extracted soy protein, non-extracted soy protein and casein supplemented with soy extract compared with those animals fed casein. These studies show that dietary protein source and consumption of an extract of soy can influence blood thyroid hormone concentrations in both rats and hamsters.

10.10 The effects of soy protein isolate, soy protein concentrate (250 g/kg diet) or casein supplemented diets on thyroid hormone concentrations was assessed for 35 days in male hamsters (Potter *et al*, 1996). Serum T_4 concentrations were significantly increased in animals fed soy protein isolate compared to controls. In a study by Mitsuma *et al* (1998), T_3 was increased and T_4 decreased in old (18 month) rats fed a soybean supplemented diet (isoflavones concentrations not determined) compared with rats fed a control diet.

10.11 Ikeda *et al* (2000) reported that a soybean supplemented (isoflavone concentrations not determined) and iodine deficient diet synergistically stimulated growth of the thyroid gland in female rats. Thyroid gland weights were significantly increased in rats fed an iodine deficient diet, with or without soybeans and T_4 levels were significantly reduced in these groups compared with controls. Serum TSH levels were increased in rodents fed a soybean or an iodine deficient soybean diet. Histologically, rodents fed an iodine deficient soybean diet displayed diffuse follicular hyperplasia of the thyroid and indices of proliferation were significantly higher. In a further study, ingestion of a soy supplemented diet (isoflavones concentrations not determined) to iodine deficient female rats resulted in increased thyroid gland weight (Ikeda *et al*, 2001).

10.12 A study by Son *et al* (2001) reported that dietary supplementation with isoflavones (400 or 2000 mg/kg diet) did not alter thyroid weights in female rats. However, thyroid weights were increased in rodents fed dietary 20% (w/w) soy or an iodine deficient diet. A further increase in thyroid weight was observed in rodents fed an iodine deficient diet supplemented with dietary soy. Serum T_4 levels were increased by dietary soy but decreased in iodine deficient diets combined with both soy and isoflavone diets. TSH was increased by the iodine deficient diet and further increased in combination with dietary soy but not isoflavones. However, Chang & Doerge (2000) found no differences in T_3, T_4, TSH concentrations or thyroid gland weight or histopathology in rats continuously fed a soy diet (60 mg genistein/kg diet) compared with control animals.

Human studies

10.13 The effect of soy on thyroid function in animals has prompted investigations into the possible effects of soy and isoflavones on the thyroid gland in humans.

Effects in infants

10.14 In the 1950s and 60s, twelve cases of altered thyroid function in infants, associated with consumption of soy-based infant formula were reported (Hydovitz, 1960; Rawson & Rall, 1955; Shephard *et al*, 1960; Van Wyk *et al*, 1959). The reports however, were mostly of goitre, with no overt signs of hypothyroidism caused by iodine deficiency. Van Wyk *et al* (1959) suggested there were goitrogenic agents in the soy formula as heating or extraction with an organic solvent prior to feeding prevented development of goitre.

10.15 However, studies have suggested that the presence of goitrogens in formula may not have been the only cause of altered thyroid function. Van Middlesworth (1957) suggested that increased faecal mass due to a high fibre diet may also alter-enterohepatic circulation of thyroxine. This in turn may lower thyroid hormone concentrations and increase iodine demand. A study by Pinchera *et al* (1965) reported increased loss of ^{131}I-labelled thyroxine in an athyreotic hypothyroid patient fed a soy formula (51% loss) when compared to a cow's milk formula (31.6% loss). These results indicate that the soy diet is responsible for the increased loss of orally administered thyroxine.

10.16 As a result of these studies, changes to soy-based formulae were implemented in the 1960s. The goitrogenic effects of soy-based infant formula were subsequently overcome by using soy protein isolate instead of soy flour, reducing the goitrogenic constituents of the formula and by supplementation of the formula with iodine (Fomon, 1993). Since this change in processing and formulation there have been no reports of goitre in infants fed soy-based formula in peer reviewed literature.[12]

Effects in adults

10.17 Ishizuki *et al* (1991) examined the effects of consuming 30 g of soybeans/day (isoflavone concentration not determined) on the thyroid function in 2 groups of Japanese men. In the first group, subjects (n=20) consumed soybeans daily for 1 month. Serum concentrations of thyroid hormones were unchanged but TSH levels were slightly elevated ($p < 0.01$). In the second group, soybeans were administered daily for 3 months. No change in serum thyroid hormone concentrations was found. However, TSH concentrations were raised and diffuse goitre and hypothyroidism was evident in half the subjects. These symptoms disappeared within 1 month of cessation of soybean consumption.

10.18 Duncan *et al* (1999a) reported a decrease in T_3 ($p=0.02$) concentrations in premenopausal women receiving 128 mg isoflavones/day. However, the authors conclude that as no effects were seen on total or free T_4 or TSH, the results were unlikely to be physiologically important.

10.19 Duncan *et al* (1999b) reported changes in thyroid binding globulin (TBG) levels in postmenopausal women following supplementation with a diet containing a high (132 mg/day) or low (65 mg/day) isoflavone content compared to an isoflavone-free diet. A decrease in TBG levels was observed

[12] The Working Group is aware and has reviewed a number of anecdotal reports regarding the effects of phytoestrogens on the thyroid gland. However, no conclusions can be made from these reports as they have not been published in peer reviewed scientific literature or subjected to medical assessment.

following supplementation with the high isoflavone diet ($p < 0.05$) however, the low isoflavone diet was reported to produce an increase ($p < 0.05$) in TBG. In addition, the control diet was also shown to have reduced ($p=0.03$) TBG levels from baseline. The authors conclude that while the changes are significant, they may not be physiologically relevant.

10.20 In a double blind trial, postmenopausal women consumed diets supplemented with soy protein (0, 56 or 90 mg isoflavones/day) for 6 months (Persky *et al*, 2002). T_4 concentrations were higher in the group fed 56 mg isoflavones/day ($p < 0.05$) compared with controls. T_3 ($p=0.03$) and TSH ($p=0.01$) concentrations were higher in the group fed 90 mg isoflavones/day at 6 months compared with controls. The authors suggest that isoflavones may affect levels of thyroid hormones but considered the changes in hormone concentrations to be of too small a magnitude to be clinically important.

10.21 In a study by Jayagopal *et al* (2002), postmenopausal women with type 2 diabetes (n=32) ingested a soy supplemented diet (132 mg isoflavones/day) or placebo for 12 weeks in a randomised cross-over trial. A small decrease (2.5%, $p=0.004$) in T_4 concentration was found in subjects after dietary supplementation with soy compared with placebo. There was no change in TSH or T_3 concentrations. No changes in oestradiol, FSH, LH, SHBG, testosterone, dehydroepiandrosterone sulfate or androstenedione concentrations were found.

Iodine status of the UK population

10.22 As reported alterations in thyroid function have been associated with lowered iodine intake, it is possible that people most likely to be affected are those living in geographical areas where dietary iodine intakes are not ideal. To protect against iodine deficiency, the Committee of Medical Aspects of Food Policy (COMA) has recommended a Reference Nutrient Intake (RNI) for adults of 0.14 mg/day and between 0.05-0.14 mg/day for children. In the UK, cows' milk is the main source of dietary exposure to iodine and a survey carried out during 1998-1999 found that the average iodine concentration of milk was 0.31 mg/kg (MAFF Surveillance Information Sheet, No. 198, 1999). No specific information on the intake of iodine from vegetarian or vegan diets is available.

10.23 In 1997, the average UK exposure to iodine was 0.25 mg/day (Food Standards Agency, Information Sheet 05/00, 2000). This is within the range of estimates from the total diet surveys (TDS) reported in 1985 (0.28 mg/day), 1991 (0.17 mg/day), and 1995 (0.21 mg/day). This comparison shows that while there is some variation in estimates of iodine exposure in different years, there is no consistent increase or decrease in intake over time. The estimated average UK exposure to iodine (0.24 mg/kg) is higher than that from the New Zealand TDS (0.1 mg/kg) and to a lesser extent the Chinese TDS (0.17 mg/day). All the estimated iodine exposures from this survey, including that for the high-level adult consumers, are below the JECFA PMTDI for iodine of 0.017 mg/kg bw/day, which is equivalent to 1.0 mg/day for a 60 kg person.

Possible mechanisms of isoflavone-thyroid interactions

10.24 It has been hypothesised that phytoestrogens may interact with the thyroid gland by a number of potential mechanisms.

Interaction with thyroperoxidase

10.25 Isoflavones have a similar chemical structure to T_3 and T_4, which suggests they may be active in the thyroid (see Figure 10.2). Indeed *in vitro* studies demonstrated that the isoflavones, genistein, daidzein and biochanin A inhibited thyroperoxidase (TPO), an enzyme involved in the synthesis of T_3 and T_4 (Divi *et al*, 1997). These compounds acted as alternative substrates for iodination (Divi & Doerge, 1996; Divi *et al*, 1997). This effect was reversed following the addition of iodine. TPO catalyses the oxidation of iodide to iodine radical allowing iodination of tyrosine during synthesis of T_3 and T_4. If inhibition of TPO were to occur *in vivo*, concentrations of thyroid hormones would be reduced and potentially stimulate the production of TSH.

Figure 10.2 The structural similarity of the thyroid hormones T_3 and T_4, genistein and daidzein.

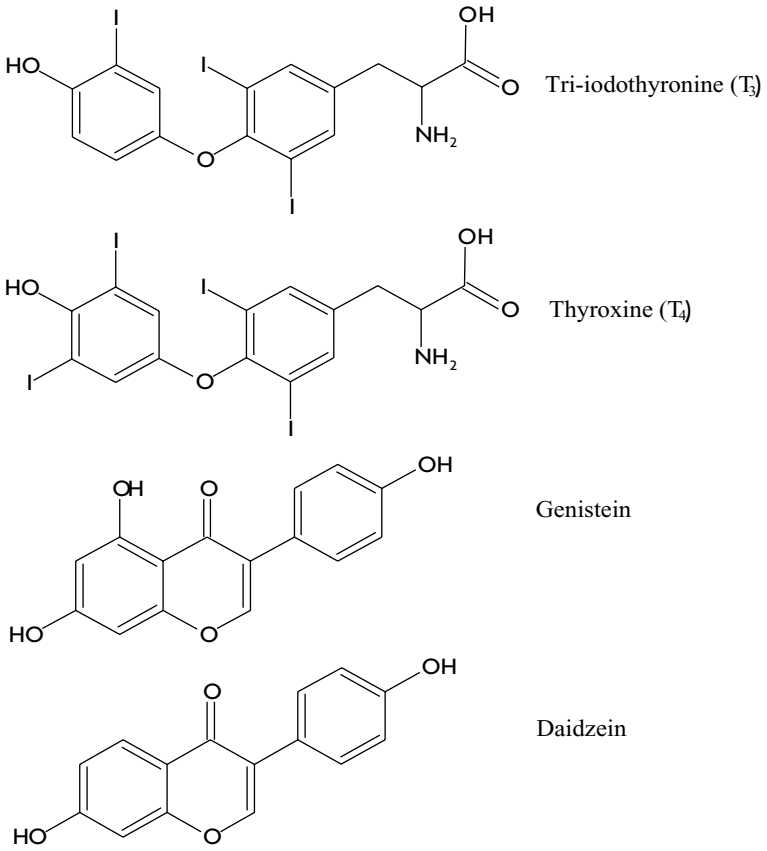


Table 10.2 Inhibition of thyroperoxidase (TPO) catalysed thyroid hormone synthesis by phytoestrogens.

Compound	Concentration (μM)	% Inhibition
Genistein	3.2	50
Daidzein	7.6	50
Biochanin A	6	15
Flavanone	>1500	2
Flavone	>2000	7

Data from Divi *et al* (1997); Divi & Doerge (1996).

10.26 Chang & Doerge (2000) have reported that dietary supplementation with genistein (5-500 mg/kg), commencing *in utero* through weaning until termination of the study 20 weeks later, resulted in a dose-dependent reduction in microsomal thyroperoxidase (TPO) activity in rats. A reduction in TPO activity was also observed in rats fed a standard soy-diet, containing 60 mg/kg total isoflavones, compared to those fed a soy-depleted diet, containing 1 mg/kg total isoflavones. There were no differences in T_3, T_4, TSH concentrations or thyroid gland weights or histopathology between the two groups. The authors suggest that whilst reductions in TPO activity occurs concomitant to soy isoflavone consumption, the remaining activity is sufficient to maintain normal thyroid homeostasis.

Interaction with thyroid binding globulin

10.27 Thyroid binding globulin (TBG) is a plasma protein involved in the inactivation and transport of T_3 and T_4. It has been hypothesised that phytoestrogens could potentially increase TBG concentrations. Any such increase could transiently increase the binding capacity for thyroxine thus lowering free thyroxine concentrations. In turn, TSH secretion would increase as the body attempted to restore free thyroxine concentrations. In an euthyroid state, the thyroid could accommodate this however, if thyroid function was compromised (e.g. autoimmune disease) or if replacement therapy was not increased, the concentration of free thyroxine would be expected to be lower.

10.28 While it is possible that phytoestrogens may alter the concentrations of TBG there is no data to suggest phytoestrogens act by this mechanism to produce clinical effects.

Potential thyroid-phytoestrogen interactions

10.29 Whilst currently there is very little research into the possible outcomes of phytoestrogen interactions with the thyroid gland, a number of potential interactions are possible and have been outlined below:

In utero exposure to phytoestrogens

10.30 There are no data that suggest maternal ingestion of phytoestrogens during pregnancy has an influence on the development of the thyroid gland. A study by Hunter *et al* (2000) reported that, in Scotland, the prevalence of congenital hypothyroidism in the young (<22 years) is low, affecting only 0.135% of the population. Toublanc (1992) reported that countries, such as Japan (when data are matched for number of people surveyed) which consume relatively higher quantities of soy than the UK, do not have a significantly higher incidence of congenital hypothyroidism than either the UK or other developed countries, where iodine deficiency is uncommon. These findings are in contrast with those expected if soy adversely affected the development of the thyroid gland.

Maternal ingestion of phytoestrogens

10.31 Maternal thyroid function during early pregnancy is an important determinant of early fetal brain development. The fetal thyroid is unable to produce T_4 prior to weeks 12-14 of gestation thus, overt maternal hypothyroidism, as seen in iodine-deficient areas, is associated with severe neurological impairment in the offspring. The results from two studies (Haddow *et al*, 1999; Pop *et al*, 1999) suggest that low maternal plasma thyroxine concentrations during early pregnancy are associated with a 4 point reduction in overall IQ performance. However, at birth and subsequently, the children in these studies had normal thyroid function.

10.32 It is possible that low iodine intake coupled with the increased metabolic demands of pregnancy and increased thyroxine need, as well as soy product ingestion, may lead to compromised thyroid function. In this situation, the fetus may receive reduced levels of thyroxine during early gestation. This situation could adversely affect the neurological development of the fetus.

Vegan diets and exposure to phytoestrogens

10.33 Key *et al* (1992) reported elevated TSH levels (p=0.001) in men consuming a vegan diet (n=48) compared to men consuming an omnivorous diet (n=53). Three of the vegan subjects with the highest TSH levels reported taking kelp supplements, a rich source of iodine. However, even after excluding these subjects, the TSH concentration was significantly higher in vegans (p=0.012). The findings suggest a vegan diet may not supply an adequate intake of iodine and thus, vegans may be more susceptible to the goitrogenic effects of soy. Individuals with restrictive diets for example, in infants where high soy intake is coupled with the exclusion of dairy produce and fish may also be more susceptible to goitrogenic effects (Labib *et al*, 1989).

Interaction with thyroxine medication

10.34 Phytoestrogens could potentially interact with thyroxine medication, which is given to patients diagnosed with congenital hypothyroidism. As previously outlined, phytoestrogens could alter TBG concentrations and increase the binding capacity for thyroxine. If a fixed dose of thyroxine is used in treatment, ingestion of large amounts of phytoestrogens could lower the amount of thyroxine available in the free (active) form.

10.35 Alterations in the concentration of TBG may not be the only mechanism by which phytoestrogens could disrupt thyroxine treatment (Chorazy *et al*, 1995; Jabbar *et al*, 1997). Studies in infants with congenital hypothyroidism have found that those fed soy-based infant formula have an increased requirement for thyroxine (above expected replacement levels). Indeed, a study by Jabbar *et al* (1997) estimated that as much as an 18-25% increase in thyroxine might be required. This increased requirement disappeared, however, once the soy-based formula was discontinued. In addition, clinicians and parents of these children noted marked alterations in bowel habit, which also resolved following removal of the soy-based formula (Pinchera *et al*, 1965). This observation led the authors to speculate that increased removal of thyroxine in the faeces was the most likely explanation for the reduced thyroxine concentration.

10.36 In 1998, following recommendations from the New Zealand Ministry of Health, clinicians were advised to closely monitor thyroxine levels in infants with hypothyroidism who were fed soy-based infant formula or high levels of other soy-containing infant foods. It was suggested that in these infants, a higher than usual level of thyroxine would be required to maintain a euthyroid state (New Zealand Ministry of Health, 1998).

10.37 It is also possible that the use of isoflavone-containing dietary supplements may interfere with thyroid medication used to maintain a euthyroid state in hypothyroid individuals. No data are available on estimated intakes of phytoestrogen from dietary supplements, however dietary exposure levels will increase very significantly if individuals take these supplements.

Thyroid autoimmunity

10.38 A study by Fort *et al* (1990) primarily investigating diabetes also reported an increased prevalence in thyroid disease amongst children fed soy-based infant formula as infants. This was a retrospective telephone recall study comparing cases of hypo- and hyperthyroidism in children with their siblings and other non-related controls. Of the infants fed soy-based formula, 31% developed autoimmune thyroid disease compared to 7% of controls. A cause and effect relationship was not established. The study itself was limited by a number of confounding factors including recall bias by patients, which compromises the accuracy of the information obtained. In addition, the small group numbers do not allow the results to be adjusted for age, sex, or parental health status and dietary habits, which may also have contributed to the child's autoimmune disease.

Thyroid cancer

10.39 A study in rodents has suggested that increases in serum TSH concentrations are associated with an increase in thyroid cancer (Thomas & Williams, 1999). However, few studies have investigated possible associations between dietary phytoestrogens and thyroid cancer.

10.40 Kimura *et al* (1976) demonstrated that feeding an iodine deficient, defatted soybean diet to female rats for 6-12 months resulted in the development of hyperplastic goitre. In addition, thyroid carcinomas were observed histologically. The authors did not identify whether the soybeans were responsible for promoting changes induced by iodine deficiency or if there were carcinogenic substances in the soybean.

10.41 In contrast, Son *et al* (2000a and b) investigated the effects of dietary genistein (25, 250 mg/kg diet) and a dietary isoflavone mixture (400 mg/kg diet) on thyroid carcinogenesis in a two-stage rodent model of thyroid cancer. No differences in weight or histopathological lesions of the thyroid between the dietary groups in either male or ovariectomised female rats were observed despite pre-treatment with a thyroid cancer promoter. The authors conclude these compounds do not exert a modifying effect on thyroid carcinogenesis in rodents.

10.42 In humans, it is not clear whether elevated TSH increases the risk of thyroid cancer. Thyroid cancer is more common in women than men and younger women are more likely to develop thyroid cancer. This suggests that oestrogen may play a role in the development of thyroid cancer. However, it is not clear whether phytoestrogens can induce similar effects.

10.43 A retrospective case-control study examining the relationship between phytoestrogen consumption and risk of thyroid cancer was conducted in San Francisco (Horn-Ross *et al*, 2002). Assessment of the dietary patterns of women (cases= 608, controls= 558) was conducted using a food frequency questionnaire and phytoestrogen intake was estimated using a previously published database (Horn-Ross *et al*, 2000). The menopausal status of women in the study was also recorded (70%= premenopausal; 20%= postmenopausal for case and control groups, respectively). The results of the study suggested that increased consumption (highest versus lowest quintile) of unfermented soy-based foods and phytoestrogens was associated with a decreased risk of developing thyroid cancer (see Table 10.3).

Table 10.3 Phytoestrogen consumption and thyroid cancer risk.

Data set [a] shown in regular font is adjusted for age, race/ethnicity, and daily calorific intake. Data set [b] shown in bold are adjusted for age, race, ethnicity, daily caloric intake, goitre or thyroid nodules, radiation to the head or neck, and family history of proliferative thyroid disease.

Phytoestrogen	Adjusted data [a]			Adjusted data [b]		
	OR	95% CI	p value	OR	95% CI	p value
Genistein	0.65	0.42 – 1.0	0.02	0.70	0.44 – 1.1	0.14
Daidzein	0.60	0.39 – 0.92	0.02	0.65	0.40 – 1.0	0.15
Formononetin	0.65	0.44 – 0.96	0.03	0.78	0.51 – 1.2	0.21
Secoisolariciresinol	0.47	0.31 – 0.73	0.0005	0.56	0.35 – 0.89	0.009

OR: Odds Ratio, CI: Confidence Interval.

10.44 However, following correction for confounding variables, including family history of thyroid disease, the strength of the association between consumption of phytoestrogens and the lowered risk of developing thyroid cancer was reduced (as indicated by an increase in p values).

Key Points

- A number of scientific publications have drawn attention to the potential for soy-based infant formulae to affect thyroid function. They have focused largely on the effect of the formula on thyroid function tests and their potential interactions with thyroxine replacement therapy.

- Goitrogenic effects in infants fed soy flour-based infant formula were reported when it was first introduced in the 1960s. This problem was subsequently overcome by using soy protein isolate instead of soy flour thus, reducing the goitrogenic constituents of the formula and by supplementation of the formula with iodine. There have been no reports of goitrogenic effects from soy-based infant formula in peer reviewed literature since the 1960s.

- The experimental data suggest that relatively high levels of dietary soy may have a goitrogenic effect in rodents deficient in dietary iodine. However, data from human studies suggest that dietary soy or isoflavones are unlikely to affect thyroid function in normal individuals with adequate iodine intake.

- It is possible that the isoflavone component of soy-based infant formula may have the capacity to inhibit thyroid function in infants. However, it has not been established whether the levels of free isoflavones in the plasma of infants fed soy-based infant formula are sufficient to significantly influence thyroid function.

- Due to the potential interactions between phytoestrogens and the thyroid gland, it is possible that the thyroid function of hypothyroid individuals consuming high levels of phytoestrogen- or goitrogen-rich foodstuffs and supplements may be adversely affected.

- There is a theoretical possibility that under circumstances in which the thyroid status of the mother is compromised, maternal exposure to high levels of phytoestrogens may impair normal development of the fetus.

- Elevated levels of serum TSH have been associated with an increase in thyroid cancer in rodents. The limited epidemiological evidence suggests that phytoestrogen exposure is not associated with thyroid cancer risk in humans.

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11. Phytoestrogens and the central nervous and immune systems

Effects of phytoestrogens on the central nervous system

Introduction

11.1 Oestrogen receptors are expressed in the central nervous system (CNS) (Kuiper *et al*, 1998; Shughrue *et al*, 1997). Oestrogens are known to be active in a number of areas of the brain and spinal cord, where they are thought to influence behaviour, movement, cognition, pain sensitivity and have a protective effect on the development of neurodegenerative diseases (McEwen, 1999). Therefore phytoestrogens may also exert similar effects in the CNS.

Transfer of phytoestrogens to the CNS

11.2 In humans, the blood brain barrier is not fully developed at birth and for this reason the CNS may be more accessible to phytoestrogens *in utero,* or at birth. However, few studies have examined the transfer of phytoestrogens from the peripheral blood to the CNS. The published data indicates that transfer across the blood brain barrier in rodents is relatively inefficient (at least in adults), as concentrations of isoflavones in the CNS are several orders of magnitude lower than in peripheral blood. A study by Lephart *et al* (2000) showed that plasma levels of isoflavones were 36-fold greater than in the brain of adult rats given dietary isoflavones (600 mg/kg) for 35 days.

11.3 A study by Chang *et al* (2000), measured genistein in the serum and tissue of rats exposed to genistein (0-500 mg/kg of feed) *in utero*, *via* maternal milk and in the diet. Endocrine-responsive tissues including brain showed dose-dependent increases in total genistein concentrations. Serum isoflavone concentrations in females and males were similar (e.g. 8 versus 6 μmol/L, respectively at the highest dose) but were orders of magnitude less concentrated in brain tissue. The levels in females were higher compared with males (e.g. 7.3 versus 0.7 pmol/mg protein, respectively).

Biochemical effects of phytoestrogens on the central nervous system (see Table 11.1)

11.4 Phytoestrogens have been shown to modulate a number of biochemical processes in the CNS (Lephart *et al*, 2002). Expression of brain-derived neurotrophic factor (BDNF) was increased in the brain of ovariectomised adult female rats fed on a soy bean or oestradiol supplemented diet for 8 weeks (Pan *et al*, 1999a; 1999b).

11.5 Calcium binding proteins in the brain such as calbindin and calretinin bind calcium and are thought to play a role in preventing neuronal cell death. Thus, it has been suggested these proteins may play a protective role in neurodegenerative disorders. In a study by Lephart *et al* (2000), lower levels of calbindin and calretinin were found in the brain of adult male rats fed an isoflavone supplemented diet (600 mg/kg) for 5 weeks. No effects on aromatase or 5α-reductase in the brain were observed. Similarly a study by Taylor *et al* (1999) reported that calbindin levels were reduced in the fetus of female but not male rats fed an isoflavone supplemented diet (200 mg/kg). However, Kim *et al* (2001) found that chronic (3 year) exposure to soy in the diet suppressed phosphorylation of tau, a microtubule-associated

protein in the brains cynomolgus monkeys. Phosphorylation of tau has been implicated in neurodegeneration.

11.6 No significant effects on aromatase levels or sex hormone concentrations were evident in the brain of adult male rats fed an isoflavone supplemented diet (600 mg/kg) for 29 days (Weber *et al,* 2001).

11.7 Cyclooxygenase-2 (COX-2) is an enzyme responsible for the metabolism of compounds involved in the inflammatory response associated with neurodegenerative disease. In a study by Lund *et al* (2001a), an increase in COX-2 was found in the brain tissue of adult male, but not female, rats fed an isoflavone supplemented diet (600 mg/kg) during gestation, lactation through to adulthood.

11.8 The effect of coumestrol on oestrogen responsive receptors in the brain was examined in ERα knock out and wild type ovariectomised mice. The mice were fed coumestrol (200 mg/kg diet) for 10 days from PND 45 and some animals were also treated with physiological levels of oestradiol. Coumestrol treatment had no effect on progesterone receptor expression in the brain but did reduce plasma LH levels. In contrast, oestradiol treatment increased progesterone receptor expression and decreased plasma LH levels. The increase in progesterone receptors seen with oestradiol was attenuated when animals were co-administered with coumestrol and oestradiol. The effects of coumestrol and oestradiol were markedly reduced in ERα knock out animals suggesting the effects may be mediated by ERα (Jacob *et al*, 2001).

11.9 These studies suggest that dietary phytoestrogens can modulate proteins involved in calcium binding and inflammation in the CNS. It is unclear if these changes have consequent functional effects and the implications for human health are unknown.

Behavioural effects of phytoestrogens (see Table 11.1)

11.10 A number of studies have shown that phytoestrogens can act on the CNS of rodents to cause behavioural effects. Patisaul *et al* (2001) showed that administration of a diet supplemented with isoflavones (46 mg/kg diet) for 5-6 days reduced mating behaviour in ovariectomised female rats primed with oestrogens but had no effect in unprimed animals. The isoflavone diet increased ERß mRNA expression in the brain, whilst oestradiol treatment both alone or in combination with the isoflavone diet lowered the mRNA levels of ERß.

11.11 Maze learning is used to assess visual-spatial memory (VSM) in rats with males normally outperforming female animals. Lund *et al* (2001a) reported an effect on maze learning in rats fed a phytoestrogen diet. The normal pattern of VSM was reversed in animals after lifelong exposure to an isoflavone supplemented diet (600 mg/kg) with male rats showing a reduction in VSM and females having an increased VSM. In a further experiment, rats were fed the isoflavone diet until PND 80 at which time they continued on this diet or were switched to an isoflavone-free diet. The females continuously fed the isoflavone diet outperformed those switched to the isoflavone free diet. The opposite effect was observed in the males.

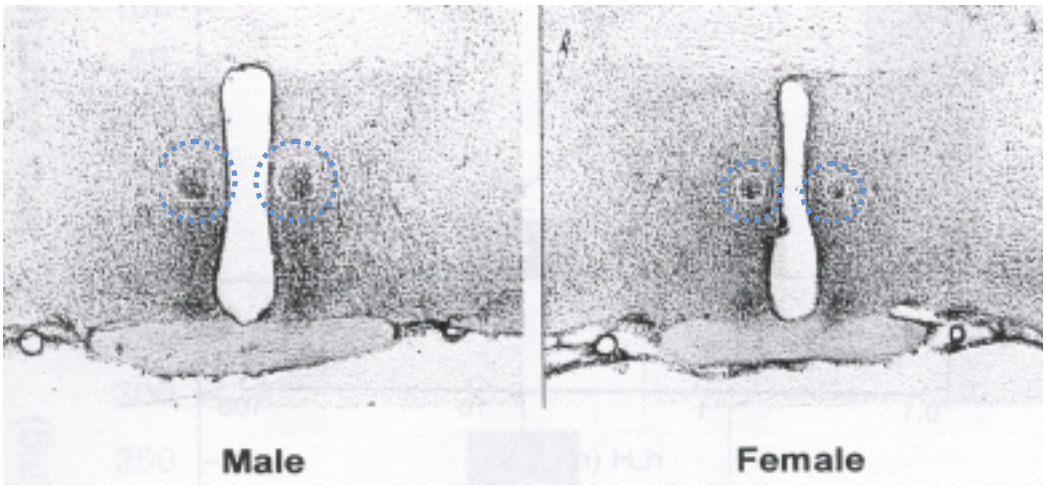
11.12 No effect on locomotor activity was observed in adult ovariectomised mice treated with coumestrol (10 µg/kg bw/day) by subcutaneous injection for 12 days (Garey *et al*, 2001). In contrast, locomotor activity was increased in oestradiol treated animals. However in co-administration experiments, coumestrol treatment lowered oestradiol-induced locomotor activity.

11.13 In a study by Flynn *et al* (2000), pregnant rats were fed genistein (0-100 mg/kg bw/day) through gestation and lactation, and offspring continued on these diets after weaning. The offspring exposed to genistein exhibited no significant differences in sexual dimorphic behaviours (open field activity, play behaviour, running wheel activity and consumption of saccharin and salt solutions) other than females in the top dose group, which displayed a preference to drinking salt solution.

Effects of phytoestrogens on the sexually dimorphic nucleus preoptic area (see Table 11.1)

11.14 In rodents, the sexually dimorphic nucleus of the preoptic area (SDN-POA) is located in the hypothalamic region of the brain. This area of the brain controls sex-specific patterns of sexual behaviour, converts androgens to oestrogens and controls gonadotrophin secretion. The SDN-POA develops in response to endogenous oestrogens during neonatal development. The volume of the SDN-POA is larger in male rats compared with females. During the neonatal period the volume of the SDN-POA is responsive to the administration of exogenous oestrogens and can be used as a sensitive marker of *in vivo* oestrogenic activity (Rhees *et al*, 1999). The structure of the hypothalamus is very different in rodents and humans and the human equivalent of the rodent SDN-POA has not been well defined (Byne, 1998).

Figure 11.1 SDN-POA in the rat.
Vertical section through SDN-POA in an untreated male and female rat. The SDN-POA (circled) lies on either side of the third cerebral ventricle. SDN-POA volume is larger in male than female rats.



11.15 Faber & Hughes (1993) examined the effects of neonatal exposure of genistein on the development of the SDN-POA and pituitary sensitivity to gonadotrophin releasing hormone (GnRH) in ovariectomised female rats. Genistein (0-1000 μg/day) was administered subcutaneously from PND 1-10. The volume of the SDN-POA was increased in the two highest treatment groups (500 and 1000 μg genistein/day). Pituitary responsiveness assessed by GnRH stimulated release of LH, was suppressed in a dose-dependent manner with genistein treatment (⩾ 100 μg/day).

11.16 A similar study, but in male and female neonatal rats was conducted (Lewis *et al*, 2002). Male and female rats were dosed subcutaneously with genistein (0.2 or 4 mg/kg bw/day) from PND 1-6 and orally (4 or 40 mg genistein/kg bw/day) from PND 7-21. Dose equivalence studies showed that the subcutaneous doses would provide plasma concentrations equivalent to oral doses of 4 or 40 mg genistein/kg bw/day, respectively. The SDN-POA volume was slightly increased in female animals in the highest treatment group at PND 44-57, but still remained significantly lower than the corresponding males. There were no effects on SDN-POA volume in male rats and no effect on basal or GnRH stimulated LH secretion were reported at either dose level.

11.17 Male and female rats were exposed to an isoflavone supplemented diet (600 mg/kg diet) throughout gestation, lactation and thereafter until PND 80 when they continued on this diet or were switched to an isoflavone free diet (Lund *et al*, 2001b). Serum isoflavone concentrations were >15 μM when measured in adulthood. An increase in SDN-POA volume was evident at PND 120 in males fed continuously on an isoflavone diet compared to those on an isoflavone-free diet. No such differences were reported in female animals. This study shows that dietary isoflavones can alter SDN-POA volume in adult male rodents.

11.18 The effect of neonatal exposure to coumestrol on SDN-POA development and pituitary responsiveness was studied in neonatal rats (Register *et al,* 1995). Both sexes received subcutaneous injections of coumestrol (0.1, 1 or 10 μg/day) on PND 1-10 and were castrated on PND 21. No effects were observed in any of the treatment groups, other than a reduction in GnRH-induced LH secretion from the pituitary in females in the highest treatment group.

Table 11.1: Effects of phytoestrogens in the central nervous system of rodents.

Study	Results	Author
Biochemical effects		
Ovariectomised adult rats fed soy beans for 8 weeks.	⇧ brain-derived neurotrophic factor.	Pan *et al* (1999a, 1999b)
Fetal exposure to isoflavones (200 mg/kg maternal diet).	⇩ calbindin in females but not males.	Taylor *et al* (1999)
Continuous exposure of male and female rats to isoflavones (600 mg/kg diet) through gestation, lactation to adulthood.	⇧ cyclooxygenase-2.	Lund *et al* (2001a)
Adult male rats fed isoflavones (600 mg/kg diet) for 5 weeks.	⇩ calbindin and calretinin. No effects on aromatase or 5α-reductase.	Lephart *et al* (2000)
Adult male rats fed isoflavones (200 mg/kg diet).	No effect on brain aromatase or sex hormone concentrations.	Weber *et al* (2001)
Ovariectomised mice fed coumestrol (200 mg/kg diet) for 10 days.	⇩ LH. No effect on progesterone receptor levels.	Jacob *et al* (2001)
Behavioural effects		
Ovariectomised adult rats fed isoflavones (46 mg/kg diet) for 5-6 days.	⇩ mating behaviour.	Patisaul *et al* (2001)
Continuous exposure of male and female rats to isoflavones (600 mg/kg diet) through gestation, lactation to adulthood.	⇩ visual-spatial memory in males. ⇧ visual-spatial memory in females.	Lund *et al* (2001a)
Ovariectomised adult mice injected s.c. with coumestrol (10 μg/kg bw/day) for 12 days.	No effect on locomotor activity.	Garey et al (2001)
Continuous exposure of male and female rats to genistein (100 mg/kg bw/day) through gestation, lactation to adulthood to PND 77.	No effects on sexual dimorphic behaviour except ⇧ salt consumption in females.	Flynn *et al* (2000)

s.c. – subcutaneous; LH – lutenising hormone; PND – post natal day

Table 11.1: Effects of phytoestrogens in the central nervous system of rodents. *(continued)*

Study	Results	Author
Effects on SDN-POA volume		
s.c. injection of genistein (0-1000 μg/day) to female ovariectomised rats on PND 1-10.	⇩ LH (⩾ 100 μg/day). ⇧ SDN-POA volume (⩾ 500 μg/day).	Faber & Hughes (1993)
Male and female rats administered genistein (4 mg/kg bw/day s.c.) on PND 1-6 and (40 mg/kg bw/day oral) on PND 7-21.	No effect on LH. ⇧ SDN-POA volume in females, no effect on males.	Lewis *et al* (2002)
Continuous exposure of male rats to isoflavones (600 mg/kg diet) through gestation, lactation to adulthood.	⇧ SDN-POA volume.	Lund *et al* (2001b)
s.c. injection of coumestrol (10 (g/day) to castrated male and female rats on PND 1-10.	⇩ LH in females. No effect on SDN-POA volume.	Register *et al* (1995)

PND – post natal day; LH – lutenising hormone; s.c. – subcutaneous; SDN-POA – sexually dimorphic nucleus – preoptic area.

The effects of phytoestrogens on cognitive function

11.19 To date, two studies have been published on the effect of soy on human cognitive function. The first study utilised subjects from the longitudinal Honolulu Heart Program study (White *et al*, 2000). The participants (Japanese-American men living in Hawaii, born between years 1900 to 1919) were initially recruited in 1965, monitored for heart disease, stroke and cancer and interviewed on their food consumption on two occasions (1965-1967 and 1971-1974). In addition to these parameters, cognitive function was also assessed (between 1991 and 1993) in a number of individuals (n=3734, aged between 71-93 years). A stratified random sample of men from this group (n=948) underwent further evaluation to investigate the causes of cognitive function impairment. A number of subjects were accompanied by their partners (n=502) who were also assessed. Dietary assessments were conducted by food frequency questionnaire, which recorded the daily or weekly consumption pattern of 26 specific food and drink items including tofu. This data was also used to estimate tofu consumption of the participant's partners.

11.20 Cognitive function was assessed using cognitive abilities screening instrument (CASI) and where possible, neuropathology reports from autopsies and neuroimaging were also evaluated. Males who consumed tofu more frequently in midlife had higher rates of cognitive impairment compared with peers who consumed tofu less frequently ($p=0.006$). A similar association was found in women. However, this association was based on their proxy assessment of tofu consumption. Calculation of odd ratios (which provides an estimate of a change in risk) showed that subjects who reported consuming $>$ 2 tofu servings/week had odds ratios (OR) in the range 1.6-2.85 for markers of cognitive impairment [i.e. poor cognitive test scores (OR 1.62, CI 1.06-2.46), low brain weight (OR 2.08, CI 0.97-11.47) and ventricular enlargement (OR 2.85, CI 0.73-11.16)] when compared with those eating $<$2 servings/week. The authors conclude the results are suggestive of an association in this population between tofu consumption in midlife and diminished cognitive function later in life (White *et al*, 2000). However, this study is reliant on the accuracy of the tofu intake data and the results may have been influenced by inaccuracies resulting from the imprecise nature of the methodology employed.

11.21 In the second study, File *et al* (2001) investigated the effects of soy on cognitive function in a 10-week placebo-controlled intervention trial of student volunteers aged between 22-30 years (n=15 male, n=12 female). Subjects consumed omnivorous diets of equivalent calorific content containing 0.5 or 100 mg total isoflavones/day. Both groups were matched for age, IQ, measures of anxiety and depression and caffeine intake. Tests of cognitive function were assessed relative to a pre-study baseline and compared with the placebo group. Tests included measures of attention, short- and long-term memory and mood. Subjects in the high isoflavone group showed small improvements in tests of short and long term memory ($p<0.05$), mental flexibility ($p<0.05$) and were rated as more restrained in a self-assessment of mood ($p<0.05$).

Key Points

- Oestrogens are active in the central nervous system (CNS) and thought to influence behaviour, movement, cognition, pain sensitivity and protect against development of neurodegenerative diseases.
- A small number of studies have examined the transfer of phytoestrogens from the peripheral blood to the CNS in rodents. The data suggest the blood brain barrier effectively restricts phytoestrogen transfer to the central nervous system in adult rodents.
- Studies in rodents suggest that dietary exposure to isoflavones (> 200 mg/kg diet) can decrease the concentrations of proteins in the brain associated with protective effects against neurodegenerative diseases. However, the functional effects of these changes have not been determined and their implications for human health are unknown.
- Few studies have been conducted on the effects of phytoestrogens on rodent behaviour. However, these suggest dietary isoflavones can alter some sexually dimorphic behaviour in rodents such as decreasing mating behaviour (females) and increasing (females) or decreasing (males) visual-spatial memory.
- Studies have shown that isoflavones can alter the structure of the sexually dimorphic nucleus of the preoptic area (SDN-POA), an area of the rodent brain controlling sexual behaviour. However, the human equivalent of the rodent SDN-POA has not been well defined thus, the implication of these findings to humans is unclear.
- Two studies have investigated the effect of soy or isoflavones on cognitive function in humans. One shows a weak association between consumption of a soy-based food (tofu) in mid-life and cognitive impairment in later life. However, this study is reliant on the accuracy of the tofu intake data used and the results may have been influenced by inaccuracies resulting from the imprecise nature of the methodology employed. The other, a small placebo-controlled study, suggests short-term consumption of high levels of isoflavones (100 mg/day) may slightly improve memory.

Phytoestrogens and immune function

Influence of sex hormones on immune function

11.22 It is well established that sex hormones can have a substantial impact on the immune system and on the integrity of immune function. Oestradiol is known to affect the development and organisation of lymphoid tissues and the activity of various cellular vectors of immune function. In general terms, females display more vigorous immune responses than males (Ansar Ahmed *et al*, 1985; Grossman, 1984). A feature of such gender differences is that in many instances autoimmune diseases are more common in women than in men (Ansar Ahmed *et al*, 1999; Jacobson *et al*, 1997; Olsen & Kovacs, 1996). As reviewed by Ansar Ahmed (2000), the same gender differences are apparent in various animal models of autoimmune diseases where it can be shown that oestrogens (or anti-androgens) may increase the susceptibility of relatively disease-resistant male mice.

11.23 Oestrogens can modulate immunological responses and the mechanisms for these effects are complex. For example, in one investigation using mice it was found that oestrogens could simultaneously activate macrophage production while depressing cell-mediated immunity (Luster *et al*, 1984).

11.24 There is evidence that oestrogen receptor activation can influence lymphoid development. In investigations by Erlandsson *et al* (2001), it was shown that in male mice ERα, but not ERβ, is required for normal development of lymphoid tissue. Mice lacking ERα (ERKO mice) exhibited hypoplasia of both the thymus and spleen and had increased numbers of immature ($CD4^+/CD8^+$) thymocytes.

Diethylstilboestrol immunotoxicity

11.25 Diethylstilboestrol (DES) is thought to cause disorders of the immune system in perinatal mice. Reversible thymic and splenic atrophy, decreased T-helper cell numbers and impaired natural killer cell activity were found in perinatal mice exposed to DES. Human studies also suggest that *in utero* exposure to DES can affect T-cell and natural killer cell activity (reviewed in Giusti *et al*, 1995). An increased incidence of autoimmune disease has also been reported in people exposed *in utero* to DES (Golden *et al*, 1998; Vingerhoets *et al*, 1998). Given these observations, it is possible that phytoestrogens could, in principle, affect the immune system or immune function however, few studies have examined this issue.

In vitro studies of immune function

11.26 Genistein (37 μM) has been shown to inhibit human T-cell proliferation, interleukin-2 (IL-2) production and IL-2 receptor expression *in vitro* (Atluru & Atluru, 1991). Daidzein (0.01-10 μM) increased the proliferation of mouse splenocytes after activation with either concanavalin A or lipopolysaccharide in a dose-dependent manner (Wang *et al*, 1997).

11.27 Sakabe *et al* (1999) proposed that oestrogens may regulate the function of the thymus gland as thymus epithelial cells were found to express oestrogen receptors. Inhibition of thymic hormone (thymosin-α1) production was shown after oestradiol (>30 pM), genistein (3 nM) and coumestrol (3 nM) administration.

In vivo studies of immune function

11.28 Several investigations have sought to determine whether phytoestrogens influence the integrity of immune and inflammatory responses in animal models.

11.29 There is some evidence to suggest that isoflavones may display anti-inflammatory properties. Sadowska-Krowicka *et al* (1998) reported that genistein (0.1 mg/kg bw/day) administered by subcutaneous injection for 7 days had a modest anti-inflammatory effect in a guinea pig model of inflammatory bowel disease. In separate investigations, Regal *et al* (2000) examined the influence of soy enriched diet on challenge-induced respiratory hypersensitivity reactions in sensitised guinea pigs. Although dietary exposure to soy was associated with a reduction in eosinophil accumulation, there was an increase in the volume of bronchoalveolar lavage fluid.

11.30 Studies have reported increased activity of aspects of the immune function after exposure to the isoflavones, daidzein and genistein. In the first of these, Zhang *et al* (1997) investigated the effect of daidzein on immune function *in vivo*. Adult mice of both sexes were given daidzein (10, 20 or 40 mg/kg bw/day) by gavage for 7 days. A significant increase in relative thymus (but not spleen) weight was seen at two dose levels (20 and 40 mg/kg bw/day). Mice given daidzein (20 and 40 mg/kg bw/day) also exhibited increased phagocytic activity and splenocyte cytolytic activity. A significant increase in circulating T-lymphocyte number was evident in both these treatment groups. This suggests that daidzein at doses > 20 mg/kg bw/day can alter immune function in mice.

11.31 A study by Guo *et al* (2001) examined the effect of genistein on immune function in a mouse tumour model. Adult female mice received genistein (0, 2, 6 or 20 mg/kg bw/day) by oral gavage for 28 days. A dose-dependent increase in body, liver, spleen and lung weight was reported. No effect on thymus weight was reported. Genistein treatment (6 and 20 mg/kg bw/day) significantly inhibited lung tumour formation but this was shown *in vitro* not to involve inhibition of tumour cell proliferation. *In vitro* assays of immune cell function showed increased activity of cell-mediated immunity mediated by cytotoxic T-cells and natural killer cells. IgM and IgG antibody numbers were not altered by treatment with genistein. The suggestion was that genistein may exhibit anti-tumour activity *via* a cellular immune mechanism.

11.32 In a further study by Guo *et al* (2002a), pregnant rats were fed genistein (0, 300 or 800 mg/kg diet) during gestation and lactation. Dietary genistein had no effect on maternal spleen or thymus weight or thymocyte numbers. However, a reduction in splenic lymphocyte numbers was reported in the highest treatment group. The offspring were evaluated immunologically at PND 22. Dietary genistein had no effect on relative thymus or spleen weight but a dose-dependent reduction in the numbers of a subset of thymocytes ($CD4^+CD8^-$ cells) was apparent in male and female offspring. Natural killer cell activity was increased in male, but decreased in female offspring exposed to genistein (Guo *et al*, 2002a).

11.33 In another study by Guo *et al* (2002b), pregnant rats were fed genistein (25, 250 or 1250 mg/kg diet) from day 7 of gestation to the birth of their offspring and through lactation until PND 21 of the offspring. After weaning, the offspring were given the same feed as their mothers until PND 62. At the end of treatment, no dose-dependent changes in relative spleen or thymus weights were reported in the mothers or the offspring. However, increases in natural killer cell activity and in T cell number were evident in the mothers and the offspring, respectively.

11.34 The effect of genistein on various immune parameters was investigated in castrated adult mice used as a surrogate to model neonatal exposure (Yellayi *et al*, 2002). Adult ovariectomised female mice were placed on a phytoestrogen-free diet and subsequently injected for either 7 or 21 days with genistein (2-200 mg/kg bw/day). A dose-dependent reduction in thymus weight was reported. In parallel experiments, castrated male mice injected with genistein (200 mg/kg bw/day) also displayed a significant reduction in thymus weight. Alterations in the phenotypic characteristics of thymocyte populations, a systemic lymphopenia and compromised humoral immune responses, were apparent in ovariectomised juvenile animals injected with genistein (8-80 mg/kg bw/day) for 5 weeks.

11.35 In further experiments, dietary genistein (1000 or 1500 mg/kg diet) administered to juvenile ovariectomised mice for 12 days also produced a dose-dependent reduction in thymic weight of similar magnitude to that observed after injection of 8 mg genistein/kg bw/day. The serum concentrations of genistein after dietary and subcutaneous exposure (8 mg genistein/kg bw/day) were approximately 1 and 4 μM, respectively. Thus, the authors conclude that exposure to physiologically relevant levels of genistein is associated with significant changes in thymic development and specific immune abnormalities in mice (Yellayi *et al*, 2002). However, the use of castrated animals in addition to species differences make it difficult to draw conclusions regarding the human health implications of these data.

Human studies of immune function

11.36 An association between soy, and immune function has been reported by Zoppi *et al* (1983). Infants in the first year of life were fed either breast milk (n=27), or one of four different types of artificial feed including soy infant formula (n=7-9 for the feeding groups). The antibody response to bacterial and viral antigens after vaccination was monitored in each group. At 5 months infants fed human milk (1.6 g protein/kg bw/day) or high protein cows milk (4.4 g protein/kg bw/day) had "protective" antibody levels. Antibody responses were lower ($p<0.05$) in infants fed on low protein cows' milk (1.8 g protein/kg bw/day) and soy-based formula (4.6 g protein/kg bw/day). However, it is not possible to attribute the change in immune response to phytoestrogens, soy or the protein content of the infant formula.

11.37 More recently the influence of soy-based infant formulae on vaccine responses, morbidity and cellular immune parameters have been reported (Cordle *et al,* 2002; Ostrom *et al*, 2002). In these studies, comparisons were made between new-born term infants fed soy infant formula (n=186) and a cohort (n=81) where infants that were breast-fed exclusively until at least two months of age. Antibody responses to vaccines were measured, as was the distribution of immune cell sub-populations in peripheral blood. The authors concluded that infants fed soy formula showed no decrement in immune function, all antibody responses were within normal ranges and immune cell sub-populations were comparable between groups (Cordle *et al*, 2002; Ostrom *et al*, 2002).

11.38 One study has examined the effects of dietary soy on immune function in adults. In this randomised cross-over trial (23 men and 18 postmenopausal women), acute-phase proteins and proinflammatory cytokines were measured in serum after consumption of control, soy (73 isoflavones/day) or alcohol washed soy (10 mg isoflavones/day) diets for 1 month. No changes in these parameters were evident, other than an increase in interleukin-6 concentration ($p < 0.013$) in women consuming the high isoflavone diet compared with the control diet (Jenkins *et al*, 2002).

Key points

- Oestrogens are involved in the development and maintenance of normal immune function.
- *In vitro* studies show that phytoestrogens can influence some immune responses.
- Rodent studies on the effects of isoflavones on immune function have produced inconsistent results, reporting stimulatory, suppressive or no effects on the immune system. The relevance of these observations for human health is uncertain.
- One study of soy infant formula fed infants suggested isoflavones may adversely effect infant immunity. However, two more recent reports of a larger study indicate that there were no differences in immune response between infants fed soy formula and those receiving breast milk.

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12. Phytoestrogens and osteoporosis

Introduction

12.1 Osteoporosis is characterised by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and risk of fracture (COMA, 1998). Bone mass is lost when an imbalance between the cells responsible for the formation of bone (osteoblasts) and those responsible for the resorption of bone (osteoclasts) occurs in favour of bone resorption. This imbalance results in bone structural failure and an increased likelihood of fracture.

12.2 Although heritable factors account for variability in bone health, secondary factors such as diet, physical activity and hormonal status are also known to be important. Studies in adolescents have indicated that bone mass accumulation during puberty appears to be critical in the development of peak bone mass which is thought to be achieved by early adulthood (Bachrach, 2001; Janz, 2002). Hence, bone status in pre- and peripubertal life is generally accepted to be a major determinant of osteoporosis in later life.

12.3 A range of factors are required to maintain good bone health, many of which are dependent on oestrogen. Osteoclastic activity is enhanced by oestrogen deficiency. Hence, osteoporosis is a particular problem in postmenopausal women, due to the acceleration of bone loss associated with reduced levels of oestrogen. Hormone replacement therapy (HRT) has been shown to be effective in preventing bone loss (Felson *et al*, 1993).

12.4 The different character and incidence of osteoporosis and hip fractures in populations consuming relatively high levels of soy has prompted investigations into the oestrogenic activity of phytoestrogens and osteoporosis (Ito *et al*, 2001). Studies in animal models have shown that phytoestrogens can have a beneficial role in preventing bone loss following ovariectomy. However, the human data are limited and results of intervention trials in both pre- and postmenopausal women have reported inconsistent results. Large, long-term studies in humans would be required before the efficacy of phytoestrogen containing foods or supplements can be confirmed.

Animal studies

Isoflavones

12.5 Data from studies in rodents have demonstrated consistently that following ovariectomy, treatment with soy or soy-isoflavone enriched diets improves retention of bone mass (Anderson & Garner, 1998; Arjmandi *et al*, 1996, 1998a, 1998b). For example, Ishimi *et al* (2000) showed that following ovariectomy, subcutaneous administration of 0.7 mg/day genistein to mice for 4 weeks markedly prevented bone loss and improved bone mineral density (BMD) at the distal femoral metaphysis.

12.6 Picherit *et al* (2000) showed that 10 mg/kg bw/day daidzein given for 3 months was more effective than genistein at the same dose in preventing bone loss in ovariectomised rats. Coumestrol has also been shown to improve femur bone mineral density in a similar rat model (Draper *et al*, 1997).

12.7 Studies combining genistein supplementation and resistance exercise training have demonstrated these treatments have synergistic effects in preventing bone loss. Genistein treatment and exercise equally increased femoral BMD of ovariectomised rats by 5%, whereas combined treatment of genistein and exercise resulted in 8% increase (Nakajima *et al*, 2001). Beneficial effects in preventing bone loss were also observed following combined treatments in a similar study in ovariectomised mice (Wu *et al*, 2001).

12.8 Although the mechanism of action of phytoestrogens on bone health is still unclear, it has been suggested that several mechanisms may be involved. Phytoestrogens may exert osteoprotective effects by mimicking oestrogen to inhibit osteoclastic bone resorption (Ishimi *et al*, 1999; Picherit *et al*, 2001; Rassi *et al*, 2002) or by stimulating osteoblastic bone formation (Fanti *et al,* 1998). Other studies have indicated that phytoestrogens may stimulate osteoblastic-derived paracrine factors that modulate osteoclast formation and activation (Spelsberg *et al*, 1999; Viereck *et al,* 2002) or suppress osteoclastic activity via tyrosine kinase inhibition (Blair *et al*, 1996). However, non-oestrogenic mechanisms or other properties of soy may also play a role. For example, enhanced intestinal absorption of soy-derived calcium may contribute to the bone conserving effects of soy (Omi *et al*, 1994).

Lignans

12.9 A few studies have investigated the effects of lignans on osteoporosis. Ward *et al* (2001a; 2001b) examined the effect of flaxseed and purified lignans on bone strength in rats. In this study pregnant rats were fed a phytoestrogen-free diet or diet supplemented with either 10% flaxseed or the equivalent amount of the purified flaxseed lignan secoisolariciresinol diglycoside (SDG). Offspring were exposed throughout pregnancy and lactation after which they continued on the corresponding maternal diets until adulthood. Offspring were examined after postnatal days (PND) 50 and 132 to represent adolescent and adult age groups, respectively. Femur weights and length did not change significantly on either diet or in any of the age groups.

12.10 A reduction in bone mechanical strength was observed in adolescent male rats fed the 10% flaxseed diet. No significant changes in bone mineral content (BMC) were noted in any males examined. The lack of effect on bone strength in groups fed a diet containing equivalent amounts of SDG indicates that an unidentified component, other than the flaxseed lignan, may be responsible for reduction of bone strength. This reduction was not sustained into adulthood suggesting that consumption of flaxseed is not detrimental to overall adult bone health in males (Ward *et al*, 2001a).

12.11 In contrast, adolescent female offspring fed SDG showed increased bone strength when compared with controls. This increase was not sustained until adulthood. Significant decreases in bone area and BMC were also noted in females on the SDG supplemented diet. BMC decreased by 8% and 14% in adolescent and adult females, respectively. This suggests that female rat bone is more sensitive to the oestrogen-like action of lignans during early life when endogenous levels of sex hormones are low, but by adulthood the increased bone strength does not persist when compared with controls (Ward *et al*, 2001b).

Human studies (see Table 12.1)

Epidemiological studies

12.12 A limited number of studies have been conducted in women to examine the relationship between phytoestrogen intake and osteoporosis. These studies have shown a beneficial effect of phytoestrogens on bone mass in the lumbar spine of peri- and postmenopausal women. No studies have been conducted in men.

12.13 A prospective study (ten years) among Dutch postmenopausal women (n=70) investigated the association between long-term urinary excretion of phytoestrogens (as markers of habitual dietary intake) and the rate of postmenopausal bone loss. Multivariate linear regression analyses showed no evidence for the preventative effects of low dose unsupplemented dietary intake of phytoestrogens (approximately 0.35 mg/g urinary creatinine as determined by excretion rates) on the rate of early postmenopausal cortical bone loss. However, enterolactone excretion was significantly higher ($p < 0.05$) in subjects with a high rate ($\geqslant$ 2.5%/year) of bone loss (Kardinaal *et al,* 1998).

12.14 A three year prospective study using food frequency questionnaires (FFQs) to study the relationship between soy intake and maintenance of BMD in the spine was carried out in a population of Hong Kong Chinese women (n=132). Despite the limitations of assessing soy intake and physical activity using food frequency and leisure questionnaires, significant differences ($p < 0.05$) in spinal BMD regression were found among subjects in the highest (mean ~15 mg isoflavones/day) and lowest (mean 1.4 mg/day) soy intake groups. Mean loss in spinal BMD was 3.5% and 1.1% for low and high soy intake groups respectively. Age and body size were shown not to be confounding factors (Ho *et al*, 2001).

12.15 This is in contrast to findings in case-control study of postmenopausal osteoporotic Korean women who had lower urinary enterolactone levels ($p < 0.05$) than controls when corrected for body mass (Kim *et al,* 2002). However, total urinary phytoestrogen excretion showed no correlation with BMD in these women (n=75) following a 24 hour analysis. The authors also note that the lower urinary enterolactone levels may also be due to the Korean diet, which includes large amounts of rice instead of bread.

12.16 In a cross-sectional study of American women (n=3302), the association between dietary soy isoflavones (as measured by genistein intake) and BMD was investigated by Greendale *et al* (2002). In this study women aged 42-52 years were recruited from four ethnic backgrounds: Caucasian (n=1003), African-American (n=497), Chinese (n=200) and Japanese (n=227). FFQs were used to determine dietary habits and estimate isoflavone intake. African-American and Caucasian women reported low or no genistein intakes (mean 0.5 and 1.4 mg genistein/day, respectively) and analysis of the association between genistein and BMD was not pursued for these women.

Table 12.1 The effect of dietary soy or phytoestrogens on bone health and markers of bone retention in women.

Study design	Dose mg/day	Duration	Effect	Reference
Soy foods				
Prospective, cohort PostM (n=70)	nd	10 year follow up	No association between urinary excretion of phytoestrogens and post cortical bone loss.	Kardinaal *et al* (1998)
Prospective cohort, PeriM (n=132))	7.4-48.3	3 years	⇧ BMD in lumbar spine.	Ho *et al* (2001
Case control, 24hr urinary analysis PostM (n=75)	nd	nd	⇩ enterolactone excretion in osteoporotic subjects. No association between BMD and total phytoestrogen intake.	Kim *et al* (2002)
Cross sectional (n=3302 women)	0.5 (African-American & Caucasian) 10.7 (Japanese & Chinese)	nd	⇧ BMD in femoral neck and spine in Japanese & Chinese women.	Greendale *et al* (2002)
Cross sectional (n=995)	nd	nd	⇧ BMD with soy consumption.	Tsuchida *et al* (1999)
Cohort PostM (n=478)	35-65	nd	⇧ BMD in lumbar spine associated with high intake groups.	Somekawa *et al* (2001)
Cross-sectional cohort PreM (n=298) PostM (n=357)	20.1-58.6	nd	⇧ BMD in the lumber spine for high isoflavone intake group. No association between BMD and lignan, coumestrol or flavonoids intake for either PreM or PostM.	Mei *et al* (2001)
Cross-sectional (n=208)	0-13.9	nd	No effect on BMD	Kritz, Silverstein & Goodman-Gruen (2002)
Cross-sectional PostM (n=87)	32	nd	No effect on parameters of bone health.	Nagata *et al* (2002)
Cross-sectional PostM (n=85)	12.6	nd	⇧ BMD.	Horiuchi *et al* (2002)
Randomised, DB,PC PeriM (n=69)	80.4	6 months	Significant positive effects on percentage change (loss) in both BMD & BMC in lumbar spine.	Alekel *et al* (2000)

Table 12.1 The effect of dietary soy or phytoestrogens on bone health and markers of bone retention in women. *(continued)*

Study design	Dose mg/day	Duration	Effect	Reference
Dietary supplements				
Randomised, DB, PC, PostM (n=66)	90	6 months	⇧ BMD & BMC in the lumber spine.	Potter *et al* (1998)
Randomised, DB, PC, PostM (n=30)	54	1 year	⇧ BMD markers of bone formation. ⇩ markers of bone resorption.	Morabita *et al* (2002)
Single group intervention, no PC PostM (n=42)	60	3 months	Osteocalcin, ⇩ N-telopeptide Neither dose-dependent.	Scheiber *et al* (2001)
DB, PC, Pre, Peri & PostM (n=97)	40	1 year	⇧ pyridinium crosslinks in preM and periM only. No changes in Post M.	Atkinson *et al* (2003)
Randomised, DB, PostM (n=46)	28.5-85.5	6 month	⇧ BMD at the two highest doses only. Low dose gave no significant results.	Clifton-Bligh *et al* (2001)
Randomised, Crossover PreM (n=14) PostM (n=17)	8-130	3 months	Small changes in markers of bone formation or bone resorption.	Wangen *et al* (2000)
DB, PC Pre M (n=15)	90	1 year	No effect on BMD or BMC.	Anderson *et al* (2002)
Flaxseed				
DB PC PostM				
(n=36)	nd	3 months	No changes in markers of bone resorption.	Lucas *et al* (2002)
Mixed soy & linseed				
R, DB, PC (n=52)	52	3 months	No changes in BMD or BMC.	Dalais *et al* (1998)

PreM: Premenopausal women; PostM: Postmenopausal women; PeriM: Perimenopausal women BMD: Bone mineral density; BMC: Bone mineral content; DB: Double blind; PC: Placebo controlled; nd Not determined

12.17 Japanese and Chinese women reported higher genistein intakes of 10.7 and 5.8 mg genistein/day. Following ethnic-specific analysis, a positive dose-related association between genistein intake and BMD in the femoral neck (p=0.02) and spine (p=0.028) was observed in premenopausal Japanese women. This association was not seen in perimenopausal Japanese women. No association between genistein and BMD was found at any genistein intake or at any menopausal status in the Chinese women (Greendale *et al*, 2002).

12.18 In a cross-sectional study of healthy middle-aged (40 to 49 years) Japanese women, dietary intake of calcium was assessed by a FFQ on dietary calcium sources including milk, diary products, fish and soybeans. A total of 995 women were recruited and BMD of the metacarpal was measured (Tsuchida *et al*, 1999). An independent association of weekly calcium intake from soy was noted (p=0.03). The authors suggest that calcium intake from soybeans and possibly from the isoflavone content may affect BMD in middle-aged Japanese women.

12.19 Using FFQs, a cross-sectional study in postmenopausal Japanese women (n=478) questioned subjects on their weekly, monthly, and yearly consumption of soy products (Somekawa *et al*, 2001). Subjects were also questioned on soy consumption when they were 40 years old. The study demonstrated that following adjustment for type of soy product, increased BMD was found to be weakly correlated with intake of fermented soybeans ($p < 0.01$) and soybean curd ($p < 0.01$). There was no correlation between BMD and other soy products. Following adjustment for weight and years since menopause, urinary analysis demonstrated that BMD was found to be significantly higher ($p < 0.001$) in women with high (> 65 mg/day) than low (< 35 mg/day) isoflavone intakes. While it is possible there are additional components in these soy products that prevent bone loss or stimulate bone formation (such as calcium or vitamin K_2) the results of this study suggest relatively high level consumption of soy products is associated with increased BMD in postmenopausal women.

12.20 The relationship between dietary phytoestrogen intake and BMD was investigated in a cross-sectional study by Mei *et al* (2001) in southern Chinese women, aged 19-86 years based in Hong Kong (n=357 postmenopausal; n=298 premenopausal). Dietary phytoestrogen intake was estimated using a FFQ and isoflavone, coumestrol, lignan and flavonoid intakes were calculated using published databases.

12.21 The results were adjusted to allow for the following confounding factors: age, height, weight, and years since menopause, smoking, alcohol consumption, HRT usage and daily calcium intake. Data indicated that postmenopausal women with a relatively high intake (~47.4 mg/day) of isoflavone had significantly higher BMD values at the lumbar spine and the neck of the thighbone (p=0.05) than those with a low intake (~18.6 mg/day). No significant association between dietary phytoestrogen intakes and BMD values were found in premenopausal women. There was also no association between lignan, coumestrol or flavonoid intake and BMD in either pre- or postmenopausal women (Mei *et al*, 2001).

12.22 In this study significant reduction in markers of bone formation and resorption, including osteocalcin and urinary N-telopeptide were evident in postmenopausal women with the relatively high isoflavone intakes suggesting that the higher BMD is due to reduced bone turnover. Calcium intakes were significantly greater in postmenopausal women, especially in the mid (21.9 mg/day) to high (47.4 mg/day) isoflavone groups. Isoflavones may enhance the utilisation of calcium in bone formation resulting in increased BMD in these women. Despite limitations in using FFQs and confounding factors including higher calcium intake by postmenopausal women, overall the study suggests relatively high habitual intake of isoflavones is associated with higher BMD at the lumbar spine and hip region in postmenopausal women (Mei *et al,* 2001).

12.23 A cross-sectional study of 208 postmenopausal American women (aged 45-74 years) showed that dietary isoflavone consumption may be protective against bone loss (Kritz-Silverstein & Goodman-Gruen, 2002). Participants were recruited from various ethnic backgrounds including White, Hispanic, Asian, American-Indian and Black. A FFQ on the usual consumption of foods in the past year was completed. This included soy-based foods (e.g. tofu and soya milk) as well as consumption of other isoflavone-containing foodstuffs such as beansprouts and soy sauce. Measurements of hip and spine BMD were taken along with biomarkers of bone resorption (urinary N-telopeptide) and formation (serum bone alkaline phosphatase).

12.24 Consumption of genistein was found to range from 0-13.9 mg/day (mean of 1.3 mg/day) with Asian women most likely to report consumption >1mg genistein/day. A small but non-statistically significant association (p=0.07) was found between total isoflavone consumption and total spine BMD. After adjustment for age and obesity, women with the highest daily intake of dietary genistein were found to have 18% lower urinary N-telopeptide concentrations (p=0.01) as compared with women who reported no daily genistein consumption. Multiple regression analyses also showed significant associations between urinary N-telopeptide and genistein (p=0.08), daidzein (p=0.09) and total isoflavone intake (p=0.09). No other statistically significant differences were found between individual or total isoflavones and markers of bone formation and BMD. The authors of this study suggest normal, unsupplemented dietary isoflavone consumption may be protective against bone loss in postmenopausal women through a reduction in bone resorption (Kritz-Silverstein & Goodman-Gruen, 2002).

12.25 In a cross-sectional study of 87 postmenopausal Japanese women, the association between BMD, soy intake and serum isoflavonoid concentrations was investigated by Nagata *et al* (2002). Intake of soy-based products and isoflavones was assessed over a year using a semi-quantitative questionnaire. These were followed up with measurement of serum genistein, daidzein, serum bone ALP and BMD. Mean intake of soy products was estimated to be 62 g/day with intake of soy-derived isoflavones of 32 mg/day. However, there were no significant correlations between soy product and isoflavone intake with any of the bone health parameters measured after controlling for covariates including age, intake of vegetable fat and carbohydrate.

12.26 In a similar cross-sectional study of 85 postmenopausal Japanese women, soy protein intake was significantly associated with BMD in the lumbar spine (Horiuchi *et al*, 2000). In this study, 60% of participants were osteopenic or osteoporotic and dietary intakes were assessed over a period of three days. Intakes of soy protein (12.6 g/day) were positively associated with lumbar BMD (p=0.038) and with urinary deoxypyridinoline crosslinks (p=0.034). However, no association was found with serum alkaline phosphatase or intact osteocalcin.

Intervention studies

Soy or isoflavones

12.27 A number of intervention trials have investigated whether dietary supplementation with soy or isoflavones has a beneficial effect on BMD and BMC. However, intervention studies have not addressed the possibility that exposure to phytoestrogens at an earlier life stage or over several life stages (as would have occurred in populations that have traditionally consumed soy) has conferred some protective change on factors of bone health.

12.28 A randomised, double-blind placebo-controlled intervention trial showed that bone loss from the lumbar spine of perimenopausal women (n=69) was reduced following daily dietary intake, of soy protein isolate containing 80.4 mg isoflavones, for 6 months (Alekel *et al*, 2000). Regression analyses showed that isoflavones were positively associated with the change in BMD (5.6%, p=0.023) and BMC (10.1%, p=0.0032). No significant changes were observed in women receiving soy protein isolate containing 4.4 mg isoflavones.

12.29 A randomised, double-blind placebo-controlled intervention trial (n=66) by Potter *et al* (1998) demonstrated that isoflavone supplementation resulted in increases in both the BMC and BMD of the lumbar spine. In this study, hypercholesterolemic postmenopausal women were first placed on a low fat, low cholesterol diet for at least 2 weeks. Subjects were then randomly assigned to one of three treatment groups and received 0, 56 or 90 mg isoflavones/day, for 6 months. Significant changes ($p < 0.05$) in BMC and BMD were only noted in women receiving 90 mg isoflavones/day.

12.30 In a randomised, double-blind placebo-controlled intervention trial (n=52) the effects of a high and low phytoestrogen diet was examined in postmenopausal women. Participants were assigned to one of three dietary treatment groups: soy, linseed or wheat for a period of 12 weeks and received 45g/day supplementation in the form of bread. Mean total isoflavones ingested from soy-based bread was calculated to be approximately 53 mg/day. No significant changes in BMD were observed for any dietary treatment group. However, a significant increase of 5.2% (p=0.03) in BMC was detected at the end of the treatment period for participants on soy supplementation. No significant changes in BMC were observed for the linseed and wheat treated groups (Dalais *et al*, 1998).

12.31 In a 1 year double-blind placebo-controlled intervention trial of postmenopausal women, the effect of treatment with dietary genistein (n=30, 54 mg/day), HRT (n=30) or placebo (n=30) on BMD and biomarkers of bone formation and resorption were compared (Morabito *et al*, 2002). Treatment with genistein reduced the excretion of pyridinium cross-links (markers of bone resorption) at 6 months (approximately 55%, $p < 0.001$) and 12 months (approximately 44%, $p < 0.001$) similar to that observed for HRT. Treatment with genistein increased serum osteocalcin and bone specific ALP concentrations (markers of bone formation) at 6 months (approximately 19%, $p < 0.005$) and 12 months (approximately 32%, $p < 0.005$). However, HRT reduced the concentrations of these markers of bone formation. In addition, after 12 months of treatment with genistein or HRT increases in BMD were evident in the femoral neck and lumbar spine (both approximately 3%, $p < 0.001$) compared with control.

12.32 Other intervention trials have also investigated the effect of dietary supplementation on biomarkers of bone formation and resorption. In a single group intervention trial of 42 postmenopausal women conducted by Scheiber *et al* (2001), subjects consumed approximately 60 mg soy-derived isoflavones/day for 3 months in addition to their normal diet. Increases in serum osteocalcin (10.2%, $p < 0.025$), marker of bone formation and decreases in urinary N-telopeptide (13.9%, $p < 0.002$), marker of bone resorption, were observed. However, no significant correlation between serum levels of phytoestrogens and any bone markers (serum skeletal alkaline phosphatase, urinary cross-linked N-telopeptides of type I collagen) was reported. The absence of a placebo control group and the short duration of this study limits any conclusions that can be attributed to isoflavone intake.

12.33 Dietary soy (90 mg isoflavones/day) had no effect on BMD or BMC in a 1 year long double-blind placebo-controlled intervention trial of premenopausal women (placebo group n=13; treatment group, n=15) (Anderson *et al*, 2002).

12.34 Two randomised crossover intervention studies, one in premenopausal (n=14) and one in postmenopausal (n=17) women conducted by Wangen *et al* (2000) which do not support the hypothesis that dietary isoflavones *per se* exert beneficial effects on bone turnover in women. These studies consisted of three treatment periods where subjects consumed 8, 65 or 130 mg isoflavone/day for 3 months, separated by washout periods of approximately three weeks. Increased levels of plasma deoxypyridinoline crosslinks, indicating an increase in bone resorption, were observed in premenopausal women. Decreased levels of biomarkers for bone formation correlating with an increase in isoflavone consumption were also noted in postmenopausal women. However, these changes were small in magnitude and were not considered clinically significant.

Flaxseed

12.35 A double-blind, placebo-controlled intervention trial in postmenopausal women showed that flaxseed supplementation did not alter biomarkers of bone metabolism (Lucas *et al*, 2002). In this trial postmenopausal women (aged < 65years) consumed either 40 g/day ground flaxseed (n=20) or a wheat-based comparative control (n=16) for 3 months. All subjects also received a supplement containing calcium and Vitamin D, and a 7-day FFQ indicated that women in both groups had similar dietary intakes before and after the study. No changes in urinary deoxypyridinoline, helical peptide, IGF and IGFBD3 (markers of bone resorption) were noted.

Red clover extracts

12.36 A double-blind, placebo-controlled intervention trial showed that a red clover dietary supplement containing 40 mg isoflavones/day (genistein, daidzein, formononetin and biochanin A) significantly reduced ($p=0.025$) markers of bone resorption, as measured by urinary pyridinoline crosslinks, in pre- and perimenopausal women (n=97). This effect was not observed in postmenopausal women and no significant changes in any other markers, urinary or plasma were noted (Atkinson *et al*, 2003).

12.37 In a randomised, double-blind intervention trial of 46 postmenopausal women, a red clover extract, containing a combination of genistein, daidzein, formononetin and biochanin A and changes in proximal radius and ulna BMD were monitored and compared to the pre-treatment baseline levels. All subjects went through a single-blind placebo phase (1 month) before receiving either 28.5 mg, 57 mg or 85.5 mg total phytoestrogens daily for a 6 month period. This was then followed by a 1 month single-blind washout phase. Significant increases in BMD, although not dose-dependent, were reported at 57 mg/day (4.1%, p=0.002) and 85.5 mg/day (3.0%, p=0.023). No significant response was noted for the group receiving 28.5 mg/day. However, these results should be treated with caution, as a simultaneous control group was not included and all subjects received 1000 mg calcium supplement throughout the study (Clifton-Bligh *et al*, 2001).

Ipriflavone

12.38 A synthetic isoflavone, ipriflavone (7-isopropoxyisoflavone) has been used as a treatment for inhibiting bone resorption in postmenopausal women (Ohta *et al*, 1999; Hale *et al,* 2000, Scheiber & Rebar, 1999). However, the efficacy of ipriflavone has been disputed in a recent 4 year prospective, randomised, double-blind placebo-controlled study (n=240 controls, n=234 cases) which indicated that ipriflavone does not prevent bone loss or bone metabolism (Alexandersen *et al*, 2002).

Key points

- Studies in rodents have consistently shown that phytoestrogens have a beneficial role in preventing bone loss following ovariectomy and that soy isoflavones and other phytoestrogens are effective in conserving bone in rodent models of osteoporosis.

- Epidemiological studies suggest that intakes of phytoestrogens are associated with higher bone mineral density in populations consuming relatively large amounts of soy.

- Intervention studies in humans have been limited, relatively short-term and no studies have been performed in men. To date, the beneficial effects of phytoestrogens on bone mineral density and bone mineral content in postmenopausal women have been small but statistically significant and confined to the lumbar vertebrae with the exception of one study that reported effects in the femoral neck and lumbar vertebrae. In short-term studies it is more likely that an effect will be seen in the spine, rather than the hip as the turnover of bone in the hip is slower than that in the spine.

- Bone status in pre- and peripubertal life is a major determinant of osteoporosis in later life. Short-term intervention studies in adulthood have not addressed the possibility that exposure to phytoestrogens at an earlier life stage or over several life stages (as would have occurred in populations that have traditionally consumed soy) has conferred some protective change.

- There is a paucity of long-term human studies investigating phytoestrogen intake in relation to changes in bone mass or markers of bone loss. Large, long-term studies in humans would be required before the efficacy of phytoestrogen-containing foods or supplements can be confirmed.

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13. Phytoestrogens and the cardiovascular system

Introduction

13.1 This chapter reviews the current data on the possible effects of phytoestrogens on the cardiovascular system. Although many risk factors, such as high blood pressure and diabetes, are associated with cardiovascular disease (CVD), the underlying basis for clinical cardiovascular disease is a combination of atherosclerosis (excessive accumulation of lipids and smooth muscle cells in the artery) and thrombosis (development of fibrinous clots) (COMA, 1994).

13.2 Hormonal status is known to play a role in the development of cardiovascular disease. For example, in women, arterial pressure generally increases after menopause suggesting that ovarian hormones, mainly oestrogen, plays a cardioprotective role. However, a recent large prospective study found that one form of hormone replacement therapy (HRT) which contains oestrogen and progestin increases the risk of CVD (Writing Group for the Women's Health Initiative Investigators, 2002).

13.3 High levels of lipoprotein (a) (Lp(a)) are acknowledged as a primary predictor of heart disease. Lp(a) levels have been shown to be lowered by up to 35% by oestrogen and other sex steroids (Shewmon *et al*, 1994). Apolipoproteins, such as Apo B and Apo E, also play an important role in the development of cardiovascular disease. Apolipoproteins are the protein components of lipoproteins, which remain after the lipid part has been removed. They play a role in lipid transport and metabolism.

13.4 As well as indirect effects on lipid metabolism, oestrogens are also known to have cardioprotective effects directly on vasculature and arterial compliance (Sudhir *et al*, 1996; Honore *et al*, 1997; Nestle *et al*, 1997). Studies suggest that oestrogen induced cardiovascular protection might be mediated *via* increased synthesis of vascular nitric oxide, a free radical messenger which inhibits platelet aggregation, cell adhesion and relaxes the underlying smooth muscle cells (Darblade *et al*, 2002; Pendaries *et al*, 2002).

13.5 Mortality rates due to coronary heart disease (CHD) are generally higher in Western populations compared with Eastern (e.g. Chinese and Japanese) populations. In 1986, mortality rates in men and women aged 40-69 years were 300 and 100 per 100,000, respectively in America compared with only 50 and 15 per 100,000 respectively in Japan (Beaglehole, 1990).

13.6 Reduction of plasma cholesterol can reduce the risk of CHD. A reduction of 1%, on a population basis, can translate to a 2-3% reduction in risk of CHD (Bingham *et al,* 1998; COMA Annual Report, 1999). This can be achieved by decreasing the amount of saturated fat in the diet.

13.7 Knight & Eden (1996) observed that the incidence of cardiovascular disease was not only lower in Asian compared with Western countries, but also among vegetarians relative to omnivores. Many dietary factors are known to play a protective role in cardiovascular disease. However, authors suggested that phytoestrogens, present in higher concentrations in Asian and vegetarian diets, may also be cardioprotective.

13.8 Many studies have investigated the cardioprotective effects of dietary soy and isoflavones. However, these have not addressed the possibility that exposure to phytoestrogens at an earlier life stage or over several life stages (as would have occurred in populations that have traditionally consumed soy) has conferred some protective change in these populations. In addition, although there are many risk factors that contribute to the development of cardiovascular disease (blood pressure, thrombosis, atherosclerosis etc) most studies have investigated the effects of soy and isoflavones on plasma lipid profiles and plasma lipoprotein concentrations.

Phytoestrogen containing foods

13.9 Dietary inclusion of phytoestrogen rich foods, such as soy, linseed and flaxseed has been demonstrated to lower plasma cholesterol levels in humans. Decreases of up to 12.5% have been observed in plasma LDLC levels after three weeks dietary supplementation with soy and linseed foods, with maximal effects achieved in individuals classified as hypercholesterolemic (Ridges *et al*, 2001). Lower plasma lipid profiles seen in Hong Kong Chinese subjects have also been associated with the higher intake of soy and soy foods in this population (Ho *et al*, 2000). In a cross-sectional study of postmenopausal women (n=208) the intake of isoflavones calculated from a food frequency questionnaire, was positively associated ($p=0.05$) with HDL cholesterol (Goodman-Gruen & Kritz-Silverstein, 2001).

13.10 It has been proposed that phytoestrogens are responsible for the cardioprotective properties of soy, but to what extent is unclear (Setchell, 1985; Clarkson *et al*, 1995). Others have suggested that the protein composition of soy may be partially responsible (Erdman & Fordyce, 1989; Mackey *et al*, 2000). A meta-analysis of 38 published trials of soy consumption indicated that soy protein intake (47 g/day) was associated with reductions in total cholesterol (9.3%), LDLC (12.9%) and triglycerides (10.5%) ($p < 0.001$). However, no significant change in HDLC was observed (Anderson *et al*, 1995).

13.11 Components other than phytoestrogens may also be responsible for the hypocholesterolaemic effects of these foods. For example, an increase in dietary fibre will increase faecal excretion of cholesterol and bile acids. A population based, case-control study of acute coronary events in middle aged Finnish men reported a significantly lower mean serum enterolactone concentration in cases than in controls (18.2 and 23.5 nmol/L, respectively). Men in the highest enterolactone quartile had a 65% lower risk than those in the lowest quartile (Vanharanta *et al*, 1999). The authors noted that a high serum enterolactone concentration might not be associated with CHD *per se*, but may be a biomarker and reflect a protective diet high in fruit, vegetables, cereals and fibre.

13.12 Phytosterols in soy are structurally similar to cholesterol and although they are poorly absorbed from the intestine, human studies have shown that they can lower levels of serum or plasma total cholesterol and LDLC (Ling & Jones, 1995).

13.13 It has also been suggested that the protective effect of linseed against coronary heart disease might be mediated, at least in part, by its α-linolenic acid and not its lignan content (Allman *et al*, 1995; Harris, 1997; Pang *et al*, 1998; Tarpila *et al,* 2002).

Lipid Profiles

Human studies

Soy supplementation

13.14 In a cross-sectional cohort study of postmenopausal American women (n=939), dietary intake of phytoestrogens was associated with an improvement in cardiovascular risk profile. Women aged 59 (7.5 years were subdivided according to phytoestrogen intake as assessed by food frequency questionnaires. The median total daily intake of isoflavones was 0.16 mg/day, with high and low consumers ingesting > 0.24 and < 0.1 mg/day, respectively. The median total daily intake of lignans was 0.58 mg/day with high and low consumers ingesting > 0.79 and < 0.41 mg/day, respectively. No significant differences were reported in total cholesterol, LDLC or HDLC between high and low dietary phytoestrogen (isoflavones and lignan) intake groups. However, significant improvements in plasma triglycerides ($p < 0.05$) were seen between the high and low phytoestrogen intake groups (de Kleijn *et al*, 2002).

13.15 In a cohort study by Somekawa *et al* (2001), lifetime consumption of dietary isoflavones were not associated with a significant change in the lipid profiles of pre- and postmenopausal East Japanese women aged 44-80. The women were assigned to two groups according to years since menopause (n=269 early postmenopausal, < 5 years since menopause; n=209 late postmenopausal, >5 years since menopause). Each group was also subdivided according to isoflavone intake and the subjects reported weekly, monthly and yearly consumption of soy products. Although the results indicated isoflavone intake increased slightly with age it was not significant (53.3 mg isoflavone/day for early and 55.5 mg/day late menopausal groups). No significant differences were observed in total serum cholesterol, LDLC, HDLC, triglycerides, Lp(a) and apolipoproteins B (apo B) and apo E between pre- and postmenopausal groups.

13.16 A randomised, placebo-controlled intervention study in healthy asymptomatic postmenopausal Italian women (aged 36-60) investigated the effects of a soy rich diet on serum lipids and other biomarkers of cardiovascular health. Subjects in the diet intervention group (n=34) consumed approximately 47 mg isoflavones/day mainly from soy milk. Although an improvement in total cholesterol:HDLC ratio was seen in the intervention group, no statistically significant differences in serum total HDLC, LDLC or triglycerides were observed. However, a high drop out rate in and low compliance to the intervention diet was reported (Chiechi *et al*, 2002).

13.17 In a single-group intervention trial by Scheiber *et al* (2001) no significant changes from baseline in total cholesterol, LDLC, VLDLC, HDLC peroxidation or triglycerides were reported, in postmenopausal women (n=42) following consumption of approximately 60 mg soy derived isoflavones/day. In this single open-group clinical intervention, women consumed three daily servings of whole soy foods for 3 months. An increase was noted in the mean HDLC levels (3.7%) ($p < 0.05$) and a decrease (5.5%) ($p<0.05$) in total cholesterol:HDLC ratios. However these effects cannot be attributed to isoflavone intake as the study lacked a placebo control group.

13.18 A randomised, double-blind cross-over study (n=21) by Sirtori *et al* (1999) showed a decrease (approximately 7%, $p < 0.05$) in total cholesterol and LDLC associated with soymilk consumption in patients with severe hypercholesterolaemia (mean 8.74 mmol cholesterol/L) when compared to patients consuming and cows' milk. In a randomised double-blind crossover trial, men (n=34) were fed soy protein isolate (69 mg isoflavones/day) or alcohol extracted soy protein isolate (3.4 mg isoflavones/day) for 6 weeks (Urban *et al*, 2001). Mean plasma cholesterol was reduced (approximately 3%, p=0.036) from baseline after ingestion of intact soy protein isolate. However, no differences in serum lipid concentrations was seen in a randomised cross-over study of hyperlipidemic individuals (n=25) fed soy (168 mg isoflavones/day) compared to controls (Jenkins *et al*, 2000). In a small placebo-controlled intervention study of men (n=10) consuming soymilk (1 L/day) for 4 weeks, no significant effects on plasma cholesterol or triglycerides were evident (Mitchell & Collins, 1999).

13.19 A randomised, double-blind placebo-controlled intervention trial in normotensive men and postmenopausal women (n=213) receiving 40 g soy protein/day for 3 months was reported by Teede *et al* (2001). Individuals received the equivalent of 118 mg isoflavones/day from the isolated soy protein. Reductions in total cholesterol, LDLC, HDLC, the ratio of LDLC:HDLC and triglycerides were observed. However, overall there was no improvement in vascular function. Potential adverse effects noted were a decline in endothelial function (males only) and an increase in Lp(a) in both males and females.

13.20 The effect of dietary soy protein on Lp(a) in normolipidemic subjects was investigated in a randomised, placebo-controlled, crossover trial by Meinertz *et al* (2002). Median plasma Lp(a) concentrations were reduced up to 60% in subjects (6 men and 6 women) following intake of extracted soy protein ($p < 0.01$) and casein diets ($p < 0.001$), whereas an intact soy protein diet produced insignificant changes. Consumption of the extracted soy protein diet also lowered triglyceride levels ($p < 0.01$). However, overall, none of the dietary treatments resulted in significant changes in plasma total cholesterol or LDLC levels. The authors suggest that the changes noted were due to unusually high intakes of soy and casein protein (mean 96 and 170 g/day for men and women, respectively) which exceeded what is normally consumed in solid-food diets.

13.21 A randomised, double-blind, placebo-controlled intervention trial showed soy protein consumption led to significant decreases in non-HDLC (Baum *et al*, 1998; Potter *et al*, 1998). Hypercholesterolaemic, postmenopausal women (n=66), maintained on a low-fat-low-cholesterol diet, received 40 g protein/day from either non-fat dry milk, isolated soy protein containing 1.39 mg isoflavones/g or isolated soy protein containing 2.25 mg isoflavones/g, for 6 months. Significant increases in HDLC ($p < 0.05$) and mononuclear cell LDL receptor mRNA ($p < 0.05$) were seen in both isolated soy protein groups ($p < 0.05$), but not in the non-fat dry milk group.

13.22 In a randomised, double-blind placebo-controlled, intervention trial of male and female hypercholesterolemic patients (n=156), Crouse *et al* (1999) reported a dose-related reduction of plasma total cholesterol (4%, p=0.04) and LDLC (6%, p=0.01) following soy supplementation. Subjects consumed 25 g of soy protein isolate treated to provide either 3, 27, 37 or 62 mg isoflavones/day for 9 weeks. Reductions in total cholesterol and LDLC were significant only at the top dose (62 mg isoflavones) relative to casein controls. No changes in triglyceride concentrations or HDLC were noted.

13.23 A randomised, double-blind, placebo-controlled intervention trial in Finnish adults demonstrated significant decreases in plasma total cholesterol (8.3%, p=0.049) and LDLC (13.2%, p=0.014) concentrations compared with a placebo group following treatment with isolated soy protein. A total of 60 hypercholesterolaemic men (aged 45-70years) and women (postmenopausal aged 45-70years) were recruited in this study. The treatment group (n=30) received a liquid supplement of 52 g isolated soy protein (192 mg isoflavones/day for 6 weeks). However, small but significant decreases in total cholesterol (5.1%) and LDLC (8%) concentrations were also noted in the placebo groups. The onset of concentration change in cholesterol levels was more rapid in the treatment compared with the placebo group and all subjects returned to pre-study levels during the 4 week post-treatment wash-out period (Puska *et al*, 2002).

13.24 In an intervention trial in hypercholesterolemic male subjects (n=20), a significant improvement in plasma lipid profiles was seen following a soy protein rich diet compared with baseline measurements. Subjects had 60% animal source proteins replaced by soy protein for 6 weeks. Total plasma cholesterol ($p < 0.001$), LDLC ($p < 0.001$), apo B ($p < 0.039$), and triglyceride ($p < 0.001$), levels were decreased (Yildirir *et al*, 2001).

13.25 In a randomised cross-over trial, normocholesterolemic premenopausal women (n=13) showed reductions in LDLC (up to 10%, $p < 0.05$) when compared to pre-treatment baseline levels. Subjects were maintained on soy diets (approximately 130 mg isoflavone/day) for a total of 3 menstrual cycles. Although isoflavone consumption did not significantly affect total cholesterol or HDLC levels, a lowering in the ratios of total cholesterol:HDLC (10.2%, $p < 0.005$) and LDLC:HDLC (13.8%, $p < 0.002$) was also reported (Merz-Demlow *et al*, 2000).

13.26 In a randomised, double-blind placebo-controlled, cross-over trial significant declines in total cholesterol (6% lower) and LDLC (7% lower) were also observed in non-hypercholesterolaemic, non-hypertensive, perimenopausal women (n=51) when fed a soy diet compared with an isocaloric carbohydrate placebo diet. The subjects consumed 20 g of soy protein (containing 34 mg total phytoestrogens), as either a single daily dose or split into two daily doses, for 6 weeks. No significant effects on triglycerides or HDLC were observed (Washburn *et al*, 1999).

13.27 In a randomised, double-blind placebo-controlled, intervention trial, no improvement in plasma lipids or lipoproteins that could be attributed to dietary soy protein was noted in perimenopausal women (n=21). Normocholesterolemic and mildly hypercholesterolemic women received 40g soy protein /day for 24 weeks in the form of a muffin containing either 80.4 mg isoflavone/day (n=24), 4.4mg isoflavone/day (n=24) or whey protein control (Dent *et al,* 2001).

13.28 In a randomised double-blind cross-over trial, hypercholersterolaemic postmenopausal women were fed soy milk (80 mg isoflavones/day, n=31), alcohol extracted soy (3 mg isoflavones/day, n=33) or cows milk (2 mg isoflavones/day, n=30) for 12 weeks (Gardener *et al*, 2001). Reductions (not statistically significant) in LDL-cholesterol were reported in both soy fed groups compared to the group fed cows milk. There were no changes in blood HDL-cholesterol or triglyceride concentrations between treatment groups.

13.29 In a randomised cross-over trial of post menopausal women (n=18), changes in plasma LDL-cholesterol (6.5% reduction, p=0.02) were seen following consumption of an isolated soy protein drink supplemented with 7.1, 65, and 132 mg isoflavones. The soy drink containing 63 g of protein, was consumed daily for 13 weeks and each treatment was separated by a 26-day washout period. There were no significant changes in any of the other plasma lipids or lipoprotein concentrations plasma total cholesterol, HDL-cholesterol, triglycerides, Apo B or Lp(a) (Wangen *et al*, 2001).

13.30 This is in agreement with a randomised, placebo controlled intervention trial in healthy men (n=20) which showed no changes in plasma total cholesterol or HDLC following consumption of a soy protein isolate beverage powder (60 g/day for 28 days). The authors suggest this was because subjects were normocholesterolemic at start of the study (Gooderham *et al*, 1996).

13.31 It has also been suggested that a component of soy protein may affect the regulation of peripheral LDL receptors. This has been demonstrated by the effect of dietary soy protein in type II hypocholesterolaemic patients with differing apo E genotypes. In apo E3 and E4 genotype patients, peripheral LDL receptors are fully functional but receptor activity is suppressed. When these patients supplemented their diet with soy protein, plasma cholesterol levels were significantly lowered, possibly due to soy protein activation of LDL receptors. Patients with apo E2 genotype, characterised by defective binding to LDL receptors, were found to respond poorly to dietary soy supplementation and no significant effect on plasma cholesterol levels was observed (Gaddi *et al*, 1991; Sirtori and Lovati, 2001).

13.32 In contrast, there was no significant correlation between apo E3 and E4 genotypes and the lipid lowering effects of isolated soy protein in a study by Vigna *et al* (2000). However, there was a significant reduction in apo B levels in the two apo E2 genotype subjects treated with isolated soy protein when compared to apo E2 genotype placebos (p=0.05). In this double-blind placebo-controlled trial, 104 postmenopausal Italian women (aged 53.3 (3.3.years), consumed 60g isolated soy protein/day for 12 weeks. Significant reductions in total cholesterol and LDLC concentrations were seen in both placebo and treatment groups, but only the treatment group demonstrated a significant reduction in apo B (6% lower) and LDLC:HDLC ratios (8% lower). Changes in LDLC and apo B were particularly prominent in hypercholesterolemic subjects. Lp (a) plasma levels were not significantly changed by either treatment.

Table 13.1: Effect of phytoestrogen supplementation on risk factors for cardiovascular or coronary heart disease and atherosclerosis in humans.

Diet	Isoflavone Dose[a]	Duration	Study design	Effect							Reference
Intervention	mg/day			Total C	LDLC	HDLC	TriGly	Lp(a)	ApoB	BP	
Soy foods											
Normal soy rich diet	54 (mean)	–	Cohort, FFQ, normal postM women (n=478)	nc	nc	nc	nc	–	nc	–	Somekawa *et al* (2001)
Soy and Linseed Containing foods	10–31.5	8 weeks	Pilot study, mildly hypercholesterolemic women (n=20)	⇩	⇩	nc	nc	–	–	–	Ridges *et al* (2001)
Whole soy foods	60	12 weeks	Single open-group prospective normal postM women (n=42)	nc	nc	⇧	nc	–	–	–	Scheiber *et al* (2001)
Soy milk	NS	4 weeks	PC intervention, Healthy male volunteers (n=10)	nc	–	–	nc	–	–	–	Mitchell & Collins (1999)
High protein soya drink	NS	4 weeks	R, DB, CO hypercholesterolemic (n=21)	⇩	⇩	–	–	–	–	–	Sirtori *et al* (1999)
Soy protein (36 g/day)	168	3 weeks	R, DB, hyperlipidaemic men and women (n=25)	nc	nc	–	nc	–	–	–	Jenkins *et al* (2000)
Soy protein isolate or alcohol extracted soy protein isolate (20 g soy protein)	69	6 weeks	R, DB, CO men (n=34)	⇩	–	–	–	–	–	–	Urban *et al* (2001)
Soy protein isolate drink (60 g soy protein)	NS	4 weeks	R, PC healthy men (n=20)	nc	nc	nc	nc	–	–	–	Gooderham *et al* (1996)
Soy protein drink (52 g soy protein)	96	6 weeks	R, DB, PC hypercholesterolemic men (n=60) & post M women (n=30)	⇩	⇩	nc	nc	nc	nc	–	Puska *et al* (2002)
Soy protein (20 g/day)	NS	6 weeks	Intervention, hypercholesterolemic men (n=20)	⇩	⇩	nc	⇩	–	⇩	–	Yildirir *et al* (2001)

Table 13.1: Effect of phytoestrogen supplementation on risk factors for cardiovascular or coronary heart disease and atherosclerosis in humans. *(continued)*

Diet	Isoflavone Dose[a]	Duration	Study design	Effect							Reference
Intervention	mg/day			Total C	LDLC	HDLC	TriGly	Lp(a)	ApoB	BP	
Soy protein 20g/day	34	6 weeks	R, DB, CO healthy periM women (n=51)	⇩	⇩	nc	nc	–	–	⇩	Washburn *et al* (1999)
Soy milk (42 g/day)	80	12 weeks	R, DB, PC hypercholesterolemic post M women (n=31)	nc	nc	nc	nc	–	–	–	Gardener *et al* (2001)
Soy protein drink (96 or 170g protein/day)	406 (men) 229 (women)	3 x 4weeks	R, CO, PC healthy men (n=6) and pre M women (n=6)	nc	nc	nc	⇩	⇩	–	–	Meinertz *et al* (2002)
Soy protein muffin (containing 40g soy protein)	80	24 weeks	R, DB, PC normo & hyper cholesterolemic peri M women (n=69)	nc	nc	nc	nc	nc	nc	nc	Dent *et al* (2001)
Soy protein (60g/day)	76	12 weeks	R, DB, PC healthy postM women (n=104)	⇩	⇩	⇩	⇩	nc	⇩	⇩	Vigna *et al* (2000)
Soy protein (40g/day)	118	12 weeks	R, DB, PC in normotensive men and postM women (n=213)	⇩	⇩	⇩	⇩	⇧	NS	⇩	Teede *et al* (2001)
Soy protein (25 g/day)	62	12 weeks	R, DB, PC mildly hyper-cholesterolaemic men and women (n=156)	⇩	⇩	nc	nc	–	–	–	Crouse *et al* (1999)
Soy (75-100g/day)	10-129	12 weeks	R, CO, PC, healthy preM women (n=13)	nc	⇩	nc	nc	nc	nc	–	Merz-Demlow *et al* (2000)
Isolated soy protein drink 63g/day	7-132	3 x 13 weeks	R, CO, healthy postM women (n=18)	nc	⇩	nc	nc	nc	nc	–	Wangen *et al* (2001)
Soy protein (40g/day)	56-90	24 weeks	R, DB, PC hypercholesterolemic postM women (n=66)	nc	⇩	⇧	nc	–	nc	–	Baum *et al* (1998) Potter *et al* (1998)

Table 13.1: Effect of phytoestrogen supplementation on risk factors for cardiovascular or coronary heart disease and atherosclerosis in humans. *(continued)*

Diet	Isoflavone Dose[a]	Duration	Study design	Effect							Reference
Intervention	mg/day			Total C	LDLC	HDLC	TriGly	Lp(a)	ApoB	BP	
Supplements											
Isoflavone tablet	62	4 weeks	R, DB, PC, healthy postM women (n=23)	nc	nc	nc	nc	–	–	–	Uesugi *et al* (2002)
Isoflavone tablet (subterranean clover)	55	8 weeks	R, DB, PC, normo-cholesterolaemic (n=46 men & 13 postM women)	nc	nc	nc	nc	nc	–	–	Hodgson *et al* (1998)
Isoflavone tablet (red clover)	40-80	2 x 5 weeks	PC, CO, postM and periM women (n=17)	nc	nc	nc	nc	–	–	nc	Nestel *et al* (1999)
Isoflavones tablet	80	5-10 weeks	PC, CO, postM and periM women (n=21)	nc	nc	nc	nc	–	–	nc	Nestel *et al* (1997)
Isoflavone tablet Isoflavone tablet	80	2 x 8 weeks	R, DB, PC, CO healthy postM women (n=20)	nc	nc	nc	nc	nc	nc	nc	Simons *et al* (2000)
(red clover)	40-80	12 weeks	R, DB, PC, mild & moderate hypercholesterolaemic postM women (n=93)	nc	nc	nc	nc	nc	nc	nc	Howes *et al* (2000)
Isoflavone tablet	100	16 weeks	R, DB, PC, periM women (n=80)	⇩	⇩	nc	nc	–	–	–	Han *et al* (2002)
Isoflavones tablet	150	24 weeks	R, DB, PC parallel, elderly, moderately hypercholesterolemic postM women (n=36)	nc	–	nc	nc	–	–	–	Dewell *et al* (2002)
Flaxseed											
Ground Flaxseed 1.3g/100g & flaxseed oil 5g/100g	NS	4 weeks	R, DB, CO healthy volunteers (n=8)	nc	nc	nc	nc	–	–	–	Tarpila *et al* (2002)
Lignans (defatted flaxseed)	NS	2 x 3 weeks	R, CO, PC hyperlipidemics (n=22men & 9 postM women)	⇩	⇩	nc	–	⇩	⇩	–	Jenkins *et al* (1999)

LDLC: low density lipoprotein cholesterol; Total C: Total cholesterol; Lp(a): Apolipoprotein A-I; BP: Blood pressure; NS: not stated; HDLC: high density lipoprotein cholesterol; TriGly: triglycerides; ApoB: Apolipoprotein B; R: randomised; nc: No change; DB: double-blind; PC: placebo-controlled; CO: crossover; FFQ: food frequency questionnaire.

Isoflavone supplementation

13.33 Efforts have been made to determine if the isoflavones in soy are hypocholesterolemic agents. Anderson *et al* (1995) suggested that isoflavones may contribute up to 70% of the hypolipidemic effect of soy. This was based on experiments conducted in rhesus monkeys fed soy isolates with and without isoflavones (Anthony *et al*, 1996). However, clinical findings with regard to isoflavones, have been inconsistent and inconclusive (see Table 13.1).

13.34 A randomised double-blind, placebo controlled study by Han *et al* (2002) demonstrated a significant decrease in total cholesterol and LDLC in menopausal women (n=80) receiving 100 mg isoflavones/day for a period of 16 weeks compared with baseline ($p < 0.001$) and the placebo group ($p < 0.01$). However, no changes in HDLC and triglyceride levels were observed.

13.35 A randomised, double-blind placebo-controlled, intervention study in 46 healthy middle-aged men and 13 postmenopausal women (not on HRT) was carried out by Hodgson *et al* (1998). Diet was supplemented daily with tablets containing 55 mg of isoflavones (mainly as genistein). Isoflavone supplements were taken over an 8 week period, but no post-intervention differences in either serum lipid or Lp(a) levels were observed.

13.36 A randomised, double-blind placebo-controlled, intervention study in 20 healthy postmenopausal women (50-70 years) showed no improvement in any plasma lipid profiles or lipoprotein concentrations. Subjects received a supplement containing 80 mg isoflavone/day for 8 weeks (Simons *et al*, 2000).

13.37 Furthermore, data from a relatively short-term (5 or 10 week) placebo-controlled cross-over study found no change in plasma lipids in 21 menopausal and perimenopausal women supplemented daily with 80 mg isoflavones, containing 45 mg genistein (Nestel *et al,* 1999; 1997).

13.38 Isoflavone supplementation for 24 weeks also had no effect on serum lipid profiles in elderly (mean age 69), moderately hypercholesterolemic postmenopausal women (Dewell *et al,* 2002). In this randomised, double-blind placebo-controlled trial, elderly postmenopausal women, not on HRT, received either a 150 mg isoflavone supplement/day (n=20) or a placebo (n=16). No changes in total triacylglycerol, total cholesterol, or HDLC were observed after 8 weeks treatment. Total triacylglycerol and cholesterol remained unchanged at 24 weeks.

13.39 In a randomised, double-blind placebo-controlled trial, 23 healthy postmenopausal Japanese women receiving isoflavone supplementation (62 mg isoflavone/day, mainly as daidzin) for 4 weeks also showed no significant changes in blood serum lipids. Total serum cholesterol and LDLC decreased significantly ($p < 0.05$) from baseline levels in the treatment groups, but this decrease was not significant when compared to the placebo group (Uesugi *et al*, 2002).

13.40 A randomised, double-blind placebo-controlled trial, investigated the effects of a red clover isoflavone supplement on the lipid profiles in postmenopausal Australian women (n=93). Subjects (aged 58 ± 7.3 years) with mild to moderate hypercholesterolaemia consumed a low isoflavone diet for two weeks

before starting the 12 week continuous treatment period. Purified extract of red clover was consumed in the form of a tablet containing approximately 40 mg total isoflavones (26 mg biochanin A, 16 mg formononetin, 1 mg genistein, and 0.5 mg daidzein). Subjects were given ascending doses of isoflavones of 0, 40 mg (one tablet), and 80 mg (two tablets) total isoflavones/day, with each dosing period lasting three weeks. Urinary excretion of isoflavones was dose-dependent, but no significant changes in total plasma cholesterol, HDLC, LDLC or triglyceride levels were noted (Howes *et al* 2000).

Lignans

13.41 Few studies have been conducted to determine if lignans found in flaxseed or cereals are hypocholesterolemic agents. In a randomised, cross-over placebo-controlled trial, reductions in total plasma cholesterol, LDLC, apo B and Lp(a), but no changes in lipoprotein ratios, were observed in 29 hyperlipidemic patients fed a flaxseed-fibre supplemented diet (Jenkins *et al,* 1999). In this trial, 22 men and 7 postmenopausal women, consumed 20 g fibre/day from flaxseed (approximately 50 g partially defatted flaxseed/day) for three weeks and results were compared with a control group fed a wheat-bran diet.

Animal studies

13.42 Studies in ovariectomised cynomolgus monkeys have demonstrated that addition of soy protein to oestrogen replacement therapy improves clinical indicators related to cardiovascular disease, such as arterial dilatation and plasma lipid and lipoprotein concentrations (Wagner *et al*, 1997; Williams *et al*, 2001). However, the active component of soy protein has yet to be identified, as results from animal studies have yielded conflicting results.

13.43 Anthony *et al* (1997) showed that a diet containing intact soy (n=27) was the most effective, and a diet containing casein (n=27), the least effective in improving LDLC and HDLC profiles, in young male cynomolgus monkeys fed an atherogenic diet. Diets containing alcohol-extracted soy (i.e. isoflavones removed) had an intermediate effect, but did not differ significantly from the casein group. It was not possible to ascertain from the study whether any beneficial effects of the diet containing alcohol-extracted soy were due to the soy protein itself or residual traces of isoflavones. In addition, Nogowski *et al* (1998) reported that dietary supplementation with genistein (up to 0.1%), resulted in significant reductions of serum and muscle triglycerides, increases in free fatty acids and diminished plasma and increased hepatic cholesterol in ovariectomised rats.

13.44 In a study by Clarkson *et al* (2001), ovariectomised cynomolgus monkeys (n=189) were fed an isocaloric atherogenic diet for 26 months followed by a further 36 month treatment period. The control group (n=57) received alcohol washed soy protein. The treatment groups received intact soy protein (n=60) or conjugated equine oestrogens (n=62). Both the intact soy and equine-oestrogen treatment groups showed significant reductions in LDLC and VLDLC compared with control groups. There were no significant differences among the groups for Lp(a) concentrations. Generally animals treated with intact soy showed significant improvements in plasma lipids compared with those fed alcohol washed soy.

13.45 In contrast, a study in ovariectomised cynomolgus monkeys demonstrated that although a diet consisting of intact soy protein improved the lipid and lipoprotein profile, a diet consisting of semi-purified extract of soy rich in isoflavones added to casein-lactalbumin protein did not (Greaves *et al*, 1999). Other authors have suggested that a decrease in intestinal cholesterol absorption mediated by soy protein may be responsible for the lipid lowering effects of soy (Nagata *et al,* 1982; Potter 1998). However, results from this study could not be explained by differences in absorption of isoflavones from the two diets.

13.46 A study in gerbils suggested that consumption of an isoflavone containing extract from soy did not contribute to the hypocholesterolemic effect of alcohol-extracted soy. However, isoflavone containing extract from soy may influence lipid metabolism by altering expression for lipid-related genes, illustrated by the down regulation of hepatic apolipoprotein A-I mRNA (Touvar-Palacio *et al*, 1998).

13.47 The effects of different sources of protein and a soyflour extract on serum cholesterol levels in rats (n=24) and hamsters (n=50) was investigated by Balmir *et al* (1996). The study found that serum total cholesterol and LDLC were decreased in both rats and hamsters fed 200 g soy-protein/kg bw compared with those fed a casein-protein. Addition of ethanol-acetone soyflour extract (360 mg extract/kg protein) to casein diets also resulted in lowering of LDLC concentration compared with casein alone, in both rats and hamsters.

13.48 This is in agreement with the study by Potter *et al* (1996), which demonstrated that serum total cholesterol was lowered in hamsters, following consumption of 250 g soy protein/kg protein concentrate or isolated soy protein when compared to casein protein fed hamsters. However, serum HDLC and LDLC, as well as HDL/LDL ratios were unaffected by dietary treatment.

13.49 Alexandersen *et al* (2001) reported soy diet (6.5 mg isoflavone/day) had significant cholesterol-lowering effects in ovariectomised rabbits. This anti-atherogenic effect was comparable to that produced by oestradiol supplementation (4 mg/day), which produced serum concentrations similar to those seen in postmenopausal women on HRT.

13.50 The hypocholesterolemic and anti-atherogenic properties of flaxseed have also been attributed to its lignan constituents (Prasad *et al*, 1998; 1999). The lignan, secoisolariciresinol diglucoside, was found to reduce hypercholesterolemic atherosclerosis in rabbits when added to a high-cholesterol diet. The reduction was associated with decreased serum total cholesterol, LDLC and an increase in HDLC.

Blood pressure

13.51 Few studies have investigated the effect of soy or phytoestrogens on blood pressure and arterial compliance. However, reductions in systolic, diastolic and mean blood pressure (BP) was evident ($p < 0.05$) in normotensive men and postmenopausal women following consumption of 40 g soy protein/day (118 mg isoflavones/day) for 12 weeks (Teede *et al,* 2001).

13.52 Consumption of 60 g isolated soy protein/day (containing 76 mg isoflavones) for 12 weeks decreased ($p=0.01$) systolic blood pressure in postmenopausal women (Vigna *et al*, 2000).

13.53 A significant decrease in diastolic blood pressure was observed in healthy peri-menopausal women consuming a twice-daily soy diet (20g soy protein/day containing 34 mg isoflavones) when compared with the placebo diet for 6 weeks (Washburn *et al,* 1999).

13.54 Yildirir *et al* (2001) demonstrated that endothelial function as measured by flow mediated dilatation of the brachial artery, was improved following substitution of 60% animal protein in diet with soy protein in hypercholesterolemic male subjects (n=20).

13.55 Rivas *et al* (2002) showed that ingestion of soy milk (143 mg isoflavones/day) reduced systolic blood pressure (by 17 mmHg, $p< 0.0001$) and diastolic blood pressure (by 12.2 mmHg), $p< 0.0001$) compared with cows milk in a 3 month double-blind randomised trial of men and women (n=40) with mild to moderate hypertension.

13.56 Clinical studies have been unable to attribute the anti-hypertensive effects of soy to phytoestrogen content. In a randomised double-blind, placebo controlled study by Han *et al* (2002), supplementation of the diet with 100 mg isoflavones/day for a period of 16 weeks had no effects on blood pressure in menopausal women (n=80). Healthy postmenopausal women, with evidence of endothelial dysfunction also showed no changes in blood pressure or improvement in flow-mediated endothelium dilatation after supplementation of 80 mg soy isoflavones/day for 8 weeks (Simons *et al*, 2000).

13.57 Nestel *et al* (1997; 1999) did show a significant improvement (up to 26%, $p< 0.001$) in systemic arterial compliance when compared with placebo controls in perimenopausal women consuming 80 mg isoflavones/day. However, these effects were noted in the absence of any affect on arterial pressure.

13.58 In a randomised double-blind placebo-controlled trial of postmenopausal women (n=60), supplementation of the diet with genistein (54 mg/day) improved flow-mediated endothelial vasodilation ($p< 0.01$) compared with placebo (Squadrito *et al*, 2002). Measurement of plasma nitric oxide and endothelin-1 concentrations suggested this could be a result of increased nitric oxide to endothelin ratios.

13.59 In animal studies, consumption of a soy based diet for 5 weeks attenuated the development of hypertension when compared to casein based diets in both male and female spontaneously hypertensive rats (Nevala *et al,* 2000).

13.60 The mechanism of anti-hypertensive effects of soy is unclear although the production of vascular nitric oxide is thought to play a role. Karamsetty *et al* (2001) demonstrated that isoflavones act like oestrogen in mediating nitric oxide release in isolated pulmonary rat arteries. Enhanced arterial relaxation was seen in chronically hypoxic rats on treatment with genistein (30 μM) and daidzein (30 μM). No response was seen in normoxic rats.

13.61 In animal studies, blockade of autonomic function, removed the anti-hypertensive effects of a diet consisting of 19% whole soy meal (Martin *et al,* 2001). Although isoflavone intake was not measured, rats were reported to have plasma genistein concentrations of 0.9-1.37 μM. Dietary phytoestrogens were also found to have a beneficial role in NaCl-induced hypertensive ovariectomised rats indicating that the sympathetic nervous system also plays a role in the anti-hypertensive effects of dietary soy (Fang *et al*, 2001).

Thrombosis

13.62 Thrombosis is a risk factor of cardiovascular disease. Few studies have investigated the effects of phytoestrogens on the formation of fibrinous clots, platelet aggregation or the biochemical mechanisms involved in thrombi formation.

13.63 In a study by Dent *et al* (2001), no changes in coagulation and fibrinolytic factors such as fibrinogen, and factor VII antigen relating to soy protein intake were noted in perimenopausal women when compared to a control group fed whey protein. Normocholesterolemic and mildly hypercholesterolemic women received 40g soy protein/day for 24 weeks in the form of a muffin containing either 4.4 or 80.4 mg isoflavone/day.

13.64 No significant differences in platelet aggregation were seen in platelet rich plasma taken from men (n=20) receiving a soy protein isolate beverage (60 g soy protein isolate powder) for 4 weeks compared to casein controls (Gooderham *et al*, 1996).

13.65 Platelet activation is mediated by a number of protein-tyrosine kinase (PTK) pathways and isoflavones are known to be PTK inhibitors (see Chapter 7). A number of *in vitro* studies have demonstrated genistein can effect human and rat platelet function, by blocking PTK pathways (Kuruvilla *et al*, 1993; Helmeste & Tang, 1995; Schoene & Guidry, 1999). Hence, PTK inhibition and prevention of platelet aggregation may be a mechanism by which isoflavones exert their anti-atherogenic effects.

13.66 In addition, a study suggests that the effects of genistein on human platelet function may be due to thromboxane receptor antagonism rather than inhibition of tyrosine kinase (McNicol, 1993). *In vitro* studies have shown that genistein blocks thrombin-induced aggregation of human platelet in a dose dependent manner. Increases in intracellular calcium concentrations induced by thrombin were also inhibited by genistein (Asahi *et al*, 1992).

13.67 There are no studies to date on lignans and the development of thrombosis.

Atherosclerosis

13.68 Atherosclerosis may also be a factor in the development of cardiovascular disease. Atheromatous plaques are formed by the migration of smooth muscle cells into the artery lumen and deposits of circulating cholesterol.

13.69 In a study by Kirk *et al* (1998), soy supplemented diets (360 mg isoflavones/kg diet) reduced atherosclerosis in wildtype but not LDL receptor null mice compared with mice fed alcohol washed soy (30 mg isoflavones/kg diet) suggesting that isoflavones may act by a LDL receptor-mediated mechanism to protect against atherosclerosis. However, Adams *et al* (2002) used two genetically engineered mouse models of atherosclerosis to investigate the mechanism of soy protein isolate inhibition on atherosclerosis. In the first mouse model, which was devoid of LDL receptors and over produced apo B (LDL-/-), atherosclerosis was inhibited by both intact and alcohol washed (isoflavones removed) soy protein. In the second experimental model, mice had normal complement of LDL receptors but did not produce apo E (apo E-/-). Apo E is required to ensure efficient clearance of plasma lipoproteins. There was no association between soy consumption and plasma lipids and the anti-atherosclerotic effects seen in LDL-/- despite of an increase in plasma LDLC. Atherosclerosis was also reduced in the apo E -/- mice but to a lesser extent. These results suggest the anti-atherosclerotic effect of soy protein isolate does not require the presence of LDL receptors and is independent of plasma lipid levels.

13.70 Two primate studies suggest that dietary soy may lower atherosclerosis. Clarkson *et al* (2001) reported a non-significant lowering of the extent carotid and artery atherosclerosis in ovariectomised cynomolgus monkeys following a 36 month treatment with dietary soy compared with alcohol washed dietary soy. In a study by Anthony *et al* (1997), male cynomolgus monkeys with atherosclerosis were fed intact soy (1500 mg isoflavones/kg diet), alcohol washed soy (179 mg isoflavones/kg diet) or caesin supplemented diets. Atherosclerosis was significantly reduced in both groups of monkeys fed soy compared with caesin, however the effect was greater in those animals fed intact soy.

13.71 The anti-atherogenic properties of isoflavones without soy protein were investigated in cholesterol-fed rabbits by Yamakoshi *et al* (2000). An isoflavone-rich extract containing either 0, 3.3 or 10 g isoflavones/kg diet was fed to male rabbits for 8 weeks. A dose-dependent reduction in atherosclerotic lesions in the aorta was noted, in the absence of any change in plasma lipid concentrations. Immunohistochemical analysis revealed inhibition of LDL oxidation. The authors suggest the anti-oxidative activity of isoflavone metabolites may be the mechanism by which isoflavones exert their anti-atherogenic effect. A number of studies reviewed in Chapter 7 also show dietary soy can inhibit LDL oxidation.

13.72 The migration of smooth muscle requires PTK activity. Studies by Shimokado *et al* (1994; 1995) have demonstrated that genistein can inhibit PTK and can also inhibit the chemotaxis of rat aortic smooth muscle cells and cytoskeletal reorganisation *in vitro*. In an *in vitro* study, genistein inhibited proliferation and migration of rat smooth muscle cells (Makela *et al*, 1999). Genistein has also been demonstrated to inhibit platelet-derived growth factor (PDGF) activity. PDGF plays a role in the proliferation of smooth muscle cells and cytoskeletal reorganisation (Fujio *et al*, 1993).

13.73 A single human study has examined the effects of phytoestrogens on atherosclerosis (van der Schouw, 2002). This prospective study reported an association between aortic stiffness and increased isoflavone intake ($p=0.02$) and lignans ($p=0.03$) in women ($n=180$) that had been postmenopausal for 20-30 years. The association was less pronounced in women that had been postmenopausal for $<$ 20 years. The authors suggest that dietary phytoestrogens may have a protective effect on the risk of atherosclerosis.

U.S. Food and Drug Administration consideration of soy and CHD

13.74 The United States Food and Drug Administration (FDA) in 1999 initially refused a health claim application based on an association between dietary soy with a specified concentration of isoflavones and a reduction of heart disease risk. This was because the FDA concluded there was insufficient evidence to suggest a specific contribution of isoflavones to the cardioprotective effects of soy. However, the FDA did permit the application when the health claim was changed to associate soy protein with the reduction of heart disease. Food producers are now able to use the following health claim, or a reasonable variation, on their products in the US (FDA, 1999):

'Diets low in saturated fat and cholesterol that include 25 grams of soy protein a day may reduce the risk of heart disease. One serving of (name of food) provides (insert amount) grams of soy protein'[13]

13.75 To qualify to use this claim the foods must contain the following per serving:

- 6.25 g of soy protein
- low fat (less than 3 g)
- low saturated fat (less than 1 g)
- low cholesterol (less than 20 mg)
- Sodium value of less than 480 mg for individual foods
- Sodium value less than 720 mg if considered a main dish
- Sodium value less than 960 mg if considered a meal

Key Points

- There are many risk factors which contribute to the development of cardiovascular diseases (e.g. hypertension, thrombosis, atherosclerosis) reflecting a variety of mechanisms underlying the condition. Not all of these factors are directly affected by diet.
- The similarity of phytoestrogens to oestrogens, which have hypocholesterolaemic effects, and the lower cardiovascular disease mortality rates in populations consuming soy have prompted the suggestion that phytoestrogens are protective against cardiovascular disease.
- There is evidence from epidemiological studies and intervention trials that diets containing soy or soy protein isolates can have a hypocholesterolaemic effect in humans. There is some evidence that flaxseed (which is rich in lignans) also has a hypocholesterolaemic effect.

[13] In 2002, the UK Joint Health Claims Initiative recommended a similar health claim for soy (see website: www.jhci.co.uk).

- Intervention studies conducted in healthy and hypercholesterolemic subjects, with pure isoflavones have produced inconsistent results. Therefore it is not possible to attribute the hypocholesterolaemic effect of soy to its phytoestrogen content.

- The effects of phytoestrogens on other factors important in the risk of cardiovascular disease have not been extensively investigated. Studies measuring the effects of soy or purified isoflavones suggest that soy may reduce blood pressure but this effect cannot be attributed to the isoflavone content. Human studies have shown dietary soy has no effect on thrombosis. A single human study suggests that dietary phytoestrogens may have a beneficial effect on one parameter of atherosclerosis.

- Data obtained from animal studies suggest isoflavones either play no role or are only partly involved in the soy-associated lowering of plasma total cholesterol and/or low density:high density cholesterol ratio. Animal studies suggest that soy has a beneficial effect on atherosclerosis but it is not possible to attribute this effect to the isoflavone content.

- Short term intervention studies in adulthood have not addressed the possibility that exposure to phytoestrogens at an earlier life stage or over several life stages (as would have occurred in populations that have traditionally consumed soy) has conferred some protective change in these populations.

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14. Phytoestrogen modulation of endogenous hormones in humans

Introduction

14.1 There is much interest in the possible hormonal effects of phytoestrogens in both men and women. The majority of studies conducted in women have examined the ability of phytoestrogens to alleviate menopausal symptoms. While hormone replacement therapy (HRT) is recommended for women experiencing menopausal symptoms, there remains some concern that HRT increases the risk of breast cancer and cardiovascular disease (Writing Group for the Women's Health Initiative Investigators, 2002)[14]. As a result of these concerns, investigations into natural alternatives to HRT such as phytoestrogens have attracted considerable attention.

14.2 It has been observed that the rate of breast cancer is relatively lower in women from some populations in Asia e.g. Japanese and Chinese compared with Western populations leading to suggestions of a possible association with increased dietary intakes of phytoestrogens. Studies in premenopausal women have thus focused on the potential cancer protective effects of phytoestrogens. Markers of "protective" effects include increased menstrual cycle length and increased sex hormone binding globulin levels, which are thought to reduce the risk of breast cancer by decreasing an individual's lifetime exposure to endogenous oestrogens (see Chapter 15).

14.3 In men, increased phytoestrogen consumption has been associated with a reduced risk of prostate cancer (see Chapter 15). However, there has also been some speculation that phytoestrogens may adversely affect the fertility of men by reducing testosterone levels and sperm counts (see Chapter 9).

14.4 This chapter reviews the data from human trials. The data from studies in animals is reviewed in Chapter 9.

Hormonal effects in peri- and postmenopausal women

14.5 The menopause is associated with ovarian failure, an increased serum concentration of follicle stimulating hormone (FSH) and reduced levels of oestradiol. The perimenopausal transition, which occurs 1-5 years prior to the menopause, is characterised by irregularities in the menstrual cycle followed by shortening in cycle length and subsequent intermittent skipping of cycles. Throughout this time, there are fluctuations in oestrogen levels and women experience a number of different symptoms including hot flushes, depression, vaginal dryness and muscle/joint aches. The pathophysiology of the hot flush is uncertain but it appears that reduced oestrogen concentrations may be the primary trigger. In most Western countries, 80% of menopausal women experience hot flushes. In almost one-third of these women, hot flushes are frequent, severe and may be accompanied by sensations of tenseness, tiredness, irritability, headaches and muscle and/or joint pain. In contrast to other symptoms that are mainly experienced in the peri-menopausal period, vaginal dryness is also a problem for approximately 50% of women after the menopause occurs (Eden, 1998).

[14] Some forms of HRT contain a combination of oestrogens and progestin and thus the risk factors identified for these forms of HRT may not be the same as those when only oestrogen is administered.

14.6 Women in different populations report the symptoms of the menopause differently. Hot flushes and sweating seem to be the most commonly reported symptoms, however their prevalence is lower among women in South East Asian populations i.e. Hong Kong, Indonesia, Korea, Malaysia, the Philippines, Singapore and Taiwan when compared with their Western counterparts. In some countries, such as Malaysia, the predominant symptom reported is headache. It has been suggested that these differences may be due to cultural and dietary factors, particularly with regard to phytoestrogen intake (Eden, 1998).

14.7 A study demonstrated that dietary phytoestrogens produced mild oestrogenic effects in postmenopausal women (Wilcox *et al*, 1990). It showed that in comparison with baseline measurements, dietary supplementation with soy flour, linseed or red clover sprouts in postmenopausal women (n=25), each for 2 weeks, significantly improved vaginal cytology ($p < 0.01$). An increase in vaginal maturation index, a sensitive measure of oestrogenicity, was also observed following the soy flour ($p < 0.05$) and linseed ($p < 0.02$) diets but not the red clover sprouts. A cumulative reduction in FSH ($p < 0.05$), but not lutenising hormone (LH), was also noted during 6 weeks. However, the results of this study are difficult to interpret, as no control group was included. A randomised, placebo-controlled study by Baird *et al* (1995) assessing the oestrogenic effects of a high soy diet (165 mg total isoflavones/day) in postmenopausal women (n=97) reported a small non-significant increase in the percentage of vaginal superficial cells after 4 weeks. There was no effect on any of the other parameters measured (serum LH, FSH and sex hormone binding globulin (SHBG)).

14.8 More recently, other studies have also suggested that phytoestrogens produce weak hormonal effects in postmenopausal women. A randomised crossover study by Duncan *et al* (1999a) assessed the effects of a soy protein containing different levels of isoflavones on plasma hormone levels in postmenopausal women (n=18). The high isoflavone diet reduced the levels of oestrone sulfate ($p < 0.05$) and there was a trend towards reduced levels of oestrone and oestradiol with the high isoflavone diet. There was no effect on vaginal cytology or endometrial thickness. A randomised crossover trial by Xu *et al* (2000) investigated the effect of soy on the oxidative metabolism of oestradiol. The study demonstrated an increase in the ratio of 2-hydroxyoestrone:16α-hydroxyoestrone ($p < 0.05$) in postmenopausal women (n=18) receiving 7, 65 or 132 mg/day total isoflavones for approximately 93 days.

14.9 A cross-sectional study in postmenopausal (n=456, aged (55 years) and premenopausal (n=636 aged 20-44 years) women participating in the UK arm of the European Prospective Investigation into Cancer and Nutrition (EPIC) examined the effect of soymilk consumption on plasma sex hormone concentrations (Verkasalo, *et al*. 2001). Participants completed a FFQ to determine the frequency of soy consumption. Soymilk consumption was not significantly associated with any change in blood sex hormone concentrations in either post- or premenopausal women.

14.10 Nicholls *et al* (2002) investigated the effects of dietary phytoestrogens on gonadotrophin releasing hormone (GnRH) stimulated hormone levels in premenopausal (n=5) and postmenopausal women (n=7) fed 50 g textured soy protein (60 mg total isoflavones). In both pre- and postmenopausal women, short

term soy consumption did not effect serum hormone or gonadotrophin concentrations after GnRH administration. Increased LH secretion was observed in postmenopausal women after cessation of soy consumption.

Studies showing beneficial effects of phytoestrogens on the menopause (see Table 14.1)

14.11 Japanese women (n=1106) aged between 35-54 years (all were premenopausal at the start of the study) were studied in 6 year prospective study on the effect of soy intake on hot flushes (Nagata *et al*, 2001a). A food frequency questionnaire (FFQ) was completed at the start of the study period and women were questioned on the occurrence of hot flushes at the end of the study period. Women (n=101) reported moderate or severe hot flushes. The incidence of hot flushes was inversely associated with consumption of soy products in terms of total soy product intake (OR= 0.47, 95% CI 0.28-0.79, p=0.005) and isoflavone intake (mg/day) (OR= 0.42, 95% CI 0.25-0.72, p=0.002).

14.12 A group of Spanish postmenopausal women (n=190) were studied in a multicentre, prospective non-randomised trial of the effects of an isoflavones dietary supplement on menopausal symptoms (Albert *et al*, 2002). Each subject received 35 mg isoflavones/day over four months and menopausal symptoms including sleep disorder, anxiety, depression, vaginal dryness, loss of libido and bone pain were assessed. Incidence of hot flushes were decreased in 81% of participants ($p < 0.05$). All the other parameters studied also showed a statistically significant decrease. The study did not include a placebo group.

14.13 A study by Somekawa *et al* (2001) evaluated the effect of dietary isoflavones on menopausal symptoms of women in Japan. The women were assigned to two groups according to years since menopause (n=269 early postmenopausal, $<$ 5 years since menopause; n=209 late postmenopausal, $>$ 5 years since menopause). Each group was also subdivided according to isoflavone intake. Subjects reported weekly, monthly and yearly consumption of soy products. Menopausal symptom scores of palpitations and backache or aching joints were lower in early postmenopausal women with a higher isoflavone intake ($p < 0.05$). The severity of other menopausal symptoms tended to be less in the higher intake group although the differences were not significant. No differences in menopausal symptoms were reported between women in the late postmenopausal group.

14.14 Nagata *et al* (2001a) investigated the effect of soy products and isoflavone intake on the incidence of hot flushes in a cohort of Japanese women (n=1106, aged 35-54). Consumption of soy products and isoflavone intake was assessed by means of a semi-quantitative food frequency questionnaire. Following adjustment for confounding factors the study suggested that hot flushes were inversely associated with consumption of soy products both in terms of total soy product intake (p=0.005 for the highest tertile versus the lowest tertile) and isoflavone intake (p=0.002 for the highest tertile versus the lowest tertile).

14.15 A randomised, placebo controlled study by Brzezinski *et al* (1997) reported that following 12 weeks of intervention with a soy and flaxseed rich diet, both peri- and postmenopausal women (n=145) experienced a reduction in the severity of hot flushes (p=0.004) and vaginal dryness (p=0.005). Similarly, Albertazzi *et al* (1998, 1999) reported that following 12 weeks of supplementation with 60 g soy protein

(76 mg isoflavone aglucone/day), postmenopausal women (n=104) experienced a reduction ($p < 0.01$) in moderate to severe hot flushes. There was no difference in vaginal maturation index. In addition, a double-blind, cross-over study in perimenopausal women (n=51) found that women consuming a soy protein isolate (34 mg isoflavones) twice daily showed improvements in the severity of menopausal symptoms and hypoestrogenic symptoms compared with those receiving the supplement once daily (Washburn *et al*, 1999).

14.16 A double-blind randomised placebo-controlled trial in postmenopausal women (n=177) given a soy isoflavone supplement (50 mg total isoflavones/day) for 12 weeks found a reduction in hot flush severity with the soy supplement ($p=0.01$) compared to the placebo. There was no change in endometrial thickness nor vaginal maturation index or vaginal pH with either treatment (Upmalis *et al*, 2000). Similar results were obtained in a double-blind randomised placebo-controlled trial in postmenopausal women (n=39) receiving 400 mg/day soy extract (50 mg total isoflavones/day) by Scambia *et al* (2000). Women receiving soy had a reduction in the number of hot flushes ($p < 0.01$) and the severity of hot flushes and night sweats ($p < 0.001$). There were no oestrogenic changes in vaginal cytology, endometrial thickness or uterine artery pulsatility index.

14.17 In a double-blind placebo controlled study, Brazilian postmenopausal women (aged 45-55 years) were randomly assigned to daily treatments of an isoflavone supplement (33.3 mg isoflavones, n=40) or placebo (n=40) (Han *et al*, 2002). Menopausal symptoms were examined at baseline and after 4 months of treatment. Isoflavone treatment significantly decreased menopausal symptoms ($p < 0.01$) versus control as measured by the Menopausal Kupperman Index.

14.18 Van der Weijer & Barentsen (2002) reported a 44% reduction ($p < 0.01$) in the incidence of hot flushes in a double blind placebo controlled trial of postmenopausal women (n=30) ingesting isoflavones (80 mg/day) for 12 weeks. The Green climacteric scale score also decreased (13%) in the isoflavone treatment group compared with controls. The researchers included a 4 week blind placebo phase prior to the treatment phase to eliminate the placebo response.

14.19 In a study by Jeri (2002), a 48% reduction ($p < 0.001$) in hot flushes was found in a placebo-controlled double blind study of postmenopausal Peruvian women (n=30) ingesting isoflavones (40 mg/day) for 16 weeks.

Studies failing to show an effect of phytoestrogens on the menopause (see table 14.2)

14.20 Three cross-sectional studies by Nagata *et al* (1998; 1999; 2000b) report that dietary factors such as soy may have an effect of menopausal symptoms. The association between frequency and severity of hot flashes and soy intake was investigated in Japanese postmenopausal women (n=20, aged 40-59 years). Diet was assessed by FFQ. Fermented soy product intake but not total soy product intake was significantly inversely correlated with hot flush severity ($p < 0.05$) after controlling for age and menopausal status. Neither total soy product intake nor fermented soy product intake was significantly correlated with menopausal index score.

14.21 A randomised, double-blind crossover study did not find a clear correlation between oestrogenic changes in vaginal cells and effects on menopausal symptoms in postmenopausal women (n=52) (Dalais *et al*, 1998). This study reported that 12 weeks of dietary supplementation with 45g linseed ($p < 0.009$) or wheat ($p < 0.001$) resulted in a reduction in the rate of hot flushes with no increase in vaginal maturation index. A reduction in hot flushes was not observed following a soy diet (52 mg total isoflavones/day) although this diet did increase vaginal maturation index ($p=0.03$).

14.22 Murkies *et al* (1995) reported no benefit of dietary supplementation in a randomised, double-blind study with soy compared with wheat on the incidence of hot flushes or menopausal symptom scores in postmenopausal women (n=58). Following 12 weeks, both soy and wheat decreased the number of hot flushes ($p < 0.001$) and menopausal symptom scores ($p < 0.05$). There was no effect on vaginal maturation index.

14.23 The results from a 12 week pilot study in postmenopausal women (n=24) indicated no significant difference between the incidence of hot flushes in women receiving an isoflavone containing supplement compared with those receiving a placebo. There were no differences between groups with respect to menopausal symptom scores, levels of FSH, SHBG, thyroid binding globulin (TBG) and vaginal maturation value (Knight *et al*, 2001). An 8 week double-blind, randomised intervention study in women (n=182) with a history of breast cancer, who were given soy isoflavone supplements (150 mg total isoflavones/day) also failed to find a reduction in the incidence or severity of hot flushes (Quella *et al*, 2000).

14.24 Two studies investigating the effects of a tablet preparation of isoflavones extracted from red clover found the supplement (40 mg or 160 mg total isoflavones/day) had no oestrogenic effects on vaginal epithelium or endometrial thickness when compared with the placebo. In addition, the supplement and placebo had a similar effect in reducing hot flushes (Baber *et al*, 1999; Knight *et al*, 1999).

14.25 Postmenopausal women (n=59) were given a soy beverage containing 90 mg isoflavones (Van Patten *et al,* 2002). A control group (n=64) received a placebo rice beverage. The women recorded frequency and severity of hot flashes for 4 weeks at baseline, and for 12 weeks while consuming 500 ml of soy or placebo beverage. No significant differences between the soy and placebo groups in the number of hot flashes were observed.

14.26 Postmenopausal women (n=28, aged 52-82) consumed their habitual diets plus 0, 5 or 10 g ground flaxseed for three seven week periods (Hutchins *et al*, 2001). The flaxseed diets significantly reduced serum concentrations of oestradiol to 3.26 pg/mL and oestrone sulfate to 0.09 ng/mL and increased prolactin to 1.92 µg/L. Serum concentrations of androstenedione, oestrone, SHBG, progesterone, testosterone, free testosterone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate were not altered with flaxseed feeding.

14.27 One factor that may mitigate against the effects of isoflavones on hot flushes is their poor penetration of the blood brain barrier (see Chapter 11). This may prevent centrally mediated modulation of endogenous hormone concentrations to reduce the number and severity of hot flushes.

Table 14.1 Dietary intervention studies reporting a beneficial effect of soy or isoflavone supplementation of the diet on menopausal symptoms.

Study Design Intervention	Dose (mg total isoflavones/day)	Duration	Effect	Reference
Soy				
DB, R, parallel, PC n=104, PostM	76	12 weeks	Significant ⇩ in moderate to severe hot flushes. Exposure to soy did not alter vaginal maturation index.	Albertazzi *et al* (1998; 1999)
R, PC n=145, PeriM & PostM	NS	12 weeks	Significant ⇩ in frequency of hot flushes and vaginal dryness with phytoestrogen diet.	Brzezinski *et al* (1997)
DB, R, CO n=51, PeriM	34 (as either a single or divided dose)	6 weeks/diet	Significant ⇩ in severity of menopausal symptoms in women receiving divided dose.	Washburn *et al* (1999)
Isoflavone supplement				
DB, R, PC n=39, PostM	50	6 weeks	Significant ⇩ in the number and severity of hot flushes and the severity of night sweats.	Scambia *et al* (2000)
DB, R, PC, CO n=51, menopausal	40	3 months	Both treatment and placebo ⇩ menopausal symptoms. No oestrogenic effect on vaginal epithelium or endometrium.	Baber *et al* (1999)
DB, R,PC n=80, PostM	23.3 mg genistein/day 6.2 mg daidzein/day 3.8 mg glycitein/day	4 months	Isoflavone treatment significantly decreased menopausal symptoms as measured by the Kupperman Index.	Han *et al* 2002
DB, R, PC n=177, PostM	50	12 weeks	Significant ⇩ in number and severity of hot flushes and night sweats. No change in vaginal cytology.	Upmalis *et al* (2000)
DB, R, PC n=30, PostM	80	12 weeks	Significant ⇩ in number and severity of hot flushes and Greene climacteric scale score.	Van der Weijer & Barentsen (2002)
DB, R, PC n=30, PostM	40	16 weeks	Significant ⇩ in number of hot flushes.	Jeri (2002)

DB – double blind; R – randomised; PC – placebo controlled; CO – crossover; PeriM – perimenopausal; PostM – postmenopausal; NS – not stated

Table 14.2 Dietary intervention studies failing to show an effect of soy or isoflavone supplementation of the diet on menopausal symptoms.

Study Design Intervention	Dose (mg total isoflavones/day)	Duration	Effect	Reference
Soy				
DB, R, CO n=52, PostM	52 (soy diet)	12 weeks	Significant ⇩ in hot flushes with wheat and linseed diets. Significant ⇩ in vaginal maturation index but no effect on hot flushes with soy diet.	Dalais *et al* (1998)
DB, R n=58, PostM	NS	12 weeks	Significant ⇩ in hot flushes and menopausal symptom scores with soy and placebo (wheat flour).	Murkies *et al* (1995)
PC n=59, PostM	90	12 weeks	No ⇩ in hot flushes following treatment with soy.	Van Patten *et al* (2002)
Isoflavone supplement				
DB, R, PC n=182, breast cancer patients	150	8 weeks	No ⇩in hot flushes following treatment with soy isoflavones.	Quella *et al* (2000)
DB, R, PC n=37, PostM	40 or 160	12 weeks	No ⇩ in hot flushes between treatment and control.	Knight *et al* (1999)
DB, R, PC n=24, PostM	77.4	12 weeks	No ⇩ in hot flushes following both treatment and placebo.	Knight *et al* (2001)

DB – double blind; R – randomised; PC – placebo controlled; CO – crossover; PeriM – perimenopausal; PostM – postmenopausal; NS – not stated

Hormonal effects in premenopausal women

14.28 The interest in hormonal effects of soy in premenopausal women has centred on the potential of these compounds to alter the length of the menstrual cycle. Increasing the length of the menstrual cycle is predicted to lower the body's lifetime exposure to endogenous oestrogens, which may have the potential to reduce the risk of hormone dependent cancers such as breast cancer. However, the results of these studies have been inconsistent.

14.29 Lu *et al* (1996) reported a non-significant increase in menstrual cycle length, when compared with baseline, in women (n=6) given soymilk (200 mg total isoflavones) daily for 1 month. Menstrual cycle length remained increased one cycle after termination of soy milk consumption but returned to the original level 5 or 6 cycles later. A decrease in luteal phase oestradiol (p=0.03), luteal phase progesterone (p=0.002) and a time dependent decrease in dehydroepiandrosterone sulfate levels (p=0.03) were also reported. A crossover study by Watanabe *et al* (2000) also demonstrated that an isoflavone supplement (20 or 40 mg/day) increased menstrual cycle length in 60% of women (n=42). However, a decrease in cycle length was also experienced in 20% of these women. There was no difference in serum oestradiol during the follicular or luteal phases. A detailed analysis in 3 women showed a decrease in serum oestradiol throughout the menstrual cycle and increases in SHBG. Increases in T_3 and T_4 were also seen during the follicular phase although the levels decreased during the luteal phase.

14.30 A crossover study by Cassidy *et al* (1994) demonstrated that daily ingestion of 60g soy protein (45 mg total isoflavones/day) increased the follicular phase of the menstrual cycle ($p< 0.01$) and total cycle length by an average of 2.5 days (n=6). There was no increase in the luteal phase. Soy supplementation also reduced mid-cycle LH ($p< 0.05$) and FSH ($p< 0.01$) and increased serum oestradiol during the follicular phase ($p< 0.02$). There was no change in the levels of progesterone, SHBG or testosterone or oestradiol during the luteal or mid-cycle phases.

14.31 In contrast, a randomised cross over study by Duncan *et al* (1999b) reported that soy isoflavones (64 or 128 mg total isoflavones/day) had no significant effects on the length of the follicular, luteal or total menstrual cycle (n=14). However, the levels of LH (p=0.009) and FSH (p=0.04) were decreased during the periovulatory period with 64 mg isoflavones/day while 128 mg isoflavones/day decreased levels of free T_3 (p=0.02) and DHEA sulfate (p=0.02) during the early follicular phase and oestrone during the midfollicular phase (p=0.02).

14.32 A randomised crossover study by Martini *et al* (1999) reported no effect on any hormone parameters measured when a soy beverage (38 mg total isoflavones/day) was consumed for the duration of two menstrual cycles (n=36). In addition, there was no change in menstrual cycle length or the ratio of oestrogen metabolites 2-hydroxyoestrone:16αhydroxyoestrone.

14.33 Wu *et al* (2000) did not observe an effect on menstrual cycle length in 20 women (n=10 Asian and n=10 non-Asian) given soy foods, providing an average of 32 mg/day total isoflavones. A decrease in serum oestradiol ($p < 0.05$) was observed in Asian subjects and the authors suggest this may have reflected the increased intake of isoflavones as a non-significant increase in urinary isoflavone excretion was observed in these subjects. There was no change in SHBG, progesterone or levels of follicular oestradiol. Lu *et al* (2000a) reported that soymilk supplementation (154 mg total isoflavones/day) did not change total menstrual cycle length or length of follicular phase. There was a slight decrease in the length of the luteal phase but this was not significant. Decreases in serum oestradiol ($p < 0.01$) and progesterone ($p < 0.0001$) levels were also noted. There was no effect on LH or FSH.

14.34 The effect of isoflavones in regulating the oxidative metabolism of oestrogens has been investigated in some studies. A randomised crossover trial in premenopausal women (n=12) receiving 10, 65 or 129 mg/day total isoflavones (approximately 100 days for each diet followed by 3 weeks washout between diets) showed an increase in the ratio of 2-hydroxyoestrone:16α-hydroxyoestrone ($p < 0.05$) and a decrease in the ratio of total potentially genotoxic oestrogens:total oestrogens ($p < 0.05$) compared to the low isoflavone diet (Xu *et al*, 1998). Similar results were found in a longitudinal crossover study by Lu *et al* (2000b) who reported that consumption of soymilk (containing mean level of 158 mg isoflavones/day) increased the ratio of 2-hydroxyoestrone:16α-hydroxyoestrone ($p=0.01$) in premenopausal women (n=8) for one menstrual cycle duration.

14.35 Premenopausal women (n=34) were provided with 100 mg isoflavones/day or a placebo for one year in a double blinded trial to look at the effect of dietary phytoestrogens on the ovulatory cycle (Maskarinec *et al,* 2002). Blood samples were collected at baseline, five days after ovulation and at months 1, 3, 6 and 12 and assayed for oestrone, oestradiol, oestrone sulfate, progesterone, SHBG, FSH, and LH. Menstrual cycle length was unaffected by dietary intervention and no significant changes in sex hormone concentrations were observed.

14.36 A study by Kumar *et al* (2002) reported decreased hormone concentrations after isoflavone intake. Premenopausal women (n=68, aged 25-55 years) were given 40 mg/day genistein or a placebo over a 12-week period. Supplementation with genistein increased mean menstrual cycle length by 3.5 days ($p < 0.05$) from baseline to the third menstrual cycle. The follicular phase of the menstrual was increased by 1.5 days ($p=0.08$) with supplementation. Genistein supplementation decreased serum free oestradiol and oestrone concentrations in 54% of the women in the treatment group compared with 38% in the control group. SHBG levels increased in 41.4% of women in the experimental group compared with 37.5% of women in the placebo group. Oestrone decreased in 56% of women in the experimental group compared with 43% in the placebo group.

14.37 Nagata *et al* (1998) reported decreased oestradiol concentrations in Japanese women (n=31) given a diet supplemented with 400 ml soymilk (containing 109 mg isoflavones) compared to a control diet (n=29) over a two months intervention period of three consecutive menstrual cycles. No statistical significance between the two groups was determined, due to the small number of subjects.

14.38 Nagata *et al* (1997) reported decreased oestradiol concentrations on days 11 and 22 of the menstrual cycle of Japanese women (n=50, aged 21-42 years) consuming soy (especially tofu and miso ($p < 0.05$) as assessed by FFQ). Soya intake was estimated at 5.0 $\pm$ 3.0 g. The hormone concentrations were 73 $\pm$ 48 and 98 $\pm$ 48 pg/mL for oestradiol, and 78 $\pm$ 31 and 85 $\pm$ 32 pg/mL for SHBG, on days 11 and 22 of the cycle respectively. Intake of soy products significantly inversely correlated with oestradiol on days 11 and 22 of the cycle after controlling for age, body mass index, menstrual cycle length, and total energy intake. No significant correlation was observed between soy product intake and SHBG.

Hormonal effects in men

14.39 There is limited information on the effects of soy or soy isoflavone consumption on reproductive hormones in men. A cross sectional analysis of the relationship between soy product intake and reproductive hormones in Japanese men (n=69) was reported by Nagata *et al* (2000a). The average isoflavone intake, estimated using a food frequency questionnaire was 22 mg/day. After controlling for confounding factors, an inverse correlation was observed between soyfood consumption and serum oestradiol (p=0.009). Inverse correlations were also reported for soyfood consumption and serum oestrone (p=0.05) and serum total testosterone (p=0.05).

14.40 In a larger study, British men (n=696) also had their dietary soy intake determined by FFQ (Allen *et al*, 2001). Multiple regression was used to investigate the association between soy milk intake, and hormone levels after adjustment for confounders. Soymilk intake was not associated with changes in serum concentrations of testosterone, free testosterone, androstanediol glucuronide, sex hormone-binding globulin, or lutenising hormone.

14.41 Three dietary intervention studies have reported effects in men. A 4 week randomised crossover study in men (n=42) consuming 150 g lean meat or 290 g tofu (70 mg total isoflavones/day) demonstrated that after adjusting for weight alterations, the mean ratio of testosterone:oestradiol (p=0.05) was reduced while the concentration of SHBG (p=0.01) was increased (Habito *et al*, 2000). However, these findings were not supported by those of Nagata *et al* (2001a) who assessed the effect of soymilk on the hormonal status of Japanese men (n=35). Men consumed 400 mL soymilk (90 mg total isoflavones/day) for 8 weeks. The results indicated that although serum oestrone levels were reduced in the soy treated group (p=0.04), there was no decrease in any of the other hormones measured. A third study by Mitchell *et al* (2001) reported that daily supplementation with a soy extract (40 mg total isoflavones/day) for 2 months did not alter sex hormone and gonadotrophin levels (n=14). In addition, there were no effects on ejaculate volume, sperm concentration, sperm count or motility or testicular volume.

14.42 In a randomised dietary intervention study, serum oestrogen and androgen concentrations were determined in Japanese men (Nagata *et al*, 2001b). Subjects supplemented their diet with 400 ml of soy milk (n=35) for 8 weeks or maintained their habitual diet (n=35). Blood samples were obtained at baseline and during the intervention period every two weeks for 12 weeks. Serum oestrone concentrations were significantly lower in the soy supplemented group but increased in the control group. There were no statistical differences in oestradiol, testosterone or SHBG levels showed between the treatment groups.

Effects of phytoestrogens on diabetes

14.43 Diabetes is a disease characterised by a lack of insulin leading to uncontrolled glucose metabolism. Diabetes is either insulin (type 1) or non-insulin (type 2) dependent. It has been suggested that soy may have a beneficial effect on some factors of diabetes. A beneficial impact of soy on diabetes was first noted in a series of case studies which showed a reduction in urinary sugar excretion in diabetic patients provided with a diet including soy beans (Friedenwald & Ruhrah, 1910). More recently, epidemiological studies have suggested that diabetes is more common in Japanese-American subjects compared with Japanese subjects living in Japan. For example, diabetes appears to be 4 times more common in Japanese men living in America compared with those living in Japan (Fujimoto *et al*, 1987). Similar comparisons in Japanese women show diabetes is 6 times more common in Japanese women living in America compared with those living in Japan (Fujimoto *et al*, 1991). It has been postulated that this difference may be due to the greater exposure of those living in Japan to phytoestrogens from dietary soy compared to those living in the West.

14.44 The effect of soy on factors of diabetes has been examined in experimental studies in primates. When soy (148 mg isoflavones/day) was fed to ovariectomised cynomolgus monkeys for 7 months, insulin sensitivity and glucose effectiveness were improved especially when co-administered with oestradiol (Wagner *et al*, 1997). However, these findings were not reproduced in a similar study of male diabetic cynomolgus monkeys (Wagner *et al*, 2000).

14.45 No effects on parameters of diabetes were reported in two studies on non-diabetic humans. In a randomised cross-over trial of premenopausal women (n=14), Duncan *et al* (1999b) reported no effect on plasma insulin concentrations after consumption of soy diets with a range of isoflavone contents (10, 64 or 128 mg isoflavones/day) for 1 menopausal cycle. Similarly there was no effect in a randomised cross-over trial of postmenopausal women (n=18) consuming soy with the same range of isoflavone contents (10, 64 or 128 mg isoflavones/day) for 26 days (Duncan *et al*, 1999a).

14.46 In contrast, beneficial effects were reported in a randomised, double blind cross-over trial of postmenopausal women with type 2 diabetes (n=32) (Jayagopal *et al*, 2002). Subjects consumed soy (132 mg isoflavones/day) for 12 weeks and plasma hormone and lipid profiles were monitored. Soy treatment resulted in decreases in fasting insulin (8%, $p < 0.006$) and insulin resistance (6%, $p < 0.003$) compared with the placebo group. The authors suggest that dietary soy may improve factors involved in type 2 diabetes.

14.47 *In vitro* studies suggest that isoflavones may act on insulin secretion and glucose metabolism. Genistein (10-100 μM) increased insulin secretion in a dose-dependent manner from islet of Langerhans cells (pancreatic cells which secrete insulin). The authors postulate that genistein modulates insulin secretion *via* inhibition of tyrosine kinase. Genistin had no effect on insulin secretion (Sorenson *et al*, 1994). In addition, genistein has been shown to inhibit (K_i of 0.06 nM) α-glucosidase (an enzyme in carbohydrate metabolism) isolated from bacteria (Lee & Lee, 2001).

Key points

- Most data from studies investigating the effect of dietary supplementation of soy or isoflavones suggest that isoflavones produce weak oestrogenic effects in postmenopausal women. However, data on whether such supplementation provides relief from menopausal symptoms are inconsistent. Although studies have suggested soy may be beneficial, especially if basal intake is low or vasomotor symptoms severe, the data are equivocal, as positive results are often not statistically significant and strong placebo responses are observed. Thus, the weight of evidence does not strongly support the view that supplementation of the diet with soy or isoflavones alleviates menopausal symptoms.

- The poor penetration of isoflavones across the blood brain barrier may mitigate against centrally mediated modulation of endogenous hormone concentrations to reduce the number and severity of hot flushes.

- It has been suggested that phytoestrogens may be protective against the potentially harmful effects of endogenous oestrogens (e.g. oestrogen-dependent breast cancer) by lengthening the menstrual cycle thus, reducing the lifetime exposure of women to these compounds. Data from studies on premenopausal women suggest that supplementation of the diet with soy or isoflavones produces weak hormonal effects. However, the nature of these effects is inconsistent. Most studies show some suppression of hormone concentrations (i.e. lutenising and follicle stimulating hormone and/or oestradiol) during phases of the menstrual cycle with supplementation. Few studies have shown supplementation to result in a lengthening of the menstrual cycle.

- Reports of hormonal effects in men from dietary soy or isoflavone supplementation are inconsistent, showing no or weak hormonal effects.

- An intervention trial has suggested that dietary soy may improve some aspects of diabetes in postmenopausal women with type II diabetes. However, there may be factors in soy other than phytoestrogens, which aid glycaemic control. No studies have specifically looked at the effect of phytoestrogens on diabetes although an *in vitro* study suggests that genistein may increase insulin secretion by pancreatic cells. The relevance of these latter observations to humans is unknown.

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15. Phytoestrogens and cancer

Introduction

15.1 The incidence of a number of cancers, including those of the breast and prostate, has been found to be much higher in Western populations compared with that in countries such as Japan and China. Epidemiological and migrant studies have suggested that racial characteristics and other factors including lifestyle, diet and fat or fibre intake may play a role in the aetiology of these diseases. One notable dietary difference is the relatively high consumption of soy-based products amongst Asian populations. As such, soy has attracted much attention as a potential chemoprotective factor (Bingham *et al*, 1998; Cassidy & Faughnan, 2000). However, studies investigating the role of phytoestrogens in these diseases have been limited by the paucity of data on phytoestrogen levels in foods and dietary intakes, especially for the lignans.

15.2 It has been suggested that exposure to oestrogen during development or early life may play an important in programming hormonal homeostasis and influence an individuals later life risk of developing cancer. Few human studies on cancer have addressed the possibility that exposure to phytoestrogens at an earlier life stage or over several life stages may confer protective changes. This possibility may, in part, explain why the reduced risk of certain cancers observed amongst migrants increases with subsequent generations.

15.3 This chapter reviews the evidence from human, animal and *in vitro* studies on the role of phytoestrogens in a variety of different cancers.

Breast Cancer

15.4 Breast cancer is the most common form of cancer affecting women in the UK and the incidence is rising. Identified genetic factors account for a relatively small proportion (about 4%) of breast cancer in Western populations (Bingham *et al*, 1998). The risk of developing breast cancer increases markedly with age, although the rate of increase is less after the menopause.

15.5 Hormone related cancers such as breast cancer have been reported to vary by as much as 5- to 20-fold between populations. Studies of migrant populations indicate the highest rates of cancer are typically seen in populations following Western diets that are higher in fat and lower in fibre. The lower rates, typically seen in populations consuming a traditional Eastern (e.g. Chinese or Japanese) diet relatively low in fat and relatively high in fibre and soy (Tham *et al*, 1998).

15.6 The development of breast cancer is highly dependent on the hormones associated with ovarian function, as such hormone-related events occurring premenopausally, or even in adolescence, may

determine whether breast cancer develops postmenopausally. These hormone-related risk factors include early onset of menarche, late onset of menopause, delayed age of first pregnancy and, in postmenopausal women, an elevated free oestradiol concentration (Bingham *et al*, 1998). There is also evidence from studies of migrants, that the development of breast cancer can be influenced by environmental factors. This is exemplified in a study by Ziegler *et al* (1993) who reported that Asian-American women born in the West had a 60% higher risk of developing breast cancer than those born in the East (e.g. China and Japan). Among those born in the West, the risk of breast cancer was 50% higher in those whose grandparents had been born in the West. Among those born in the East, risk was determined by whether, prior to migration, their community had been rural or urban. Migrants from urban communities had a 30% higher risk of developing breast cancer than those from rural communities.

Epidemiological studies

15.7 Four case-control studies conducted in China and Japan have evaluated the association between soy intake and incidence of breast cancer. The results from a case-control study by Lee *et al* (1991), reported an inverse association between soy protein intake, the ratio of soy to total protein and total soy products, and the incidence of breast cancer in pre- ($p= 0.02$) but not postmenopausal Singapore-Chinese women ($n= 200$). Similarly, Hirose *et al* (1995) reported an inverse association between pre- ($p< 0.05$) but not postmenopausal Japanese women. However, no relationship between the soy intake and risk of breast cancer was established in another case-control study (Yuan *et al*, 1995). The Shanghai breast cancer study, included 1459 breast cancer cases and 1556 age-matched controls. Soy food intake, assessed by a food frequency questionnaire (FFQ), was shown to be high with over 96% of women reporting consumption at least once per week. A non-significant reduction in risk ($p< 0.10$) was observed amongst women who reported eating soy foods at least once per week (OR= 0.78; 95% CI= 0.52-1.16). Following adjustments for confounding factors, women in the highest decile compared to the lowest decile intake group were shown to have a 30% lower risk of breast cancer (OR= 0.66; 95% CI= 0.46-0.95). Stratified analysis demonstrated that the inverse association was more evident in women with a higher body mass index (BMI). The reduction in risk was also greater for women with oestrogen and progesterone receptor positive (ER+/PR+) breast cancer (OR= 0.44; 95% CI= 0.25-0.78) than those with any other ER/PR status (Dai *et al*, 2001). The Shanghai study also obtained information on adolescent (13-15 years) dietary soy food intakes and reported a inverse association between intake at this age and risk of breast cancer ($p< 0.001$) in later life. The inverse association was reported for both pre- and postmenopausal women. Details of adolescent soy food intakes were also obtained from the mothers of participant's aged $<$ 45 years. For women in the highest soyfood group, these data were also inversely associated ($p< 0.001$) with breast cancer (Shu *et al*, 2001).

15.8 The role of soy and risk of breast cancer has also been evaluated in four case-control studies conducted in the United States (US). A small case-control study by Witte *et al* (1997) reported that weekly tofu intake may be associated with lowered risk (OR= 0.5) of bilateral breast cancer in premenopausal women ($n= 488$). However, the reduction in risk was not significant. In addition, the results of a large multicentre study conducted among Asian-Americans ($n= 597$) reported that a high intake of tofu ($\geqslant$ 120 times/year) was associated with a lowered risk of breast cancer (OR = 0.85; 95% CI= 0.74-0.99) compared with low intake ($<$ 13 times/year). This finding was demonstrated in both pre- and postmenopausal women

following adjustment for age, geographical location, ethnicity and migration history. However, the association was only significant in women born in Asia and not among women of Asian origin born in the US (Wu *et al*, 1996). A case-control study of 501 Asian-American women with breast cancer and 594 control subjects also reported an inverse association between soy intake and breast cancer risk (OR= 0.65; 95% CI= 0.43-0.97) (Wu *et al*, 2002). Additionally, the odds ratios for adolescent and adult soy intake suggested that a high soy intake in adolescence is inversely associated with breast cancer risk (p trend= 0.04). The risk may be further reduced by soy intake in adulthood (p trend= 0.04). Horn-Ross *et al* (2001) found that in a study of non-Asian women with breast cancer (n= 1326) and without breast cancer (n= 1657) in the US, consumption of soy milk and soy burgers but not consumption of tofu, miso or other foods with added soy was inversely associated with breast cancer risk. In addition, the intakes of isoflavones and lignans were not associated with a decreased risk.

15.9 Two prospective investigations have investigated intakes of soy foods and breast cancer risk. The prospective cohort study by Hirayama (1986) demonstrated a significant inverse association between the intake of soybean paste soup and the risk of breast cancer in Japanese women (n= 142857). A large prospective study of 34,759 women in Hiroshima and Nagasaki in Japan investigated soy food consumption and risk of breast cancer. The risk of breast cancer is higher in women exposed to radiation, and analyses controlled for radiation dose and age at time of the bombings, as well as reproductive and other non-dietary factors. There was no significant association between breast cancer risk and consumption of soy foods, tofu and miso soup. Results were similar in a sub analysis of women not exposed to radiation (Key *et al*, 1999).

15.10 Four further case control studies in western countries and in China have compared urinary excretion of lignans and isoflavones (which is assumed to reflect intake) and the risk of breast cancer. Ingram *et al* (1997) obtained 3 day urine collections from Australian cases and controls (n= 144) and showed that high excretion of both equol (OR= 0.27; 95% CI= 0.10-0.69) and enterolactone (OR= 0.36; 95% CI = 0.15–0.86) were associated with a lowering of breast cancer risk. This effect was particularly strong for equol, which was associated with a 4-fold reduction in risk, although it is surprising that all subjects excreted equol. Enterolactone was associated with a 3-fold reduction in risk. There were no associations with the parent phytoestrogens daidzein and matairesinol, suggesting that metabolism of these compounds by the gut microflora may be an important factor in reducing the risk of breast cancer. A case control study by Murkies *et al* (2000) reported that postmenopausal women with breast cancer (n=18) had lower urinary daidzein (p= 0.03) and a trend towards lower genistein (p= 0.08) excretion compared with controls. In addition, women with breast cancer were found to have higher levels of testosterone than those in the control group (p= 0.05). There were no differences between any of the other hormone parameters measured. In a sub-study of a population based case-control study (n= 60) in China, Zheng *et al* (1999) demonstrated that women with a total urinary isoflavone excretion in the highest tertile displayed a 50% reduction in breast cancer risk compared with those in the lowest tertile (OR= 0.14; 95% CI= 0.02-0.88). Dai *et al* (2002) reported a reduction in breast cancer risk with urinary excretion of isoflavones (OR= 0.62; 95% CI= 0.39-0.99) and lignans (OR= 0.40; 95% CI= 0.24-0.64) observed for the highest *versus* lowest tertiles of excretion in a study of breast cancer cases (n= 250) and controls (n= 250) in Shanghai. Equol was not measured.

15.11 One case control study has examined serum levels of enterolactone in Eastern Finland. The risk of breast cancer was significantly reduced in women classified in the highest quintile of serum enterolactone (OR= 0.38; 95% CI= 0.18–0.77) and this was associated with higher consumption of rye products and intakes of dietary fibre and vitamin E (Pietinen *et al*, 2001).

15.12 There is one prospective investigation of urine excretion of phytoestrogens. Den Tonkelaar *et al* (2001) measured urinary excretion of genistein and enterolactone in postmenopausal women with (n= 88) and without (n= 268) breast cancer. The results showed that increased urinary excretion of genistein was weakly, non-significantly associated with a reduced risk of breast cancer (OR for highest tertile compared with the lowest tertile was 0.83; 95% CI= 0.46-1.51). Whereas, increased urinary excretion of enterolactone was weakly, non-significantly associated with an increased risk of breast cancer (OR for highest tertile compared with the lowest tertile was 1.43; 95% CI= 0.79-2.59). Equol and other phytoestrogens were not measured in this study.

Studies of breast cancer biomarkers

15.13 It is thought that an increased risk of breast cancer may be associated, at least in part, with an individual's lifetime exposure to oestrogen. It has been suggested that an increase in menstrual cycle length results in lower exposure to oestradiol and, over a lifetime, this could correlate to a lower risk of breast cancer (Henderson *et al*, 1985). The findings that menstrual cycles in Asian women consuming a relatively high soy, high-phytoestrogen diet are generally longer than in Western women, and breast cancer incidence is lower in the former group, lend strong support to this theory (Bingham *et al*, 1998). However, it has been found that irregular rather than longer menstrual cycles are generally related to lower cancer risk (Bingham *et al*, 1998; Den Tonkelaar & De Waard, 1996). Additionally, Whelan *et al* (1994) reported that extremely short or long menstrual cycles were associated with breast cancer risk (see Chapter 14).

15.14 Cassidy *et al* (1994) reported that textured vegetable protein (TVP), containing 45 mg isoflavones/day, given daily for 1 month, suppressed gonadotrophin output and increased the follicular phase ($p < 0.01$) of the menstrual cycle by 2-3 days in premenopausal women. In addition, dietary intervention with a linseed supplement has also been shown to increase menstrual cycle length. However, the effects seen in this study were not significant and were related to the luteal rather than follicular phase of the cycle (Phipps *et al*, 1993). In contrast, a study by Duncan *et al* (1999a) reported that daily supplementation with soy powder, containing 64 or 128 mg isoflavones/day, over 3 menstrual cycles had no effect on the length of either the follicular or luteal phase in premenopausal women (n= 14). Taken together, the body of evidence is conflicting and it is not possible to attribute longer menstrual cycle lengths of Asian women specifically to increased phytoestrogen intake (see Chapter 14). The effects of isoflavone and lignan supplementation on hormones and menstrual cycle in premenopausal women are summarised in Table 15.1.

15.15 Competing pathways of oestrogen metabolism result in metabolites with differing oestrogenic activities. It has been suggested that the ratio of the metabolites 2-hydroxyoestrogen and 16α-hydroxyoestrogen may serve as a biomarker of lowered breast cancer risk. A randomised, cross-over study in postmenopausal women (n= 28) reported that dietary supplementation with flaxseed (5 or 10 g/day) for 21 days significantly increased urinary excretion of 2-hydroxyoestrogen ($p < 0.0005$) thereby increasing the ratio of 2-hydroxyoestrogen:16α-hydroxyoestrogen ($p < 0.05$). There were no significant differences in urinary 16α-hydroxyoestrogen excretion. The authors suggest these results indicate flaxseed has a chemoprotective effect in postmenopausal women (Haggans *et al*, 1999).

15.16 An increased urinary 2-hydroxyoestrone:16α-hydroxyoestrone ratio was also reported in studies of premenopausal women (n= 12) (Xu *et al*, 1998) and postmenopausal women (n= 18) (Xu *et al*, 2000) following dietary supplementation with isoflavones (65 or 132 mg/day). Decreased urinary oestradiol, oestrone, oestriol total oestrogens and putatively the genotoxic oestrogen metabolites (16α hydroxyoestrone, 4-hydroxyoestrone, 4-hydroxyoestradiol) were also noted (Xu *et al*, 1998; 2000). However, in contrast, a study by Martini *et al,* (1999) in premenopausal women (n= 36) given a soy beverage, containing 38 mg total isoflavones, over two menstrual cycles did not find a significant difference in the urinary 2-hydroxyoestrone:16α-hydroxyoestrone ratio.

15.17 Other markers of a high breast cancer risk in postmenopausal women include increased levels of androstenedione, testosterone and lower levels of sex hormone binding globulin (SHBG). Studies have suggested that phytoestrogens may exert a protective effect by lowering plasma free oestrogen and androgen levels through an increase in SHBG concentration (Adlercreutz *et al*, 1987; Adlercreutz & Mazur, 1997; Duncan *et al*, 1999b; Berrino *et al*, 2001). However, the concentration of SHBG is influenced by many factors, including changes in bodyweight, making cross sectional comparisons difficult. The results of short-term intervention studies following supplementation with either flaxseed or soy failed to show any significant increases in SHBG concentration (Baird *et al*, 1995; Cassidy *et al*, 1995; Lu *et al*, 1996; Martini *et al*, 1999; Nagata *et al*, 1997 and 1998; Petrakis *et al*, 1996; Phipps *et al*, 1993).

15.18 Duncan *et al* (2000) investigated the effect of soy protein isolate (10-128 mg isoflavones/day) on the plasma hormone and SHBG concentrations of postmenopausal women (n= 14) who were divided into equol and non-equol excretors. Women who excreted equol generally displayed lower concentrations of oestrone, oestrone sulfate, androgens and prolactin. The equol excretors also had higher concentrations of SHBG and midluteal progesterone. The authors conclude that the plasma hormone profile of equol excretors is associated with a reduced risk of breast cancer. The effect of phytoestrogens on SHBG concentrations is discussed further in Chapter 7.

Table 15.1 Hormonal and menstrual cycle changes associated with soy and flaxseed dietary supplementation in pre-menopausal women.

Study design	Form	Dose (mg total isoflavones/day)	Duration	Effect	Reference
CO, non-Asian women, (n=24)	Soy protein isolate	38	6 months	Serum oestradiol, moderate ⇧ in gross cystic disease fluid protein in nipple aspirate no significant changes in serum prolactin, progesterone or SHBG	Petrakis *et al* (1996)
R, CO, (n=14)	Soy protein powder	10, 64 or 128	3 menstrual cycles + 9 days	No change in menstrual cycle length	Duncan *et al* (1999a)
R, CO, (n=18)	Soy protein powder	7, 65 or 132	3 menstrual cycles + 9 days	pre-ovulatory LH and FSH (65 mg/day), ⇧ SHBG ⇩ T3 and DHEA sulfate in early follicular phase and ⇩ oestrone in the mid follicular phase, ⇧ SHBG (132 mg/day)	Duncan *et al* (1999b)
R, CO, 3 week washout, (n=12)	Soy protein powder	10, 65 or 129	3 menstrual cycles	⇩urinary excretion of oestradiol, oestrone, oestriol and total oestrogens and oestrogen metabolites (16α-hydroxyoestrone, 4-hydroxyoestrone, 4-hydroxyoestradiol), ⇧ in 2-hydroxyoestrone: 16α-hydroxyoestrone ratio	Xu *et al* (1998)
R, PC, Japanese women, (n=30/group)	Soy milk	109	3 menstrual cycles	Non-significant ⇩ serum oestrone and oestradiol in follicular phase, non-significant ⇧ menstrual cycle length	Nagata *et al* (1998)
R, CO, (n=36)	Soy beverage	38	2 menstrual cycles	No significant differences in serum oestrone, oestradiol, SHBG, DHEA-sulfate, prolactin or progesterone concentrations. No changes in menstrual cycle length or the ratio of 2-hydroxyoestrone:16α-hydroxyoestrone	Martini *et al* (1999)
CO, (n=6)	TVP	45	1 month	⇧ follicular phase of menstrual cycle, ⇩ gonadotrophin levels	Cassidy *et al* (1994)
CO, (n=18)	Flaxseed powder	NS	3 menstrual cycles	Significant ⇧ in luteal progesterone:oestradiol ratio, non-significant ⇧ in luteal phase, no effect on serum prolactin, DHEA sulfate, SHBG, oestradiol, oestrone or progesterone	Phipps *et al* (1993)

TVP – textured vegetable protein, NS – not stated R – randomised, CO – crossover, PC – placebo-controlled

15.19 A study investigated the effect of dietary soy on the proliferation rate of histologically normal breast epithelium in premenopausal women who had previously been diagnosed with either benign or malignant breast disease (n= 48). All subjects were randomly assigned to either their normal diet or a diet supplemented with 60 g soy/day (45 mg total isoflavones/day) for 14 days. Following soy supplementation, biopsies of normal breast tissue were taken and proliferating cells were assessed by thymidine labelling index (TLI) and immunocytochemical staining of the protein Ki67. In a preliminary report of this study by McMichael-Phillips *et al* (1998), a strong correlation between Ki67 and TLI (both markers of cellular proliferation) was observed (r= 0.868; p≤ 0.001). After adjusting for day of menstrual cycle and age of the patient, the proliferation rate of breast epithelium in the soy-treated group was found to be significantly increased as measured by TLI (p= 0.028) and Ki67 (p= 0.008). Progesterone receptor expression was also significantly increased in the soy treatment group (p= 0.04). The results of the study suggest short-term dietary soy supplementation can induce proliferation in breast tissue of premenopausal women with breast disease.

15.20 However, the effects on breast epithelial cell proliferation reported by McMichael-Phillips *et al* (1998) were not substantiated when the study was expanded. In the enlarged study of premenopausal women (n= 84), no effect on proliferation, oestrogen or progesterone receptor status, Bcl-2 expression, apoptosis or mitosis in breast epithelial cells was observed. However, the levels of apolipoprotein D were significantly lowered and expression of the oestrogen responsive gene pS2 was increased in nipple aspirate (p≤ 0.002), suggesting a weak oestrogenic effect on the breast (Hargreaves *et al*, 1999).

15.21 Increased mammographic density has been associated with a 4- to 6-fold increased risk of breast cancer (Atkinson *et al*, 1999). It is not known whether phytoestrogens can reduce mammographic density in a similar way to tamoxifen. However, a randomised, placebo controlled study investigating the effect of an isoflavone supplement (40 mg isoflavones/day) has suggested a significant ($p < 0.05$) reduction in density in women aged 56-65 compared to age matched controls (Atkinson & Bingham, 2002). Similar results were obtained from a cross-sectional study in Singapore-Chinese women (Jakes *et al*, 2002). Women (n= 406) were asked to self-report dietary intake of soy and soy isoflavones. Following adjustment for confounding factors, comparison of the highest and lowest quartiles of dietary intakes found that the highest intakes were associated with low-risk mammographic parencymal patterns (OR= 0.41, 95% CI= 0.18-0.94; OR= 0.44, 95% CI= 0.2-0.98 for soy and isoflavones, respectively).

15.22 In contrast, in a cross-sectional study, Maskarinec & Meng (2001) reported a positive correlation between self-reported soy food intakes and percentage breast density in women living in Hawaii (p= 0.04).

Animal studies

15.23 Animal studies have provided evidence that soy may have a protective role in breast cancer (Barnes *et al*, 1990; Connolly *et al*, 1997; Hawrylewicz *et al*, 1991; Troll *et al*, 1980). These effects were attributed to the isoflavones, as dietary treatment with isoflavone free soy abolished the protective effects (Barnes *et al*, 1990). In contrast, a study by Cohen *et al* (2000) showed no significant differences in tumour incidence, latency, multiplicity or volume between female mice treated with 10 or 20% (w/w) intact soy protein or 20% (w/w) isoflavone-depleted soy protein for 18 weeks. Although a non-significant trend

towards inhibition was observed in tumour volume and latency. Constantinou *et al* (2001) investigated the effect of dietary supplementation with intact and isoflavone depleted soy protein isolate (SPI-n and SPI-d, respectively) (16% w/w), dietary genistein and daidzein either singly (200 mg/kg diet) or in combination (100 + 100 mg/kg diet) on DMBA induced mammary cancer in rats. SPI-n and SPI-d caused a non-significant reduction in tumour incidence. Mean tumour multiplicity was reduced and tumour latency was increased by both diets although the SPI-d diet was more effective. Diets containing genistein and daidzein did not reduce tumour incidence. Tumour multiplicity was significantly reduced only by daidzein. Similarly, Gallo *et al* (2001) reported no effect of a standardised soy extract on the incidence and multiplicity of chemically-induced mammary tumours in rodents.

15.24 The protective effect of soy protein isolate or whey on chemically induced breast tumours in rats was investigated by Hakkak *et al* (2000) over two generations. A 1-day advance in age of vaginal opening was observed in soy protein isolate ($p < 0.05$) compared to whey or control animals. Both whey and soy protein isolate caused a reduction in tumour number and increased tumour latency in both the F_1 and F_2 generations compared to controls. Animals receiving whey exhibited a reduction in tumour incidence. However, only animals in the subsequent generation fed soy protein isolate had a reduced tumour incidence.

15.25 Gotoh *et al* (1998) demonstrated that inclusion of 10% (w/w) miso, 10% (w/w) soybean in the diet significantly decreased the number of chemically-induced mammary tumours per animal in young female rats. In addition, inclusion of biochanin A in the diet significantly decreased the incidence (50 mg/kg; $p < 0.01$) and multiplicity (10 and 50 mg/kg) of mammary tumours. In contrast, Appelt & Reicks (1999) reported that 13 weeks of dietary supplementation with soy (30-810 mg isoflavones/kg diet) did not significantly reduce the incidence of chemically induced breast tumours in rats.

15.26 The timing of exposure appears to be a critical factor in determining the efficacy of the chemoprotective effects of isoflavones. Neonatal (5 mg/animal on post natal day (PND) 2, 4 and 6) and prepubertal (500 mg/kg bw on days 16, 18 and 20) subcutaneous administration of genistein reduced the number and delayed the appearance of chemically induced mammary tumours in a rat model of breast cancer. In rats treated prepubertally, serum genistein levels were 4 μM and 102 nM on PND 21 and 50, respectively. Genistein was not measured in animals treated neonatally (Lamartiniere *et al*, 1995 and 1998; Murrill *et al*, 1996). A further study by Hilakivi-Clarke *et al* (1999b) also reported that prepubertal exposure of rats to dietary genistein (1 mg/kg bw/day) decreased the number of tumours per animal but with no overall change in tumour incidence. In addition, a decrease in tumour growth was noted.

15.27 A dose-related decrease in chemically induced mammary tumours was observed in rats following perinatal exposure to genistein (from conception to PND 21) through the maternal diet (25 or 250 mg genistein/kg diet). Female offspring were also found to have significantly reduced numbers of mammary terminal end buds (TEBs) at PND 21 and 50 correlating with the reduced number of mammary tumours observed. The DNA labelling index, a marker of cellular proliferation, was unchanged following perinatal exposure to genistein although the total number of proliferating cells was reduced. The authors conclude that by altering mammary gland differentiation early in life, genistein can lower the number of TEBs, which may reduce later susceptibility to breast cancer (Fritz *et al*, 1998).

15.28 A study by Constantinou *et al* (1996) found a 27% reduction in mammary tumour multiplicity in rats aged > 35 days following intraperitoneal administration of genistein (0.8 mg/day) for six months.

15.29 Yang *et al* (2000) reported that *in utero* (5, 25 mg/day subcutaneously) exposure to genistein on PND 16-20 of gestation or neonatal exposure (12.5 mg/day subcutaneously) on PND 15 and 18 did not alter mammary gland growth, cell proliferation or tumour latency. However, the incidence of chemically induced mammary tumours was greater in animals exposed to genistein compared to controls. Lamartiniere *et al* (2002) demonstrated that perinatal exposure of female offspring to 250 mg daidzein/kg diet did not inhibit the development of chemically induced mammary tumours.

15.30 In contrast, Hilakivi-Clarke *et al* (1998) reported that exposure to genistein (20 μg) *in utero* on gestational days 15-20 increased the density of TEBs in mammary glands compared to control mice. Hilakivi-Clarke *et al* (1999a) found that following *in utero* exposure, by subcutaneous administration of genistein (20-300 μg/day) to the mothers on days 15-20 of gestation, offspring were more susceptible to chemically induced mammary tumours compared to controls. The authors suggest *in utero* exposure to genistein may increase the incidence of mammary tumours in the offspring.

15.31 These data suggest that in rodents, exposure to genistein during critical periods of postnatal life can alter mammary gland development and render the adult animals less susceptible to chemically induced mammary tumours. Taken together, the data from Constantinou *et al* (1996), Fritz *et al* (1998), Hilakivi-Clarke *et al* (1998, 1999a and b), Lamartiniere *et al* (1995, 1998 and 2002), Murrill *et al* (1996) and Yang *et al* (2000) suggests that an early postnatal exposure to phytoestrogens may be the most sensitive period for an optimal anticancer effect. However, *in utero* exposure may increase susceptibility to mammary cancer later in life.

15.32 A study by Ju *et al* (2001) demonstrated that dietary exposure to genistein (⩾ 250 mg/kg) stimulated growth of MCF-7 tumours implanted into athymic, ovariectomised female mice although to a much lesser extent than oestradiol. The same dose of genistein was also found to increase tumour cell proliferation and expression of the oestrogen-responsive gene pS2. Similar results were reported by Allred *et al* (2001b) following exposure of mice to genistein (750 mg/kg diet) or genistin (1200 mg/kg diet). Tumours regressed over a 9-week period following removal of genistein or genistin from the diet. Increases in cell proliferation and pS2 expression in subcutaneously implanted MCF-7 tumours were also demonstrated following dietary exposure to soy protein isolate containing 150-330 mg/kg genistein (Allred *et al*, 2001a). Charland *et al* (1998) reported that intraperitoneal injection (18 mg, 5 times per week) of a soybean extract increased the weight and volume of implanted tumours in mice. In contrast, Santell *et al* (2000) reported that dietary exposure to genistein (750 mg/kg) did not alter growth of implanted breast tumour cells in athymic mice.

15.33 Ju *et al* (2002) implanted ovariectomised athymic mice with MCF-7 cells and 2.5 mg oestradiol implants. Mice were also implanted with 2.5 or 5.0 mg tamoxifen and the diet contained 1000 mg/kg dietary genistein. Tumour growth was increased in oestradiol treated mice after 16 and 32 weeks. Tamoxifen (2.5 and 5.0 mg) treatment significantly inhibited oestradiol stimulated tumour growth compared to control. The size of tumours in genistein treated animals were significantly different from controls, showing that genistein ablates the inhibitory effect of tamoxifen on oestradiol stimulated tumour growth. Expression

of oestradiol responsive pS2 and PR and cyclin D1 mRNA were increased by dietary genistein after oestradiol and tamoxifen administration, indicating that genistein can negate tamoxifen's inhibitory effect. Dietary genistein treatment significantly lowered plasma oestradiol concentrations in the 2.5 and 5.0 mg tamoxifen treated groups.

15.34 A study by Uckun *et al* (1998) reported increases of > 200% in the tumour diameter of human breast cancer xenografts following subcutaneous administration of genistein (1 mg/kg bw/day) to mice. Hsieh *et al* (1998) reported that dietary genistein (750 mg/kg diet) increased the number and size of mammary gland terminal end buds of ovariectomised, athymic mice. Genistein was also found to increase the growth of implanted MCF-7 tumours in these animals.

15.35 In contrast, Shao *et al* (1998) reported that subcutaneous administration of genistein (0.1-0.5 mg/kg bw) produced dose-dependent inhibition in the growth and volume of MCF-7 and MDA-MB-231 tumours implanted into nude mice. Genistein was also shown to dose dependently upregulate tissue *c-fos* and *c jun* expression and p21 mRNA and protein levels. Stimulation of apoptosis was also demonstrated. In MDA-MB-231 xenografts, genistein was shown to inhibit angiogenesis by decreasing vessel density and reduce tumour levels of vascular endothelial growth factor (VEGF) and tumour growth factor (TGF)-β1.

15.36 Constantinou *et al* (2000) treated both ER negative and ER positive cells *in vitro* with genistein (30 μmol/L) for 2 or 6 days before implanting them subcutaneously into nude mice. The study showed mice injected with ER positive cells treated for 6 days had a longer tumour latency period whereas those injected with ER negative cells treated for 6 days did not develop tumours. Both ER positive and ER negative cells treated for 2 days did not alter tumour latency.

15.37 Mice received test diets containing either fermented soy bean extract (100, 200, 400 mg total isoflavones/kg diet), genistein (200 mg/kg diet), daidzein (200 mg/kg diet) or genistein and daidzein (100 + 100 mg/kg diet) for 21 days. All diets, with the exception of that containing a combination of genistein and daidzein, reduced the ratio of 16α-hydroxyoestrone: 2-hydroxyestrone in urine. These findings suggest the test diets may exert a cancer protective effect by shifting the metabolism of oestradiol toward inactive rather than genotoxic metabolites (Kishida *et al*, 2000).

15.38 Day *et al* (2001) reported that dietary exposure to genistein (1000 mg/kg diet) did not protect against development of DMBA-induced mammary cancer in ERα wild-type mice. Mammary adenocarcinoma was observed in 56% of these animals. However, tumour development was not observed in ERα knockout mice. The authors conclude induction of DMBA-induced mammary tumours is ERα-dependent.

15.39 A number of studies have investigated the effects of flaxseed and the lignan, secoisolariciresinol diglycoside (SDG) on mammary tumour development. Flaxseed contains lignans, polyunsaturated fatty acids and α-linoleic acid as well as being a source of dietary fibre and protein. Therefore, it may not be possible to attribute biological effects resulting from dietary flaxseed to the lignans. However, some studies have used purified SDG to determine if the effects are mediated by lignans.

15.40 To test whether dietary flaxseed could reduce the proliferative effect of dietary fat on the mammary gland, weaning female rats were fed high fat diets containing 0, 5 or 10% (w/w) flaxseed for 4 weeks (Serraino & Thompson, 1991). Non-dose dependent reductions in terminal end bud proliferation in mammary tissue were reported in flaxseed-treated animals compared with control animals. No such effect was observed in other mammary cell types (terminal duct, alveolar bud). In a separate experiment, the dietary groups were also administered with a mammary carcinogen. Non-dose dependent reductions in terminal end bud, terminal duct and alveolar bud cell types were reported in animals following four week dietary supplementation with flaxseed compared with control animals. Additionally, nuclear aberrations in mammary cells were inversely correlated with urinary lignan excretion (r= 0.94). The authors suggest that dietary flaxseed may reduce the risk of mammary cancer by lowering mammary gland cell proliferation and nuclear aberrations.

15.41 Female rats fed on a 0 or 5% (w/w) flaxseed supplemented diet were administered a single dose of a mammary carcinogen. After treatment with the carcinogen some of the animals were switched to the other diet. Mammary tumour number and growth were recorded in each dietary group. Tumour size was significantly decreased only in the rats fed flaxseed prior to treatment with the carcinogen. This effect was less pronounced in animals fed flaxseed continuously. The authors speculate that the effect of flaxseed on the mammary gland may be related to oestrogenic or antioestrogenic components in the flaxseed (Serraino & Thompson, 1992).

15.42 A study examined chemoprotective effects of lignans in female rats fed SDG (1.5 mg/day, equivalent to 5% (w/w) flaxseed diet) orally for 20 weeks following treatment with a mammary carcinogen (Thompson *et al*, 1996a). Mammary tumour number and growth were recorded in each dietary group following treatment with the carcinogen. The number of mammary gland tumours, per tumour bearing rat and per number of rats, was lower in the group of animals fed SDG compared with controls. The authors suggest that dietary SDG has a chemoprotective effect on tumour development in the mammary gland.

15.43 In a study focussing on the later stages of mammary carcinogenesis, female rats were divided into 5 groups, 13 weeks after treatment with a mammary carcinogen: SDG (1.5 mg/day, equivalent to 5% (w/w) flaxseed diet), 1.8% (w/w) flaxseed oil (does not contain SDG), 2.5 or 5% (w/w) flaxseed or control diet (Thompson *et al*, 1996b). After 7 weeks on the experimental diets, the size of established tumours was decreased ($>$ 50%) in all groups compared with the control group and was negatively correlated with lignan excretion. New tumour incidence was lower in the SDG and 2.5% (w/w) flaxseed groups compared with the control group. The authors suggest that dietary SDG reduces the incidence and growth of mammary tumours.

15.44 In a further study, female rats treated with a mammary carcinogen were fed a diet supplemented with 2.5 or 5% (w/w) flaxseed or orally administered SDG (equivalent to that present in the flaxseed diets) for 22 weeks (Rickard *et al*, 1999). Mammary adenocarcinoma size, multiplicity or incidence was no different in the dietary flaxseed treated groups compared with the control group. Tumour multiplicity in rats given low dose SDG was significantly higher than controls, whereas that in rats given the high SDG dose was lower (not significantly). All diets appeared to decrease invasiveness of tumours.

In vitro **studies**

15.45 Elevated levels of oestrogen in plasma can stimulate the growth of mammary cancer cells. The cell line MCF-7, derived from a human breast tumour, is commonly used to assess the oestrogenicity of compounds *in vitro*. These cells express ERα and exhibit a biphasic growth response to oestrogens (see Chapter 8).

15.46 The role of ERα in mediating the proliferative response of breast cancer cells to phytoestrogens is supported by *in vitro* studies with genistein in MCF-7 cells. Genistein has been shown to transactivate a luciferase reporter gene coupled to the ERα ligand binding domain (Maggiolini *et al*, 2001; Martin *et al*, 1978; Panno *et al*, 1996). Additional evidence that genistein acts *via* an oestrogen receptor mechanism was provided by Hsieh *et al* (1998) who evaluated the effect of genistein on the expression of the oestrogen responsive gene pS2 in cultured MCF-7 cells. These results showed that, at concentrations of 1-10 μM, genistein increased pS2 mRNA expression. In addition, Jensen *et al* (2001) have reported that tumours expressing ERß but not ERα exhibit increased expression of the cellular proliferation markers Ki67 and cyclin A. This suggests ERβ is also associated with breast cell cancer proliferation.

15.47 Studies have reported that phytoestrogens have a concentration dependent effect on cell growth (Welshons *et al*, 1987; Wang & Kurzer, 1997 and 1998; Hsieh *et al*, 1998). These studies report that genistein has a biphasic effect on cell growth with low concentrations ($< 10\mu M$) stimulating DNA synthesis but higher concentrations ($> 10\mu M$) inhibiting DNA synthesis. Higher doses of genistein may be cytotoxic. Maggiolini *et al* (2001) report that genistein cytotoxicity at concentrations of $\leq 10\mu M$ to MCF-7 cells is a receptor-independent phenomenon as a similar cytotoxic effect was observed in ER-independent HeLa cells.

15.48 Genistein has also been reported to arrest the cell cycle at the G_2/M phase and stimulate apoptosis in human breast cancer cell lines (Santell *et al*, 2000; Pagalicci *et al*, 1994; Constantinou *et al*, 1998; Balabhadrapathruni *et al*, 2000).

15.49 Choi *et al* (1998) reported genistein induced cell cycle arrest due in part to reduced cyclin B1 levels and increased levels of the cell cycle regulators p21, cdc2 and cyclin dependent kinase 2 (cdk2). However, Cappelletti *et al* (2000) reported that genistein did not alter cdc2 expression suggesting that cell cycle arrest at the G_2/M phase is not due to down-regulation of the cdc2/cyclin B1 complex. Genistein (1 μM) was shown to increase the cell cycle regulators cdk2 and cyclin D1 synthesis, which stimulate cells to enter the cell cycle (Dees *et al*, 1997). Additional effects of genistein on breast cancer cells include up-regulation of p53, down-regulation of bcl-2, increased bcl-2 phosphorylation, down-regulation of c erbB-2 and inhibition of matrix metalloproteinase MMP-2 and MMP-9 secretion, changes in gene expression associated with apoptosis (Li *et al*, 1999a and b; Constantinou *et al*, 1998; Balabhadrapathruni *et al*, 2000). Genistein (15-45 μM) has also been shown to dose-dependently increase expression of the cell maturation markers intracytoplasmic casein, lipid and ICAM-1 in ER positive and ER negative breast cancer cells (Constantinou *et al*, 2000).

15.50 In a series of studies, Shao *et al* (1998a, b and c) reported that genistein (20-70 μM) dose-dependently inhibited growth of MCF-7 and MDA breast cancer cells by induction of $p21^{WAF/CIP1}$ and G_2/M cell cycle arrest. Inhibition was also characterised by transcriptional down-regulation of matrix metalloproteinase (MMP)-9. Tissue inhibitor of metalloproteinase (TIMP)-1 was found to be up-regulated as were the early intermediate genes *c-fos* and *c-jun*.

15.51 Upadhyay *et al* (2001) compared the effects of genistein (90 μM) in malignant and normal breast epithelial cells. In this study, malignant cells were more sensitive to G_2M cell cycle arrest, hyperdiploid progression and induction of apoptosis compared to normal breast cells. The difference between malignant and normal cells is thought to be mediated by a differential effect of genistein on $p21^{WAF1}$ as $p21^{WAF1}$ mRNA and protein levels were induced in normal compared to malignant cells. In addition down-regulation of $p21^{WAF1}$ by antisense cDNA transfection showed that malignant cells were more sensitive to G2/M cell cycle arrest after genistein treatment.

15.52 Rong *et al* (2001) investigated the effects of 8-prenylnaringenin on aggregation, growth and invasion of MCF7/6 cells. The E-cadherin/catenin complex suppresses invasion and is expressed in MCF-7 cells whereas it is functionally defective in the invasive cell line MCF7/6. 8-Prenylnarinegenin (10 μM or 1 μM) was shown to stimulate aggregation of MCF7/6 cells. This effect was inhibited by both an anti-E-cadherin antibody and an anti-oestrogen (0.1 μM). 8-Prenylnaringenin did not affect the invasion of MCF7/6 cells in the chick heart assay *in vitro*.

15.53 Other possible mechanisms through which phytoestrogens may influence the risk of breast cancer have also been investigated. It has been suggested that inhibition of hydroxysteroid dehydrogenase (HSD) enzymes may reduce the risk of breast cancer by lowering the concentration of endogenous oestrogen (Le Bail *et al*, 2000; Krazeisen *et al*, 2001). These enzymes have been shown to inhibit the synthesis of oestrone from androgens (3β-HSD activity) and oestradiol from oestrone (17β-HSD type 5 activity). The most potent inhibitors of 3β-HSD were genistein, daidzein and biochanin A (IC_{50}= 2.9, 10 and 10 μM, respectively). Coumestrol and formononetin were less potent ($IC_{50} > 50$ μM). In contrast, the most potent inhibitor of 17β-HSD was coumestrol ($IC_{50} > 0.2$ μM). The isoflavones genistein, daidzein, biochanin A and formononetin were less potent (IC_{50}= 1, 10 or 5 and > 50 μM, respectively).

15.54 Peterson *et al* (1998) demonstrated differences in the growth inhibition of four breast cancer cell lines following incubation with genistein and biochanin A (3.7 or 26 μM, respectively). The cell lines MCF-7 and T47D had similar sensitivity to both biochanin A and genistein whilst ZR-75-1 and BT-20 cells were 2- to 4-fold less sensitive. Further investigations were conducted to determine if the differences in inhibition were due to differences in metabolism. Two metabolites of genistein (genistein sulfate and a hydroxylated methylated metabolite) and four metabolites of biochanin A (genistein, genistein sulfate, biochanin A sulfate and a hydroxylated methylated metabolite) were detected. The IC_{50} values for growth inhibition did not correlate with any of the sulfated metabolites, but did correlate with the hydroxylated methylated metabolite of genistein. This suggests that hydroxylated methylated metabolites may be an active form of genistein in human breast cancer cells. A study by Shen *et al* (1999) suggested that genistein may have oestrogen receptor-independent antiproliferative effects on breast cancer cells. Growth inhibition of oestrogen receptor negative human breast cancer cells (MDA-MB-435) was observed on genistein treatment (IC_{50} of 27 μM).

Key points

- Most epidemiological studies have investigated soy rather than individual phytoestrogens in breast cancer. Of these, the majority of case control studies have shown a protective trend for soy foods, particularly in Asian women, but results from two prospective studies are conflicting. Studies using biomarkers of phytoestrogen intake have shown some protective effects of lignans in the Netherlands and Finland, and case control studies have also suggested a protective effects of isoflavones. There are no prospective studies in which accurate assessments of food intake and biomarkers of exposure to all phytoestrogens are available so there are insufficient data on which to confirm a causal association.

- Exposure to oestrogen during development or early life may play an important role in programming hormonal homeostasis and influence an individuals later life risk of developing cancer. The results from some epidemiological studies suggest that consumption of soy in childhood may protect against development of breast cancer later in life. This suggestion may, in part, explain why the reduced risk of certain cancers observed among migrants increases with subsequent generations.

- It is suggested that a reduction in lifetime exposure to oestrogen may lower the risk of breast cancer. The lower rates of breast cancer in Japanese and Chinese women has been associated with longer menstrual cycles which in turn has been associated with a soy rich diet. The effects of phytoestrogens on menstrual cycle length has been investigated but the weight of evidence suggests it is not possible to attribute alterations in menstrual cycle to phytoestrogen intake.

- In one study, short-term dietary supplementation induced a weak oestrogenic effect in premenopausal women with breast disease, as shown by modulation of the levels of the oestrogen responsive gene products apolipoprotein D and pS2 in nipple aspirate. However, no effect on breast cell proliferation was evident.

- The animal data on breast cancer is conflicting. A number of studies have shown that genistein has a protective effect in animal models of chemically induced cancer. However, similar experiments using tumour implant models showed that genistein stimulated the growth of implanted mammary tumours both by dietary and subcutaneous administration. Studies in animal models with chemically induced mammary cancer suggest that dietary supplementation with the lignan, secoisolariciresinol may have a chemoprotective effect on breast cancer development although the results are not consistent. Animal studies suggest that exposure to phytoestrogens in early life inhibits development of breast cancer later in life.

- Genistein has been shown to have a number of effects *in vitro* including modulation of oestrogen responsive genes, interaction with the cell cycle and alteration of cell differentiation. Genistein has also been shown to inhibit and/or stimulate cell proliferation. The biological response is dependent on the cell type and the concentration applied. However, these effects occur at much higher concentrations than those likely to occur as a result of dietary exposure. These experimental studies have provided a number of possible mechanisms through which phytoestrogens may exert their effects.

Female reproductive tract cancers

Endometrial Cancer

Human studies

15.55 Cancer of the endometrium is more common in developed countries and the pattern of hormonal risk factors is similar to that associated with the development of breast cancer. Human case-control studies have shown that the combined oral contraceptive pill is protective against endometrial cancer. However, substantial evidence suggests unopposed oestrogen replacement therapy contributes to an increased risk of developing endometrial cancer (Beral *et al*, 1999; Bingham *et al*, 1998).

15.56 In countries such as Japan, where the intake of phytoestrogens is high the incidence of endometrial cancer, relative to the UK, is low (Bingham *et al*, 1998). A population-based, case-control study conducted among Asian and non-Asian migrants in Hawaii showed that following adjustment for body mass index there was a significant inverse association between consumption of tofu and/or other soy products and the risk of endometrial cancer. The study also found similar associations with increased consumption of foods such as whole grains, vegetables, fruits and seaweed. These observations were largely independent of other risk factors, although they were limited to women who had never been pregnant or used oestrogen therapy (Goodman *et al*, 1997).

15.57 A prospective, placebo controlled trial by Balk *et al* (2002) investigated the effect of dietary phytoestrogen supplementation in postmenopausal women over 6 months. Participants received either a soy containing cereal (100 mg total isoflavones/day) or a placebo cereal. The results indicated that dietary phytoestrogens did not stimulate proliferation of the endometrium. Hale *et al* (2001) investigated the effect of supplements containing 50 mg/day red clover isoflavones, containing a high proportion of biochanin A, on the proliferative biomarker Ki67 in endometrial biopsies from perimenopausal women (n= 30). The results showed dietary supplementation for 3 months did not alter the proliferative index of Ki67.

15.58 To date, there have been no reports linking an increased risk of endometrial cancer with the consumption of phytoestrogens.

Animal studies

15.59 The effect of oral genistein (50 mg/kg bw/day) for 28 days on the growth of implanted endomertial carcinoma cells in rat uterine tissue was investigated by Diel *et al* (2001). Genistein did not affect tumour growth compared to controls.

15.60 Cotroneo & Lamartiniere (2001) investigated the effect of subcutaneous and dietary exposure to genistein on the growth of lesions in a rodent model of endometriosis. The study showed that only subcutaneous administration of genistein (16.6 or 50 mg genistein/kg bw) supported growth of the implanted tissue. In addition, subcutaneous injections of genistein were found to reduce uterine ERα numbers compared to control. Tissue growth was not supported following dietary exposure to genistein (250 or 1000 mg/kg diet).

15.61 The effect of genistein and daidzein on experimentally induced endometrial carcinoma was investigated by Lian *et al* (2001). In a two-week study, a single subcutaneous injection of genistein (33 μg/kg bw) was found to significantly decrease oestradiol induced expression of *c-jun*, interleukin-1α and TNF-α mRNA in the uteri of ovariectomised mice. In contrast, a single dose of daidzein (33 μg/kg bw) administered by subcutaneous injection inhibited expression of *c-fos* and interleukin-1α. In a longer term study mice receiving subcutaneous genistein or daidzein (0.033 mg/kg) had a lower incidence of oestrogen induced endometrial adenocarcinoma and atypical endometrial hyperplasia.

15.62 In contrast, a study by Newbold *et al* (2001) reported that mice exposed to subcutaneous exposure to genistein (50 mg/kg bw/day) on PND 1-5 developed abnormalities of the reproductive tract including cystic ovaries, absence of corpora lutea, abnormal oviducts, squamous metaplasia and atypical hyperplasia of the uterus and an increased incidence of uterine adenocarcinoma in adulthood.

Key points

- No direct associations between phytoestrogen intake and endometrial cancer have been made.
- Studies in which the diets of peri- or postmenopausal women have been supplemented with isoflavones have not shown any increases in endometrial cell proliferation.
- The results from animal studies on the effects of phytoestrogens on endometrium have produced conflicting results.

Ovarian Cancer

Human studies

15.63 A single study has investigated the association between consumption of soy products and ovarian cancer. The case-control study (n=254 cases; n=652 controls) conducted in China, investigated whether certain dietary factors had an etiological association with ovarian cancer. Using a food frequency questionnaire (FFQ), participants were asked to recall how often they consumed 120 different food items. The results found a significant inverse association ($p<0.01$; OR=0.04) between soybean products and risk of ovarian cancer (Zhang *et al*, 2002).

In vitro *studies*

15.64 Chen & Anderson (2001) investigated the effect of physiological concentrations (0.1-10 pM) of genistein and daidzein on human ovarian cancer cell lines. Genistein and daidzein were found to dose dependently reduce cell proliferation ($p< 0.001$) and cell viability ($p< 0.01$). The concentration of IL-6 was decreased whereas TGF-β1 was significantly increased ($p< 0.05$). The modulating effect of both isoflavones on these cytokines was abolished by an estrogen receptor antagonist suggesting estrogen receptors are required, at least in part, for these effects.

Vulvar cancer

15.65 Thigpen *et al* (2001) reported that mice fed a purified soy protein diet, containing 228 mg total isoflavones/kg diet, for one month had a higher incidence ($p < 0.05$) of spontaneous vulvar carcinoma than animals fed a diet containing casein. The incidence was also higher ($p < 0.05$) in animals fed the soy based diet at one and three months compared to those fed other rodents diets containing lower phytoestrogen concentrations of 8, 98 or 151 mg total genistein/daidzein. However, the incidence of spontaneous vulvar carcinoma was not significantly different for any of the diets following 6-12 months.

Prostate Cancer

Human studies

15.66 Epidemiological studies have shown that although the incidence of latent, small or non-infiltrative prostate cancer in Japan and a number of other Asian countries is similar to that in Western countries, the incidence of invasive cancer and associated mortality is far lower (Adlercreutz & Mazur, 1997). It has been proposed that the lower rate of mortality may be due to dietary phytoestrogens. It has been noted that the level of phytoestrogens in the prostatic fluid of Asian men can be up to 17 times higher than those of Western men (Morton *et al*, 1997).

15.67 The relationship between soy intake and prostate cancer has been investigated in three cohort and four case-control studies that have yielded inconsistent results. One cohort study (Hirayama, 1979) and two case-control studies (Lee *et al*, 1998; Oishi *et al*, 1988) conducted in Asia did not find an association between soy intake and reduction in the risk of prostate cancer. The data from one of these studies indicated a significantly increased risk of prostate cancer associated with the consumption of miso (Hirayama, 1979).

15.68 In contrast, the results from four North American studies support an inverse association between intake of non-fermented soy foods and prostate cancer. A prospective study (n= 12395) by Jacobsen *et al* (1998) reported frequent consumption (> once a day) of soymilk was associated with a 70% reduction in prostate cancer risk (RR= 0.3; $p < 0.05$). Severson *et al* (1989) reported that high ($\geq$ 5 times/week) tofu intake was associated with a non-significant ($p = 0.054$) reduction in prostate cancer risk (RR= 0.35) in a cohort of Japanese men (n= 7999) living in Hawaii. In a study of prostate cancer cases (n= 1619) and controls (n= 1618), Kolonel *et al* (2000) reported a decreased risk of prostate cancer between the highest and lowest quintiles of soy food intake (OR= 0.62; 95% CI= 0.44-0.89). In a case-control study, Villeneuve *et al* (1999) reported an inverse association between prostate cancer risk and tofu consumption. However, this association disappeared when the data was adjusted for confounders.

15.69 Using data from 42 countries, Hebert *et al* (1998) reported a significant inverse association between consumption of soy products and prostate cancer mortality (p= 0.0001). In addition, a study by Strom *et al* (1999) demonstrated an inverse association between consumption of foods containing coumestrol (p= 0.03) and daidzein (p= 0.07) and prostate cancer risk in US men.

15.70 Stephens (1997) reported that a prostate sample from a patient who received 160 mg phytoestrogens daily for 1 week prior to surgery showed mild patchy microvacuolations and significant tumour cell apoptosis. Similarly, a preliminary study has demonstrated that 2/3 patients who received a daily supplement containing 100 mg total isoflavones for one month prior to surgery showed signs of tumour regression and tumour cell apoptosis (FSA project T05002).

15.71 The results of studies conducted in the US and UK suggest that consumption of soy can reduce the risk of prostate cancer in men. As the development of prostate cancer is thought to be dependent on exposure to male reproductive hormones, it has been suggested the protective effect of soy may result from alterations in endogenous hormone concentrations. Demark-Wahnefried *et al* (2001) demonstrated that the levels of total serum testosterone was significantly decreased in men (n= 25) whose diet was supplemented with 30 g/day flaxseed for an average of 34 days ($p < 0.05$). No differences were observed in levels of prostate serum antigen (PSA). Similarly, a randomised, double-blind crossover study in elderly men (n= 34) with elevated PSA found that twice daily consumption of a soy beverage (70 mg isoflavones/day) for 6 weeks had no effect on PSA or the proto-oncogene p105erbB-2 (Urban *et al*, 2001).

Animal studies

15.72 The development of prostate cancer in men, and spontaneous tumours of the prostate seminal vesicle (PS-V), are thought to share common mechanisms and characteristics. Animal studies have provided supportive evidence of a protective role for isoflavones in the development of the disease. Pollard *et al* (2000a) reported that the incidence of spontaneous PS-V tumours in rats genetically susceptible to prostate cancer was suppressed in animals maintained on a diet containing 410 mg isoflavones/kg. Only 3% of animals were found to develop the tumours when fed the diet from the age of 2-24 months compared with 30% of animals maintained on a control diet. A further study demonstrated a longer latency period in development of chemically induced PS-V tumours in rats fed a diet supplemented with soy protein isolate (4 mg genistein/day). Reduced levels of testosterone and testes weights were observed in rats fed a soy meal diet (4 mg genistein/day) (Pollard *et al*, 2000b).

15.73 Landström *et al* (1998) reported that the development of transplanted prostate tumours was inhibited in rats receiving a diet containing 33% (w/w) soy flour compared to controls. Similar findings have also been reported by Zhang *et al* (1997). In addition, diets containing concentrates of soy phytochemicals, but not soy protein isolate alone, were found to inhibit the growth of tumours in mice (Zhou *et al*, 1999). Aronson *et al* (1999) demonstrated that a low-fat diet containing soy protein and isoflavones decreased the growth rate and final weight of human LNCaP prostate tumours grown in severe-combined immumodeficient mice. A 70%, non-significant, reduction in testosterone was also noted. Bylund *et al*

(2000) reported that, compared to controls, the number and size of transplanted tumours was reduced following dietary supplementation with rye-bran or soy. In addition, the amount of PSA secreted was decreased in the rye-bran and soy fed groups compared to controls although tumour cell apoptosis was increased in these groups.

15.74 It has been demonstrated that *in utero* exposure to soybeans, from the maternal diet, delayed the development of morphological changes, similar to those observed in human prostatic intra-epithelial neoplasia, in male mice oestrogenised with DES shortly after birth (Makela *et al*, 1995).

15.75 Onozawa *et al* (1999) reported that dietary supplementation with isoflavones (100 mg/kg diet) reduced the incidence of chemically induced adenocarcinomas in the seminal vesicles and prostate of rats. A study by Schleicher *et al* (1999) demonstrated that subcutaneous administration of genistein (50 mg/kg bw) every 12 hours for 31 days, significantly inhibited the growth of transplanted K1 prostate tumour cells in rats and resulted in fewer invasive tumours and metastases.

15.76 It has also been suggested that genistein reduces the risk of prostate cancer by down-regulation of the epidermal growth factor (EGF) pathway. Dalu *et al* (1998) demonstrated that genistein inhibited the expression of tyrosine phosphorylated proteins in the dorsolateral prostate of rats. Inhibition of tyrosine phosphorylated EGF receptor and ErbB2/Neu receptor expression was also demonstrated. The inhibitory effect on the EGF receptor was significant at 1000 mg genistein/kg diet.

15.77 The lack of a suitable animal model of human prostate cancer has led to the development of a transgenic model. The model, known as TRAMP (transgenic adenocarcinoma of mouse prostate), which spontaneously induces transformation in prostate tissue *in vivo* (Greenberg *et al*, 1995; Abate-Shen & Shen, 2000). TRAMP mice were fed genistein (0-500 mg/kg) from weaning until 28-30 days of age. Dietary genistein (100 mg/kg diet) significantly reduced the number of mice exhibiting poorly differentiated prostate adenocarcinoma (Mentor-Marcel *et al*, 2001).

15.78 Weber *et al* (2001) reported a significant decrease in ventral prostate weight, which was not associated with a decrease in prostate 5α-reductase activity in adult male rats following a phytoestrogen rich diet (600 mg/kg) for approximately 5 weeks. Testosterone and androstenedione plasma concentrations were significantly lowered suggesting altered regulation of 17β-hydroxysteroid dehydrogenase, the enzyme responsible for testosterone synthesis, as a possible mechanism of reduced prostate weight. The study also reported that expression of testicular steroidogenic acute regulatory peptide (StAR) protein was unchanged indicating a lack of effect on early stage of steroid synthesis and cholesterol incorporation.

15.79 Fritz *et al* (2002) reported that dietary exposure of rats to genistein from either conception until day 70 postpartum (0-250 mg/kg diet) or from day 56 to day 70 postpartum (250 or 1000 mg/kg diet) resulted in a dose-dependent down-regulation of androgen receptor and ERα and ERβ mRNA in the prostate. Increased testosterone concentrations were also reported. The increase in testosterone was significant for animals exposed from conception until PND 70 only.

In vitro studies

15.80 Shen *et al* (2000) demonstrated that genistein (20 μM) inhibited cell cycle progression at the G_1-phase in human prostate cancer cells. In addition, genistein increased expression of the cyclin-dependant kinase inhibitors $p27^{KIP1}$ and $p21^{WAF1}$ in a dose-dependent manner. Apoptosis was induced at higher concentrations of genistein (⩾ 20 μM). Mitchell *et al* (2000) reported that genistein, daidzein, equol and coumestrol inhibit cell prostate cancer cell growth at concentrations ⩽10μM). Genistein was shown to cause DNA strand breakage at ⩽ 10μM, however, daidzein did not cause similar effects at concentrations up to 500 μM. These results suggest that despite structural similarities both compounds inhibit cell growth by different mechanisms.

15.81 Genistein (4.6-37 nM) has also been shown to decrease, in a dose-dependent manner, the growth of surgically removed specimens of human benign prostatic hypertrophy and prostate cancer tissue in culture (Geller *et al*, 1998).

15.82 A study by Kyle *et al* (1997) reported that genistein (50 μM) caused apoptosis and down-regulated the expression of the protein tyrosine kinase inhibitor, focal adhesion kinase (FAK) in PC-3 and DU-145 prostate carcinoma cells. Further investigations into the mechanisms by which genistein causes apoptosis have demonstrated that it can prevent the transcription factor, nuclear factor (NF)-κB from entering the cell nucleus and activating gene transcription. In cells NF-κB is bound to inhibitory proteins known as IκB, genistein has been shown to prevent IκB phosphorylation thus inhibiting the action of NF-κB (Davis *et al*, 1999). Genistein, has also been found to cause G_2/M cell cycle arrest, significant down-regulation of cyclin B and up-regulation of $p21^{WAF1}$ in prostate carcinoma cells (Choi *et al*, 2000; Davis *et al*, 1998).

15.83 Hillman *et al* (2001) reported that genistein may potentiate the effect of radiation on prostate cancer (PC 3) cells. The study showed that pre-treatment of PC-3 prostate cancer cells with 15 μM genistein for 24 hours followed by irradiation for 3 days significantly inhibited DNA synthesis, decreased cell growth and decreased the ability of these cells to form colonies. The effects of genistein combined with radiation were greater than either treatment alone.

15.84 In contrast to the animal studies, genistein and biochanin A failed to inhibit EGF receptor tyrosine autophosphorylation in LNCaP and DU-145 prostate cell lines. Daidzein also failed to inhibit cell growth and EGF receptor tyrosine autophosphorylation, suggesting differences in chemical structure determines the inhibition of cell growth and phosphorylation *in vitro* (Peterson & Barnes, 1993).

15.85 Davis *et al* (2000) reported that treatment with genistein resulted in a similar dose-dependent inhibition of both androgen dependent and androgen independent human prostate cancer cell lines. However, genistein had a differential effect on PSA expression. In the androgen dependent cell line, low concentrations of genistein (1-5 μM) decreased PSA mRNA and protein expression whereas higher concentrations (> 10μM) were required to cause similar effects in the androgen independent cell line.

15.86 Lin *et al* (2001) have reported that the mammalian lignans enterolactone and enterodiol have been shown to inhibit growth of human prostate cancer cell lines (10-100 μM).

Key points

- The epidemiological data on soy intake and prostate cancer are inconsistent. One study in an Asian population has shown an increased risk with intake of fermented soy foods such as miso. Other studies in Asian populations report no effect. However, studies carried out in Western populations report an inverse correlation with consumption of non-fermented soy foods. Only a single study has specifically investigated phytoestrogens. The study carried out in a US cohort showed a reduced risk associated with consumption of foods containing phytoestrogens.

- In contrast, most of the research in rodents has specifically examined the effects of phytoestrogens on models of prostate disease. The concentrations used in these experiments are very high compared with likely dietary exposure levels in humans in the UK. Generally these studies have shown a protective effect either in tumour implant or chemically induced models of cancer.

- *In vitro* experiments have shown that phytoestrogens can modulate components of the cell cycle pathway and inhibit growth of prostate cancer cells and stimulate apoptosis. However, these effects were only found to occur at much higher concentrations greater than would be expected from normal dietary intakes.

Colorectal cancer

Human studies

15.87 Comparisons between international cancer rates and dietary intakes are often used as the basis for identifying dietary factors used in the aetiology of specific cancers. A cross-cultural study of 38 countries found no association between soybean intake and risk of colon cancer. The countries in this study were chosen on the basis of reliable cancer mortality data, but were not identified (McKeown Eyssen & Bright-See, 1984).

15.88 Further epidemiological studies have suggested a protective effect of soy products in colorectal cancer although the data have not been consistent. Two case control studies have shown that consumption of soy products had a protective effect on risk of rectal cancer. A case-control study conducted in China by Hu *et al* (1991) found that soybean products (bean sprouts, bean curd and other products) had a protective effect on rectal but not colon cancer risk (n= 111 colon cancer cases; n= 225 rectal cancer cases). The association with rectal cancer was graded and significant in men but not in women. Watanabe *et al* (1984) demonstrated that consumption of soybeans and soybean curd was associated with a non-significant reduction in rectal cancer (RR= 0.15 and 0.12 respectively). A reduction in the risk of colon cancer was not demonstrated. A case-control study by Nishi *et al* (1997) demonstrated that consumption of traditional Japanese soyfoods was associated with a non-significant reduction in the risk of both colon (RR= 0.96 for miso soup; 0.79 for tofu) and rectal cancer (RR= 0.79 for miso soup; 1.02 for tofu). Three other studies considered the relationship between consumption of soy and risk of colorectal polyps. Witte *et al* (1996) reported that the OR for consumption of one or more servings of tofu per week was

0.55 (CI= 0.27-1.11, p= 0.17). Le Marchand *et al* (1997) reported a relationship between colorectal cancer risk and the lowest and high quartiles of legume and soy product consumption in women (OR= 0.5; 95% CI= 0.3-0.9) but not in men (OR= 0.8; 95% CI= 0.5-1.2). However, this relationship was not evident when corrected for the fibre content of the food. Kono *et al* (1993) reported no effect at the third tertile of soy paste soup intake for large and small adenomas of the colon were 0.72 (CI= 0.37-1.40) and 0.73 (CI= 0.33-1.59), respectively.

15.89 In contrast two case-control studies have reported that intake of fermented soy products is associated with an increased risk of colorectal cancer. Haenszel *et al* (1973) reported fermented soybean intake was associated with an increase ($p < 0.05$) in colorectal cancer among Issei Japanese (RR= 1.6), but not Nisei or Hawaiian Japanese living in Hawaii. In addition, Tajima & Tominaga (1985) reported that consumption of miso soup increased ($p < 0.05$) the risk of developing rectal cancer (RR= 2.05). No associations were reported between colon cancer and consumption of miso soup or other soybean products. However, consumption of soybean products was inversely associated with rectal cancer (p= 0.03) in a case-control study (n= 181 cases and n= 653 controls) (Hoshiyama *et al*, 1993).

Animal studies

15.90 Several animal studies have examined the effect of soy and/or isoflavones on chemically induced colon cancer but have produced conflicting results. Two studies demonstrated that dietary genistein can protect against the development of chemically induced pre-neoplastic lesions of the rat colon. In a study by Steele *et al* (1995) 5 weeks of dietary supplementation with genistein (75 mg/kg bw) significantly reduced the number of aberrant crypt foci (ACF) in rats. The second study demonstrated that 12 weeks of dietary supplementation with 0.015% (w/w) genistein (as the aglucone) more effectively inhibited the formation of ACF than diets supplemented with soy flour or full-fat soy flakes, containing 0.05% (w/w) genistein derivatives (as the glucosides) (Thiagarajan *et al*, 1998). In contrast, Gee *et al* (2000) found that a soy supplemented diet had no effect on colonic crypt mitosis or apoptosis in a rat model of chemically induced colon cancer.

15.91 Sorensen *et al* (1998) demonstrated that feeding a Western diet (high fat, low fibre and calcium) containing isoflavones had no effect on the development of intestinal cancer in a murine model or intestinal cancer. In addition, a study by Rao *et al* (1997) demonstrated that although dietary administration of genistein (250 mg/kg diet) significantly increased the multiplicity of chemically induced non-invasive adenocarcinomas, and total adenocarcinomas, the incidence of colon adenocarcinoma or multiplicity of invasive adenocarcinoma was unchanged.

15.92 Davies *et al* (1999) demonstrated that soy (250 mg isoflavones/kg diet) did not protect against experimentally induced colon cancer in rats. Indeed those given isoflavones had increased numbers of small ACF, thought to be markers for the disease, at 12 weeks. However, a diet containing 30% rye-bran significantly reduced the number of colon tumours. Although there was no change in the total number of ACF at 12 weeks with the rye diet, the total number of large ACF was reduced. The authors concluded the lignans in the rye bran diet were likely to be responsible for this effect. McIntosh *et al* (1995) reported that there were no differences in the incidence of experimentally induced colon tumours in rats fed a whey-based diet (213 g/kg diet) than those fed a soybean diet (333 g/kg diet).

15.93 Hakkak *et al* (2001) investigated the effect of lifetime dietary supplementation with soy protein isolate (430 mg total isoflavones/day), compared to a casein diet on chemically induced colon cancer in F_2 generation male rats. Rats fed soy protein isolate had a reduced incidence of colon tumours compared to those receiving a casein based diet.

15.94 Studies using rodent models of colon cancer have shown that flaxseed can reduce the number of chemically induced aberrant crypts, which are considered to be early markers of the disease (Jenab & Thompson, 1996; Serriano & Thompson, 1992; Thompson, 1998). Davies *et al* (1999) reported that a rye-bran diet, containing lignans, produced a highly significant inhibitory effect on the number of experimentally-induced colon tumours in rats.

In vitro studies

15.95 Park *et al* (2001) demonstrated that genistein (10-60 μM) inhibited the growth of human colon cancer cells in a dose-dependent manner. Genistein also caused cell cycle arrest at the G_2/M phase and increased p21 protein expression and activated p21 promoter reporter constructs. Arai *et al* (2000) have also reported that genistein (10 μM) had a slight inhibitory effect on the growth of human colon cancer cells. Salti *et al* (2000) reported that genistein has a concentration dependent effect on colon cancer cell growth. Genistein increased apoptosis and caused G_2/M cell cycle arrest at concentrations $\geqslant$60 μM. Sung *et al* (1998) demonstrated that enterodiol and enterolactone (100 μM) inhibited cell proliferation of human colon tumour cell lines (LS174T, Caco-2, HCT-15 and T-84) when treated for 8-10 days. However, these cell lines were shown not to be oestrogen responsive.

Key points

- Epidemiological studies in humans have suggested that consumption of non-fermented soy products may lower the risk of colorectal cancer. Whereas, consumption of fermented soy products was generally associated with an increased risk. To date, no studies have specifically examined the effect of phytoestrogens.
- The research in animal models on isoflavones has also produced conflicting data, with studies showing that genistein had a protective or no effect against chemically induced cancer or modulated the levels of biomarkers of the disease.
- Studies suggest that dietary lignans may result in potentially beneficial changes in animal models of colorectal cancer.
- *In vitro* data are sparse but show that genistein interacts with cell cycle processes to inhibit the growth of human colon cancer cells.

Stomach cancer

Human studies

15.96 Epidemiological studies exploring the relationship between soy consumption and the risk of stomach cancer have provided inconsistent results. However, an increased risk has most often been associated with the consumption of fermented soy products, whereas, protective effects have been associated with the consumption of unfermented soy products (reviewed by Messina *et al*, 1994). This is supported by a meta-analysis by Wu *et al* (2000) of 14 studies which showed that stomach cancer was associated with high intakes of fermented soy foods (OR= 1.26; 95% CI= 1.11-1.43). In contrast, a meta-analysis of 10 studies showed that stomach cancer was inversely associated with consumption of soy foods (OR= 0.72; 95% CI= 0.63-0.82).

15.97 A study by Nagata (2000) demonstrated a significant inverse correlation between the rate of mortality from stomach cancer and consumption of soy protein in Japanese men (p= 0.04). Non-significant inverse correlation's were also noted for total soy product (p= 0.07) and isoflavone (p= 0.08) intakes. Analysis of the food intake of households in 1040 census tracts in Japan demonstrated a significant inverse association between stomach cancer and tofu consumption in men and women. Following multivariate analysis, a non-significant inverse association between consumption of miso soup and stomach cancer was also noted in the women from this study (Nagai *et al*, 1982). A large-scale prospective study (Hirayama, 1982, 1984 and 1986) which followed 122261 men and 142857 women for 13 years, demonstrated significant inverse associations between consumption of soybean paste and the risk of gastric cancer. In addition, ten case-control studies have reported significant inverse associations between stomach cancer and consumption of miso soup (Segi *et al*, 1957), soybeans (You *et al*, 1988; Sasaki *et al*, 1990; Ji *et al*, 1998; Gao *et al*, 1999), bean curd (Haenszel *et al*, 1972 and 1973; Lee *et al*, 1995; Huang *et al*, 2000) and soy-milk (Yingman & Songlin, 1986).

15.98 A cohort study in 7990 American men of Japanese ancestry living in Hawaii found no significant association between ingestion of miso soup or tofu and risk of gastric cancer. However, a protective trend associated with tofu consumption was noted (Nomura *et al*, 1990). In addition, the results from five case-control studies found no significant association between risk of stomach cancer and consumption of miso (Hirayama, 1971), bean curd (Tajima & Tominaga, 1985; Lee *et al*, 1995) or unfermented soy products (Hoshiyama & Sasaba, 1992; Ahn, 1997).

15.99 Hirayama (1971) reported that fermented soybeans increased the risk of developing stomach cancer, whereas bean curd decreased the risk. However, no significant associations between the consumption of soybeans, fried bean curd or bean paste and gastric cancer were demonstrated. Five further case-control studies also demonstrated an association between the consumption of miso soup or fermented soy paste and an increased risk of stomach cancer (Crane *et al,* 1970; Hoshiyama & Sasaba, 1992; Hu *et al*, 1991; Ahn, 1997; Lee *et al*, 1995).

Animal studies

15.100 Studies evaluating the effect of soy products or isoflavones on experimentally induced stomach cancer have yielded conflicting results. A study by Tatsuta *et al* (1999) demonstrated that subcutaneous administration of genistein (30 mg/kg bw) to rats every other day for 25 weeks significantly reduced the incidence of experimentally induced gastric cancer. Genistein also significantly decreased the labelling index and vessel counts of the antral mucosa and gastric cancers and significantly increased the apoptotic index. Kim *et al* (1985) demonstrated that inclusion of 10% maejoo (Korean fermented soybean paste) in the diet of rats significantly inhibited experimentally induced stomach tumours. However, the study by Watanabe *et al* (1999) demonstrated that inclusion of 10% miso in the diet of rats had no effect on the number of stomach tumours induced by X-ray irradiation.

In vitro *studies*

15.101 Matsukawa *et al* (1993) reported that treatment of human gastric cancer cells with genistein resulted in a dose-dependent inhibition of cell growth. Further analysis showed that genistein (40 μM) arrested the cell cycle at the G_2/M phase. Cell cycle arrest was reversed following removal of genistein from the culture medium. Daidzein did not arrest cell cycle progression at the G_2/M phase. Yanagihara *et al* (1993) showed that genistein and biochanin A inhibited growth of human stomach cancer cell lines by activation of apoptosis.

Key points

- Most of the published epidemiological studies have examined the association between soy products and the risk of stomach cancer, with only one study specifically investigating the effect of phytoestrogens.
- Currently, the epidemiological data linking soy consumption to risk of stomach cancer are inconsistent. A reduction in risk has most often been associated with unfermented soy products or isoflavones. Other published studies showed either no effect or an increased risk associated with the consumption of fermented soy products.
- In contrast, animal studies suggest that fermented soy products either significantly inhibited or had no effect on the number of experimentally induced tumours. Only one study has examined phytoestrogens and showed that genistein significantly reduced the incidence of experimentally induced gastric cancer.
- Results from *in vitro* studies suggest that the isoflavones genistein and biochanin A can inhibit growth of human gastric cancer cell lines either by cell cycle arrest or induction of apoptosis.

Lung Cancer

Human studies

15.102 Lung cancer is the leading cause of cancer-related deaths in the world. Although there are epidemiological data to suggest consumption of soy products has a protective effect in lung cancer, the information is limited. A protective effect for tofu was reported by Swanson *et al* (1992) who investigated the relationship between diet and lung cancer among male residents with primary lung cancer (n= 428) living in a mining community in Yunnan Province, China. Participants were asked to recall eating habits during adult life and report usual intake (frequency in times per day, week, month or year) of 31 food items or food groups. The results showed a inverse dose-response relationship between consumption of bean curd ($p < 0.01$) and risk of lung cancer after adjustment for confounding factors. A retrospective study in non-smoking, female lung cancer patients (n= 88) and controls (n= 137) living in Hong Kong, also demonstrated a significant inverse association between consumption of tofu/soy bean products and lung cancer risk after adjustment for confounding factors. In this study, the protective effect was greater in those with adenocarcinoma or large cell lung tumours (Koo, 1988).

15.103 Three case-controls studies reported inverse associations between soy intake and lung cancer. Takezaki *et al* (2001) reported that consumption of tofu $\geqslant$ 5 times/week reduced the risk of lung adenocarcinoma in women (OR= 0.52; 95% CI= 0.30-0.91) but not men (n= 367 cases and n= 381 controls). In a case-control study decreased risk of lung carcinoma was associated with daily tofu consumption in men (OR= 0.55; 95% CI= 0.34-0.89) and women (OR= 0.14; 95% CI= 0.02-0.89) (Wakai *et* al, 1999). A case-control study by Seow *et al* (2002) demonstrated that consumption of soy foods had a protective effect amongst lifetime non-smoking Chinese women living in Singapore (n= 176 cases; n= 663 controls). The OR for women in the highest tertile compared to the lowest was 0.53 (95% CI= 0.34-0.81). A protective effect was not observed amongst women who were smokers (n= 127 cases; n= 102 controls). Isoflavone intake was also calculated based on frequency of intake and portion size of eight common local soy foods. The adjusted OR among non-smokers for the highest tertile compared to the lowest was 0.56 (95% CI= 0.37-0.85).

15.104 In contrast, two case-control studies in lung cancer patients in China found no significant protective effect between the consumption of soybean products and the risk of lung cancer (Wu-Williams *et al*, 1990; Hu *et al*, 1997).

Animal studies

15.105 The results from animal studies have yielded more consistent results. Lee *et al* (1992) demonstrated that intraperitoneal administration of biochanin A (0.125 mg/day) 3 times/week for 6 weeks significantly prevented the development of chemically induced lung tumours in mice.

15.106 In addition, results from three studies suggest that dietary supplementation with isoflavones can lower experimentally induced lung metastasis. Li *et al* (1999) demonstrated that dietary supplementation with isoflavones (120 mg/kg diet) significantly inhibited the number of pulmonary metastases induced by murine melanoma cells in male mice. Cross-sectional area and tumour volume were also reduced. Similar findings were reported by Yan *et al* (1997) following dietary supplementation with a soy protein isolate (200 mg isoflavones/kg diet).

15.107 Menon *et al* (1998), demonstrated that orally administered genistein (54 mg/kg bw) significantly inhibited the formation of lung tumour metastases caused by melanoma cells in male mice. Levels of lung collagen hydroxyproline and serum sialic acid levels, a marker of metastasis, were also lowered following treatment with genistein. These effects were not observed following administration of daidzein. Both daidzein and genistein were shown to increase the lifespan of mice by 16% and 47.7% respectively. However, lifespan was further increased by 70%, following treatment with genistein (70 mg/kg bw).

15.108 Studies have also examined the effect of lignans on experimentally induced tumour metastasis to the lung. Mice were fed 2.5, 5 or 10% (w/w) flaxseed diets (equivalent to doses of 0.22, 0.44 and 0.94 mg SDG/day) for two weeks (Yan *et al* 1998). Animals continued on these diets for two weeks after i.v. injection with murine melanoma cells. The 5 and 10% flaxseed diets reduced the number, growth and volume of pulmonary tumours.

15.109 In a study by Li *et al* (1999d), mice were fed a diet supplemented with SDG at concentrations equivalent to 2.5, 5 and 10% (w/w) flaxseed. Animals continued on these treatments for a further two weeks after i.v. injection with murine melanoma cells. A reduction in the number of pulmonary tumours was reported in animals fed the highest dose of SDG. Dietary supplementation with SDG also decreased tumour size and volume in a dose-dependent manner.

In vitro studies

15.110 Lian *et al* (1998) reported that treatment with genistein (30 μM) resulted in dose-dependent inhibition of cell proliferation in human lung cancer cells. In addition, genistein was found to have arrested cell cycle progression at the G_2/M phase, caused up-regulation of $p21^{WAF1}$ expression and induced apoptosis. Similar results were also reported by Lian *et al* (1999) using human lung cancer cell lines expressing either a wild-type or a mutated p53. This study also found genistein increased expression of wild-type p53 whilst the level of mutant p53 remained unchanged. The apoptosis gene Bax was also found to be up-regulated whereas Bcl-2 levels remained unchanged.

Key points

- A single study has investigated the effect of phytoestrogens in lung cancer, which showed that isoflavone consumption was weakly associated with a reduction in lung cancer risk in a Chinese population. Other published studies in Chinese populations, which examined the effects of soy rather than phytoestrogens also reported an inverse association between soy consumption and the risk of lung cancer.

- Animal data have shown that genistein can inhibit development of experimentally induced lung tumours and genistein and secoisolariciresinol may inhibit experimentally induced metastasis of melanoma cells to the lung.

- Genistein has been shown to act as an inhibitor of cell growth in lung cancer cell lines and can induce apoptosis.

Main Summary

15.111 It is generally accepted that lifetime exposure to oestrogen is related to the risk of developing breast cancer. It has also been suggested that exposure to oestrogen during development or early life may play an important role in programming hormonal homeostasis and may influence the risk of developing cancer later in life.

15.112 Most studies on breast cancer (and other cancers) have not addressed the possibility that exposure to phytoestrogens at an earlier life stage or over several life stages may confer protective changes. Although the weight of evidence in humans supports a protective role for soy in terms of breast cancer risk, the human and animal data for phytoestrogens are conflicting.

15.113 Epidemiological and migrant studies have suggested racial characteristics and other factors including lifestyle, diet and fat or fibre intake may play a role in the development of cancer. Genetic polymorphisms in combination may also make a contribution to an individual's susceptibility to cancer. The interpretation of epidemiological studies is complicated also by the paucity of data on phytoestrogen concentrations in foods and dietary intakes.

15.114 The epidemiological evidence, albeit limited, suggests a protective role for soy-based foods in endometrial cancer. No studies have specifically addressed the role of phytoestrogens.

15.115 Human studies have investigated the effect of soy intake and reported prostate cancer risk, which appear to differ between Eastern and Western populations. A single study in humans has specifically investigated phytoestrogens. This study showed a reduced risk associated with consumption of foods containing coumestrol and daidzein. Animal data suggest that diets supplemented with soy or isoflavones may inhibit the development of implanted, spontaneous and chemically induced tumours of the prostate in rodents.

15.116 There is no evidence from human studies to attribute the protective effect of fibre, fruit and vegetables against colorectal and stomach cancer to their phytoestrogen content. In addition, there is little or no evidence to associate phytoestrogen intake with a reduced risk of cancer of the ovary or lung.

15.117 The data from the many epidemiological studies is limited in terms of assessing the effect of dietary phytoestrogens in the UK population. This is due to the fact that many have been conducted in Eastern (e.g. Chinese or Japanese) populations and may be confounded by ethnic differences and other dietary factors. Thus, while *in vitro* studies and investigations in animals may provide some suggestions that these compounds have protective effects for some cancers, the clinical data to convincingly support these claims are not available.

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16. International policy on soy-based infant formula

Introduction

16.1 Soy-based infant formulae are used in many countries. Following concerns that have arisen as a result of using soy-based formulae, a number of countries have issued advice regarding its use and infant feeding guidelines. These guidelines are summarised below. To date, the United Kingdom (including Northern Ireland) and New Zealand are the only countries to have issued advice with specific reference to phytoestrogens and soy-based infant formula.

United Kingdom

16.2 In 2002, figures published by the Department of Health estimated that in the UK, soy-based formulae are fed to approximately 1% of non-breast fed infants aged 4-10 weeks rising to approximately 2% of infants aged 10-14 weeks (Department of Health, 2002). The Department of Health advised that although breastfeeding was preferred, where this was not possible, infant formulae could provide an alternative source of nutrition. For the majority of bottle-fed infants cows' milk formula is suitable. However, soy-based infant formulae can be given to the small number of infants who cannot tolerate cows' milk formula. Parents who are seeking a vegetarian or vegan diet may also choose to feed their infants a soy-based formula.

16.3 In 1996, the Committee on Toxicity of Chemicals in Foods, Consumer Products and the Environment (COT) reviewed phytoestrogens with particular reference to soy infant formula and published a statement. The COT endorsed advice issued by the Department of Health that breast milk and cows' milk formulae are the preferred sources of nutrition for infants. However, they recommended that women who had been advised by their doctor or other health professional to feed their baby soy-based formulae should continue to do so (Department of Health, 1996).

16.4 The advice issued by the COT was subsequently endorsed by the Food Advisory Committee (FAC) although the FAC also recommended that, as a precautionary measure, infant formulae manufacturers should investigate ways to reduce the levels of phytoestrogens in soy-based infant formulae (MAFF, 1996). In 1999, the Panel of Child and Maternal Nutrition (Committee on Medical Aspects of Food and Nutrition Policy, 1999-2000) subsequently endorsed this recommendation. However, they noted that the clinical grounds for recommending a soy-based formula to parents were diminishing, as other more suitable hydrolysates based on cows' milk were becoming available.

New Zealand

16.5 In 1998, the Ministry of Health in New Zealand recommended that soy-based infant formula should only be used under the direction of a health professional for specific medical conditions including proven cows' milk protein intolerance/allergy, lactose intolerance, in the absence of soy protein intolerance/allergy, and galactosaemia.

16.6 It was also recommended that clinicians be made aware of the potential interaction between soy infant formula and thyroid function and that assessment of thyroid function should be considered if satisfactory growth and development is not achieved or maintained. It was also recommended clinicians monitor thyroxine replacement in infants with hypothyroidism, as these infants may require a higher than usual dose of thyroxine to maintain a euthyroid state (New Zealand Ministry of Health, 1998).

United States

16.7 In 1998, it was estimated that up to 25% of infants in the USA were fed soy-based infant formula. At this time, the American Academy of Paediatrics (AAP) recommended that soy-based formulae were a safe and effective alternative for infants if their nutritional needs were not being met by either breast milk or cows' milk formulae. The AAP also recommended the use of soy-based formula for infants with galactosaemia, hereditary lactase deficiency or for infants whose parents were seeking a vegetarian or vegan diet.

16.8 However, the AAP recommend that soy-based infant formula not be routinely used for the prevention of either infantile colic or atopic disease. In addition, it recommended against feeding soy infant formula to infants with documented cows' milk protein-induced enteropathy or enterocolitis or to pre-term infants under 1800 g (American Academy of Paediatrics, 1998).

Republic of Ireland

16.9 In 1992, it was estimated that in Ireland 5% of infants aged between 3-9 months were being fed either a soy-based infant formula or a hydrolysate formula. In 1997, the Infant Feeding Sub-Committee of the Food Safety Advisory Board recommended that 'specialised' products such as soy-based infant formulae should only be fed to infants under medical supervision for the treatment of transient lactose intolerance, galactosaemia and IgE mediated cows' milk allergy.

16.10 The Committee also suggested that soy-based infant formulae could be used as an alternative to breast milk or cows' milk formulae in infants whose parents wished to feed their child a vegetarian or vegan diet, although medical supervision was recommended in these cases to avoid the development of nutritional deficiencies.

16.11 Soy-based infant formula was not recommended for routine use in infants as mineral absorption is less predictable and the precise physiological effect of high concentrations of aluminium and phytoestrogens from soybeans is unknown. In addition, the Committee recommended that soy infant formulae are not suitable for pre-term infants. Furthermore, the Committee recommended against using soy infant formula in cases of cows' milk protein induced enterocolitis or enteropathy. Nor did it recommend the use of soy infant formula for the treatment of colic or as a preventive measure in atopic disease (Food Safety Authority of Ireland, 1999).

Australia

16.12 The Australian College of Pediatrics (ACP) revised its Position Statement on soy infant formula in 1998 and recommended that the only conditions in infancy for which soy-based infant formula should be used are galactosaemia and lactose intolerance. The ACP advised against the use of these formulae for infants with unproven symptoms of cows' milk protein intolerance or as a prophylactic in infants thought to be at risk of developing allergy (Australian College of Paediatrics, 1998).

Canada

16.13 In 1998, it was estimated that 20% of infants in Canada were fed soy-based formulae either because of a real or perceived allergy to cows' milk protein. At this time the Joint Working Group of the Canadian Paediatric Society, Dieticians of Canada and Health Canada issued a joint statement on nutrition for healthy term infants from birth to 24 months of age, intended for use by health care professionals. They recommended that soy-based infant formulae be fed only to those infants who cannot be fed dairy-based products for health, cultural or religious reasons including galactosaemia or vegan lifestyle. Vegan infants who are not breast-fed and infants unable to take cows' milk products should continue to be fed commercial soy formula until 2 years of age. They recommended against the use of soy infant formula in the management of infants with an allergy to cows' milk protein or for the prevention of atopic diseases (Health Canada, 1998).

Switzerland

16.14 In 1997, the Swiss Federal Commission on Food issued an information sheet directly to Pediatricians. The Commission recommended that except for infants with lactose intolerance, galactosaemia and allergy/intolerance to cows' milk protein, soybean products should not be routinely used in food prepared for healthy infants. In addition, the Commission recommended against the use of soy infant formula for ideological or ethical reasons (Tonz & Zimmerli, 1997).

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17. Summary

Chemistry of phytoestrogens

17.1 Phytoestrogens are natural constituents of many plants, seeds and grains and as such, they are present in many foodstuffs. The four principal groups of phytoestrogens found in food are the isoflavones, coumestans, prenylated flavonoids and lignans.

17.2 Measuring phytoestrogen concentrations in food and biological samples has proved difficult and reports on the content in foodstuffs have varied widely. The process has been further complicated by the findings that species, strain and cultivation conditions can significantly influence the phytoestrogen content. Although early studies used inadequate methods of analysis, these have improved as interest in the compounds has grown. As such, the results from more recent studies are likely to be more reliable.

17.3 Current analytical methods combine chromatographic separation and mass spectrometry. However, until recently, they have been limited by the lack of suitable analytical standards. Standards for genistein and daidzein have now been developed allowing accurate measurement of these compounds in foods and biological matrices and these compounds have, by comparison to other phytoestrogens, been relatively well studied.

17.4 In plants, notably soy, isoflavones are present as glucosides. Processing reduces the isoflavone content and can partially convert them to aglucones. The analysis of isoflavones as they appear in food has been precluded by the lack of suitable standards and concentrations are usually reported in terms of the aglucone. Analytical methods for phytoestrogens, other than the isoflavones, are less developed.

Phytoestrogen intake

17.5 Comparison of estimated dietary isoflavone intakes in Western and Eastern (e.g. Japanese and Chinese) populations illustrate that Eastern populations have a significantly higher intake of phytoestrogens. These differences are generally attributed to the usage and higher consumption of soy and soy-based foods. Estimates suggest the average Japanese consumer is exposed to approximately 25-100 mg isoflavones/day. There is only limited information on the consumption or concentrations of phytoestrogens in the UK diet. Thus, the estimates of phytoestrogen intake are uncertain. Estimates of isoflavone intake indicate an average UK consumer will ingest approximately 1 mg/person/day. However, on the basis that a significant proportion of processed foods within the UK contain soy products, it is likely that this figure is an underestimate.

17.6 In addition, particular subgroups may have higher intakes of isoflavones than the average consumer. This may include infants fed soy-based infant formulae (approximately 40 mg isoflavones/day), vegetarians consuming soy-based meat- and dairy-replacement foods (approximately 3 mg isoflavones/day). Consumers of dietary supplements or soy-rich diets are also expected to have higher isoflavone exposures. Although intake estimates for these subgroups are currently unavailable, when consideration is made on a body weight basis, infants fed soy formula are likely to have the highest exposure to isoflavones (approximately 4 mg/kg bw/day).

17.7 In 2002, figures published by the Department of Health estimated that in the UK, soy-based infant formulae are fed to approximately 1% of non-breast fed infants aged 4-10 weeks rising to approximately 2% of infants aged 10-14 weeks. However, detailed information on the prevalence of, and reasons for, soy-based infant formula feeding is unavailable. In comparison, it has been estimated that this figure is as much as 25% in the US. Although soy has, for many centuries, played an integral part in some Eastern (e.g. Chinese and Japanese) cultures as a foodstuff, it is not generally fed to infants in the first 4-6 months of life in these populations. Soy-based infant formula contains relatively high concentrations of phytoestrogens (18-41 mg/L made up formula) and mean intakes are estimated at 4 mg/kg bw/day, although other studies have indicated that consumption may be higher. The isoflavone content of human breast milk varies with maternal diet but the concentration is several orders of magnitude lower than that of soy formula fed infants (2-32 μg/L for vegan/vegetarian mothers). Whereas, commercially available cows' milk-based formula contains almost undetectable levels of isoflavones.

17.8 Foods and dietary supplements rich in phytoestrogens are increasingly marketed on the basis of their potential health benefits. In 2000, the value of the European market for isoflavones was estimated to be £64 million (€106 million). Dietary supplements containing isoflavones are an expanding market and represent 9% of the phytonutrient market value. Research from market analysts predicts that the combined phytonutrient market will increase to an estimated £98 million (€163 million) by 2008. Exposure through dietary supplementation could increase individual exposure. It is possible that exposures from these sources may equate to those of infants fed soy-based infant formula for products particularly rich in phytoestrogens.

Absorption, distribution, metabolism and excretion of phytoestrogens

17.9 Isoflavones and lignans are ingested mainly as glucosides and undergo hydrolysis by gut bacterial and mammalian enzymes. The deglucosylated compounds may then undergo further metabolism by the gut bacteria prior to absorption or excretion.

17.10 There is considerable inter-individual variation in the pharmacokinetics, metabolism and bioavailability of ingested phytoestrogens, which can be attributed, at least in part, to differences in gut microflora. In turn, the microflora may be influenced by factors including diet, antibiotic use, disease and stress. Other potential factors that may influence inter-individual variations relate to food matrix, age, gender and metabolic polymorphisms. Inter-individual differences in the gut microflora-mediated conversion of daidzein to the more oestrogenic metabolite, equol and of secoisolariciresinol and matairesinol to enterodiol and enterolactone, respectively have been reported. However, factors influencing these pathways have not been elucidated.

17.11 The data suggest that isoflavones and lignans are extensively conjugated to glucuronides and sulfates in the liver. This inhibits their ability to bind to oestrogen receptors. As a consequence, the oestrogenically active parent compounds are often relatively minor components in the blood. Isoflavones can also undergo enterohepatic recirculation.

17.12 The isoflavones and their metabolites are widely distributed within body fluids, although definitive tissue distribution studies have not been reported in humans. There is some evidence that placental transfer occurs during pregnancy in humans and concentrations similar to those in maternal plasma have been detected in umbilical cord plasma and amniotic fluid.

17.13 There is limited information on how phytoestrogens are handled in the newborn and infants. The pharmacokinetics of absorption in the neonate is unclear but it is likely to differ considerably from that of the adult, particularly as the gut microflora in neonates is not fully developed. Data on the levels of isoflavones in the blood of infants fed soy-based formula suggest that they can absorb isoflavones from such formula.

Oestrogenic activity of phytoestrogens

17.14 Phytoestrogens possess oestrogenic properties due to their structural similarities to the human hormone, oestradiol. Phytoestrogens may elicit their biological effects by binding to oestrogen receptors (ERs). Two oestrogen receptors, ERα and ERβ, have been identified. The ERs display different tissue distribution patterns and can mediate differing biological effects. Phytoestrogens have been found to bind to both ERs although a number of phytoestrogens (e.g. genistein, daidzein and coumestrol) appear to be more selective for ERβ.

17.15 Experimental studies suggest that phytoestrogens can also produce oestrogenic effects by modulating the concentrations of endogenous oestrogens. However, the evidence from *in vivo* studies for such mechanisms of action is limited.

17.16 The oestrogenic activity of phytoestrogens can be assessed by *in vitro* methods such as receptor binding, cellular proliferation and reporter gene assays. *In vivo* assays in animal models incorporate biological processes such as absorption and metabolism, which significantly influence oestrogenic potency. These models also incorporate mechanisms, which modulate the concentrations of endogenous oestrogens. For these reasons they provide a more holistic assessment of oestrogenic potency.

17.17 The relative oestrogenic potency of phytoestrogens is difficult to determine. However, if both *in vitro* and *in vivo* studies of phytoestrogen potency are taken together a rank order of oestrogenic potency may be estimated: oestradiol > coumestrol > genistein, equol > glycitein, 8-prenylnaringenin > daidzein > formononetin, biochanin A, 6-prenylnarinigenin, xanthohumol and isoxanthohumol. The oestrogenic potencies of the conjugated metabolites of phytoestrogens are much lower than those of the parent compounds.

Non-oestrogen receptor mediated effects of phytoestrogens

17.18 *In vitro* studies of phytoestrogens have indicated that they may have antioxidant properties and inhibit enzymes involved in the synthesis of thyroid hormones, as well as signal transduction, apoptosis, cell cycle and differentiation pathways. However, other than the inhibition of enzymes of thyroid hormone synthesis, the concentrations of phytoestrogens required to act by many by these mechanisms *in vitro* do not equate to dietary exposure in humans. Human studies suggest that supplementation of the diet with soy may produce antioxidant effects such as inhibition of LDL oxidation. However, these effects may not be attributable to the phytoestrogen content of soy.

17.19 There are limited data on the genotoxic potential of phytoestrogens. Studies have indicated that coumestrol and genistein may be genotoxic but at much higher concentrations than would be achieved *in vivo* from dietary exposure. A single study has investigated the *in vivo* mutagenicity of isoflavones. This study suggests that genistein at dietary levels is not mutagenic *in vivo*.

Effects of phytoestrogens on fertility and development

17.20 Studies on the effects of phytoestrogens on human development and fertility are very difficult to conduct for ethical and practical reasons. Hence most of the published research has been conducted in laboratory animals such as rodents and to a lesser extent in non-human primates. Significant species differences in sexual development and reproductive function between rodents, non-human primates and humans make the interpretation of *in vivo* experimental studies to humans extremely difficult.

17.21 The rodent data indicate that coumestrol, and to a lesser extent isoflavones and lignans, produce oestrogenic effects in both male and female rodents but effects may be more pronounced in the female rodent. Exposure during the neonatal, perinatal or prepubertal stages of development produce the most marked effects.

17.22 The rodent data on phytoestrogens are of limited use for risk assessment in humans as the significance of treatment-related alterations in end points such as advancement of vaginal opening and irregular oestrus cyclicity to humans is unclear.

17.23 Extrapolation of the data from rodent studies to humans is further complicated by the fact that the animal experiments have been carried out using much higher doses than are relevant to dietary exposures in humans. Additionally, many studies use the subcutaneous route of administration, which excludes the critical influence of gastrointestinal and hepatic metabolism.

17.24 Primate studies are more applicable in terms of human risk assessment but few have been published. Recent research has addressed the effect of soy infant formula on the sexual development of soy-based infant formula fed male infant marmosets. This study is still in its initial stages but has shown that dietary consumption of soy-based infant formula reduces the neonatal surge in testosterone and increases the number of testosterone producing cells (Leydig cells) in the testes, compared with male marmoset infants fed cows' milk formula. The human health implications of these results are unclear.

17.25 A single human study has specifically examined the effect of soy formula feeding on sexual development and fertility. The data do not provide evidence of obvious adverse clinical effects on sexual development or reproductive health of males and females, apart from small increases in the duration and discomfort of menstruation. However, this study was based on recall and did not involve any direct measurements of hormone levels or other parameters in the subjects.

The effects of phytoestrogens on the thyroid gland

17.26 Goitrogenic effects in infants fed soy flour-based infant formula were reported when it was first introduced in the 1960s. This problem was subsequently overcome by using soy protein isolate instead of soy flour thus, reducing the goitrogenic constituents of the formula and by supplementation of the formula with iodine. Since this change in processing and formulation there have been no reports of goitre in infants fed soy formula. Experimental data suggest that relatively high levels of dietary soy may have a goitrogenic effect in rodents deficient in dietary iodine. However, data from human studies suggest that dietary soy or isoflavones is unlikely to affect thyroid function in normal individuals with adequate iodine intake.

17.27 It is possible that the isoflavone component of soy-based infant formula may have the capacity to inhibit thyroid function in infants. However, it has not been established whether the levels of free isoflavones in the plasma of infants fed soy-based infant formula are sufficient to significantly influence thyroid function.

17.28 Due to the potential interactions between phytoestrogens and the thyroid gland, it is possible that the thyroid function of hypothyroid individuals consuming high levels of phytoestrogen- or goitrogen-rich foodstuffs and supplements may be adversely affected. In addition, there is a theoretical possibility that under circumstances in which the thyroid status of the mother is compromised, maternal exposure to high levels of phytoestrogens may impair normal development of the fetus.

17.29 Elevated levels of serum TSH, induced by goitrogens, have been associated with an increase in thyroid cancer in rodents. However, limited epidemiological evidence suggests that phytoestrogen exposure is not associated with thyroid cancer risk.

Effects of phytoestrogens on the central nervous system

17.30 Oestrogens are active in the central nervous system (CNS) and are thought to influence behaviour, movement, cognition, pain sensitivity, and protect against development of neurodegenerative diseases. Studies suggest the blood brain barrier effectively restricts phytoestrogen transfer to the CNS in adult rodents. However, despite this, relatively high dietary exposures to isoflavones in rodents have been shown to alter protein concentrations and structures in the brain as well as induce behavioural effects. The implications of these findings to humans are unclear.

17.31 Two studies have investigated the effect of soy or isoflavones on cognitive function in humans. One, a retrospective study, shows a weak association between consumption of a soy-based food (tofu) in mid-life and cognitive impairment in later life. However, this study is reliant on the accuracy of the tofu intake data and the results may have been influenced by inaccuracies resulting from the imprecise nature of the methodology employed. The other, a small placebo-controlled study, suggests relatively high short-term consumption of high levels of isoflavones may slightly improve memory.

Effects of phytoestrogens on the immune system

17.32 Oestrogens are involved in the development and maintenance of normal immune function. Rodent studies on the effects of isoflavones on immune function have produced inconsistent results, reporting stimulatory, suppressive or no effects on the immune system. The relevance of these observations for human health is uncertain.

17.33 One study of soy infant formula fed infants suggested isoflavones may adversely effect infant immunity. However, two more recent reports of a larger study indicate that there were no differences in immune response between infants fed soy formula and those receiving breast milk.

The effect of phytoestrogens on osteoporosis

17.34 Epidemiological studies indicate that bone mineral density is higher in populations consuming relatively large quantities of soy. Studies on rodent models of the menopause have consistently demonstrated that soy- or isoflavone-rich diets prevent bone loss. Dietary intervention studies in women have been short-term and limited in number. To date, the beneficial effects of phytoestrogens on bone mineral density and bone mineral content in postmenopausal women have been small but statistically significant and confined to the lumbar vertebrae, with the exception of one study that reported effects in the femoral neck and lumbar vertebrae. However, in short term studies, it is more likely that an effect will be seen on the spine, rather than the hip as the turnover of bone in the hip is slower than that in the spine. No studies have been performed in men. Large, long-term studies in humans would be required before the efficacy of phytoestrogen containing foods or supplements can be confirmed.

Phytoestrogens and the cardiovascular system

17.35 Lower rates of coronary heart disease mortality have been reported in populations that traditionally consume soy (such as the Japanese) which led support to the suggestion that phytoestrogens may have protective effects against cardiovascular disease. Epidemiological and intervention studies provide evidence that dietary soy can have a hypocholesterolaemic effect in humans. Intervention studies conducted in healthy and hypercholesterolemic subjects, with pure isoflavones have produced inconsistent results. Therefore, it is not possible to attribute the hypocholesterolaemic effect of soy to its phytoestrogen content. The effects of phytoestrogens on other factors important in the risk of cardiovascular disease such as blood pressure, thrombosis or atherosclerosis has not been extensively investigated. There is some limited evidence that flaxseed can have a hypocholesterolaemic effect.

Phytoestrogen modulation of endogenous hormones

17.36 Most data from studies investigating the effect of dietary supplementation of soy or isoflavones suggest that isoflavones produce weak oestrogenic effects in postmenopausal women. However, data on whether such supplementation provides relief from menopausal symptoms are inconsistent. Although studies have suggested soy may be beneficial in alleviating menopausal symptoms, especially if basal intake is low, or symptoms severe, the data are equivocal, as positive results are often not statistically significant and strong placebo responses are observed. Thus, at present, the weight of evidence does not strongly support the suggestions that soy or phytoestrogens alleviate menopausal symptoms. The poor penetration of isoflavones across the blood brain barrier may mitigate against centrally mediated modulation of endogenous hormone concentrations to reduce the number and severity of hot flushes.

17.37 It has been suggested that phytoestrogens may be protective against the potentially harmful effects of endogenous oestrogens (e.g. oestrogen-dependent breast cancer) by lengthening the menstrual cycle thus, reducing the lifetime exposure of women to these compounds. Data from studies on premenopausal women suggest that supplementation of the diet with soy or isoflavones may produce weak hormonal effects. However, the hormonal effects reported are inconsistent and few studies report increases in menstrual cycle length with dietary supplementation.

17.38 Reports of hormonal effects of dietary soy or isoflavone supplementation on men are inconsistent, showing either no or only weak oestrogenic effects.

17.39 It has been suggested that phytoestrogens may have a beneficial effect on some factors of diabetes. An intervention trial indicates that relatively high levels of dietary soy may improve some aspects of diabetes in postmenopausal women with type II diabetes. However, there may be factors in soy other than phytoestrogens, which aid glycaemic control. No studies have specifically looked at the effect of phytoestrogens on diabetes although an *in vitro* study suggests that genistein may increase insulin secretion by pancreatic cells. The relevance of these latter observations to humans is unknown.

The role of phytoestrogens in cancer

17.40 The incidence of a number of cancers, including those of the breast and prostate, has been found to be much higher in Western populations compared with that in Eastern countries such as Japan and China. Epidemiological and migrant studies have suggested that the relatively high consumption of soy-based products amongst Eastern populations may play a role in the aetiology of these diseases. However, the interpretation of epidemiological studies is complicated also by a number of confounding factors, including differences in lifestyle and diet and are constrained by the paucity of data on phytoestrogen concentrations in foods and dietary intakes.

17.41 It has been suggested that exposure to oestrogen during development or early life may play an important role in programming hormonal homeostasis and influence an individuals later life risk of developing cancer. Few human studies on cancer have addressed the possibility that exposure to phytoestrogens at an earlier life stage or over several life stages may confer protective changes. This possibility may, in part, explain why the reduced risk of certain cancers observed amongst migrants increases with subsequent generations.

Breast cancer

17.42 Most epidemiological studies have investigated soy rather than individual phytoestrogens in breast cancer. Of these, the majority of case control studies have shown a protective trend for soy foods, particularly in Asian women, but results from two prospective studies are conflicting. Studies using biomarkers of phytoestrogen intake have shown some protective effects of lignans, and case control studies have also suggested protective effects of isoflavones. There are no prospective studies in which accurate assessments of food intake and biomarkers of exposure to all phytoestrogens are available so there are insufficient data on which to confirm a causal association.

17.43 It is suggested that a reduction in lifetime exposure to oestrogen may lower the risk of breast cancer. The lower rates of breast cancer in Japanese and Chinese women have been associated with longer menstrual cycles which in turn has been associated with a soy-rich diet. The effects of phytoestrogens on menstrual cycle length have been investigated but current evidence suggests it is not possible to attribute alterations in menstrual cycle to phytoestrogen intake.

17.44 The majority of the epidemiological studies on breast cancer have examined the effects of soy rather than phytoestrogens specifically. Most studies report an inverse association between risk and soy consumption or excretion. However, some studies do not support such an association. Specific studies on the effects of phytoestrogens are also inconsistent.

17.45 In one study, short-term dietary supplementation has been shown to induce a weak oestrogenic effect in premenopausal women with breast disease as shown by modulation of the levels of the oestrogen responsive gene products apolipoprotein D and pS2 in nipple aspirate. However, no effect on breast cell proliferation was evident.

17.46 The animal data on breast cancer is conflicting. A number of studies have shown that genistein has a protective effect in animal models of chemically induced cancer. However, similar experiments using tumour implant models showed that genistein stimulated the growth of implanted mammary tumours both by dietary and subcutaneous administration. Studies in animal models with chemically induced mammary cancer suggest that dietary supplementation with the lignan, secoisolariciresinol may have a chemoprotective effect on breast cancer development although the results are not consistent. Animal studies suggest that exposure to phytoestrogens in early life inhibits development of breast cancer later in life.

Endometrial and ovarian cancer

17.47 Limited epidemiological evidence does not allow direct associations between phytoestrogen intake and endometrial or ovarian cancer risk to be made.

Prostate cancer

17.48 Epidemiological data from studies investigating the effect of soy intake on prostate cancer report inconsistent results. One study has shown an increased risk of prostate cancer is associated with the

intake of fermented soy foods such as miso. Other studies report either no effect or an inverse correlation with consumption of non-fermented soy foods. Only one study has specifically investigated phytoestrogens and found an association with a reduced risk of prostate cancer.

17.49 Research in rodents has specifically examined the effects of phytoestrogens on models of prostate disease. Generally, these studies have shown a protective effect either in tumour implant or chemically induced models of cancer. However, the concentrations used in these experiments are very high compared to likely dietary exposure levels in humans in the UK.

17.50 *In vitro* experiments have shown that phytoestrogens can modulate components of the cell cycle pathway and inhibit growth of prostate cancer cells and stimulate apoptosis. However, these effects were only found to occur at much higher concentrations than would be expected from normal dietary intakes.

Colorectal cancer

17.51 Epidemiological data suggests that consumption of non-fermented soy may lower the risk of colorectal cancer. In contrast, fermented soy products are associated with an increased risk of colorectal cancer. These studies have looked only at soy or soy products and no studies have specifically examined the effects of phytoestrogens. Experimental research in animal models have reported inconsistent results for isoflavones but the studies on lignans in animals appear more consistent, showing an inverse association between lignan consumption and risk of colorectal cancer.

Stomach cancer

17.52 Studies in humans have shown an inconsistent relationship between soy consumption and the risk of stomach cancer. However, as with colorectal data, an increased risk has most often been associated with the consumption of fermented soy products, whereas protective effects have been associated with the consumption of unfermented soy products. In contrast, animal studies suggest fermented soy products either significantly inhibit or have no effect on the number of experimentally induced tumours. Only one study has examined the effects of phytoestrogens and showed that genistein significantly reduced the incidence of experimentally induced gastric cancer.

Lung cancer

17.53 A single study has investigated the effect of phytoestrogens in lung cancer. This study showed that isoflavone consumption was weakly associated with a reduction in lung cancer risk in a Chinese population. Other published studies in Chinese populations, which examined the effects of soy rather than phytoestrogens also reported an inverse association between soy consumption and the risk of lung cancer. Animal data have shown that genistein can inhibit development of experimentally induced lung tumours and genistein and secoisolariciresinol may inhibit experimentally induced metastasis of melanoma cells to the lung.

18. Conclusions

18.1 The remit of the Working Group was to review phytoestrogens generally, rather than soy, specifically. However, the Working Group did consider the literature on soy to ensure inclusion of all relevant information.

18.2 Phytoestrogens are biologically active when administered to animals and humans and have been shown to elicit their effects via a number of mechanisms:

- Interaction with oestrogen receptors (ER) to modulate the expression of oestrogen-responsive genes[15].
- Inhibition of enzymes involved in oestrogen biosynthesis and metabolism.
- Modulation of thyroid hormone biosynthesis.
- Inhibition of protein kinases and interaction with components of the cell cycle as well as proliferation, differentiation and apoptosis pathways.
- Inhibition of topoisomerase.
- Antioxidant reactions.

Evaluation of risks and benefits of dietary phytoestrogens

18.3 Evaluation of the public health implications of phytoestrogens is complex as these compounds can elicit agonist and antagonist actions via the oestrogen receptor and non-oestrogenic effects, which are age, tissue and gender dependent. There are also significant inter-species differences in ADME and timing of sexual development making extrapolation of the effects seen in animals to humans complex.

18.4 Many of the reports on the benefits of consuming phytoestrogens are based upon observations in Eastern populations such as the Japanese and Chinese that have traditionally consumed soy. In addition, suggestions that dietary phytoestrogens do not pose significant health risks have been attributed to the lack of reports of adverse effects in these populations. However, it is uncertain whether data from Eastern populations can be extrapolated to Western populations, as there may be differences in how phytoestrogens are handled between such populations.

[15] Some of the mechanisms outlined hereafter may also be dependent on ER activity.

18.5 Given the level of complexity the Working Group *considered* it inappropriate to evaluate the public health implications of phytoestrogens to the population as a whole or communicate the implications in a single statement.

18.6 An evaluation of the risks and benefits of dietary phytoestrogens is critically dependent on the nature, timing, conditions and extent of exposure. However, currently detailed intake data for the UK population as a whole, or for specific subgroups of consumers, is very limited.

18.7 *In vitro* studies suggest that at physiological concentrations, interactions with oestrogen receptors are the primary cause of biological effects. *In vivo* studies support this view, as the principal biological effects observed on administration of phytoestrogens are similar to those of oestrogen and can be blocked by ER antagonists. Many experimental studies have used subcutaneous administration, which can significantly influence the biological activity of phytoestrogens. In addition, these studies, for the most part, use high concentrations of phytoestrogens. This makes interpretation difficult, as these experimental conditions are not equivalent to the level of dietary exposure in humans.

18.8 Many studies have used phytoestrogen-containing foods such as soy or flaxseed as a test material and assumed that phytoestrogens are responsible for the biological effects seen. However, it is impossible to exclude the possibility that there are other active components in these foods that could also contribute to the effects observed. Most research in the phytoestrogen field has focused on the isoflavones and thus, comparatively little is known about the prenylated flavonoids, coumestrol and lignans. Current analyses suggest that there are very few sources of prenylated flavonoids or coumestrol in the diet. However, the lignans are relatively common.

Does ingestion of soy-based infant formula pose any risk for human infants?

18.9 In the UK, soy-based infant formulae have been used since the 1960s and are currently fed to approximately 1% of non-breast fed infants aged 4-10 weeks rising to approximately 2% of infants aged 10-14 weeks. However, detailed information on the prevalence of, and reasons for, soy-based infant formula feeding is unavailable.

18.10 The concentration of phytoestrogens found in soy-based infant formulae is several orders of magnitude higher than that found in human breast milk. It has been estimated that intake by infants of isoflavones from soy-based formulae is approximately 4 mg/kg body weight/day. The Working Group *concluded* that infants fed soy-based formulae are the population subgroup exposed to the highest concentrations of isoflavones and that exposure via breast milk is low by comparison. No data on the transfer of lignans from the maternal diet to breast milk have been published.

18.11 There is little published information to suggest that isoflavones affect thyroid function in infants fed soy-based formulae. However, the Working Group *considered* that isoflavones may lower free thyroxine concentrations. Although a normally functioning thyroid may compensate for this, by stimulating thyroxine production, it is possible that infants with congenital hypothyroidism may be unable to

increase thyroxine production. These individuals may represent a small susceptible subgroup of the population, therefore the Working Group *recommends* that physicians and other health care workers are made aware of the potential interactions between isoflavones in soy-based infant formulae and thyroid function. The Working Group *advise* that it is appropriate to monitor thyroxine levels in infants with congenital hypothyroidism who are fed soy-based infant formulae in order to establish the susceptibility of this subgroup.

18.12 Few studies have examined the effect of isoflavones on the immune system. Studies in rodents have suggested that isoflavones may alter some parameters of immune function but the effects were inconsistent. However, the Working Group *considered* that investigations of human infants fed soy-based formulae provide reassurance that phytoestrogens in soy do not have a significant impact on the integrity of immune function in such children.

18.13 A recent study conducted in male neonatal marmosets suggests that feeding with soy-based infant formulae can alter some parameters of reproductive health during the neonatal stage. The Working Group *acknowledged* that this work is still in progress, and therefore, no definitive conclusions can be made about likely human health implications. The Working Group *advise* that future findings from this work be evaluated fully once it has been completed.

18.14 Only a single study specifically examining the long-term health effects of soy-based formula feeding on sexual development and fertility in humans has been published. The Working Group *considered* that these data do not provide definite evidence for adverse clinical effects on sexual development or reproductive health, but *noted* the association between soy-based formula feeding and small increases in the duration and discomfort of menstruation. However, the study was based on recall and did not include any direct measurements of hormone levels or other parameters in the subjects. The Working Group *acknowledged* that it was difficult to draw general conclusions from the results of a single study.

18.15 The Working Group *considered* that the findings from these studies do not provide definitive evidence that phytoestrogens present in soy-based infant formulae can adversely affect the health of infants. However, the findings, together with those from studies on the mechanism of action and biological activity of phytoestrogens reviewed in this Report, provide evidence of potential risks. For this reason, the Working Group *expressed* concern about the use of soy-based infant formulae. The Working Group *noted* that the Scientific Advisory Committee on Nutrition (SACN) *expressed* similar concern when considering evidence presented in this Report. SACN also *considered* there to be no substantive medical need for, nor health benefit arising from, the use of soy-based infant formulae. However, it was *noted* that soy-based infant formulae were the only vegan infant formula option available if babies were not exclusively breast fed[16]. In light of the concerns expressed, the Working Group *recommends* that the Department of Health review current advice on the use of soy-based infant formulae.

[16] Scientific Advisory Committee on Nutrition response to the COT Working Group on Phytoestrogens draft report on phytoestrogens & health (2003).

Are there health implications for other subgroups of the population?

18.16 The Working Group *considered* that it was of more value to identify and characterise health implications in specific population groups rather than provide an overall evaluation for the general population. At present, there are only limited data on the intake of phytoestrogens by specific population groups in the UK. However, those consuming a vegetarian or vegan diet may ingest larger amounts of soy, an assumption supported by what intake data are available. The Working Group has identified a number of population subgroups that may be expected to have a higher than average intake of phytoestrogens:

- Vegetarians and vegans (isoflavones and lignans).
- Particular ethnic groups e.g. Japanese and Chinese (isoflavones).
- Consumers of soy-based foods (isoflavones).
- Consumers of phytoestrogen-containing dietary supplements (mostly isoflavones).

18.17 The Working Group *noted* the possibility that exposure among these subgroups will vary due to the large inter-individual differences in metabolism and bioavailability of phytoestrogens and in particular, differences in gut microflora. Specific gut microflora are responsible for the conversion of daidzein to the more potent oestrogen, equol. Thus, equol-producing individuals would be expected to be exposed to a greater oestrogenic potential than non-equol producers.

18.18 Dietary supplements containing phytoestrogens and soy-enriched foods are commercially available and are promoted as having beneficial health effects on human health. Phytoestrogen supplements are marketed as 'natural' alternative treatments for a range of conditions including the menopause, osteoporosis, cardiovascular disease and a number of cancers. Specific marketing for these conditions may lead to increased consumption within certain population subgroups adding significantly to consumer exposure. However, at present, it is not possible to estimate the impact on the consumer exposure, as few data on the phytoestrogen concentrations in or consumption patterns of these products are available.

18.19 Isoflavones and lignans can cross the placenta after metabolism in the mother. However, it is not known how the fetus metabolises these compounds and there are no published human studies examining the potential effects of *in utero* exposure to phytoestrogens. Therefore the implications of this exposure are unclear. The Working Group *advise* that further research be conducted to examine the implications of consuming a phytoestrogen-rich diet during pregnancy.

18.20 The Working Group *identified* individuals with hypothyroidism as a subgroup of potential concern. Consumption of phytoestrogen supplements, or a soy-rich diet, may provide sufficient concentrations of phytoestrogens to interfere with thyroxine replacement therapy. Although no adverse effects in hypothyroid children or adults have been reported in the published literature, the Working Group *recognised* that research had not addressed this issue specifically. In view of the increasing availability of phytoestrogen-rich food and supplements in the UK, the Working Group *recommends* that research is conducted to monitor the plasma thyroxine levels of children and adults with hypothyroidism who consume large quantities of dietary phytoestrogens.

18.21 The Working Group also *acknowledged* the theoretical possibility that under circumstances in which the thyroid status of the mother is compromised, maternal exposure to high levels of phytoestrogens may impair normal development of the fetus. The Working Group *recommends* research is carried out to address this issue.

18.22 Despite the suggested benefits of phytoestrogens in lowering the risks of developing breast cancer, a study has shown that soy supplementation of the diet can induce oestrogen-responsive gene products in nipple aspirates in premenopausal women with breast disease. Although breast cell proliferation was not evident in this study, the Working Group *suggested* that until further research is carried out, women with oestrogen-dependent breast disease should be cautious in supplementing their diet with phytoestrogen-rich foods or dietary supplements. However, the Working Group *considered* that the data are insufficient to allow a quantitative recommendation so far as the phytoestrogen intake of this population subgroup is concerned.

18.23 The Working Group *considered* an epidemiological study that suggested an association between high levels of consumption of soy-based foods and decreased cognitive function in a group of Japanese-American men and women. The Working Group *concluded* that this report did not provide sufficient evidence to confirm this association as the report lacked sufficient detail and the associations may have resulted from inaccuracies in the methods employed.

Evidence for beneficial effects of dietary phytoestrogens

18.24 Epidemiological data suggests a soy-rich diet is associated with a reduction in the risk of a number of conditions, including certain hormone-dependent diseases. However, the Working Group *considered* many of these studies to be of limited value because they do not address specifically the roles of phytoestrogens. Any reported effects from such research therefore cannot be attributed with certainty to phytoestrogens, as other biologically active components may be causally responsible for the effects observed.

18.25 In addition, many of the studies were short-term intervention studies in adults that did not address the possibility that exposure to phytoestrogens at an earlier age may influence the risk of disease later in life. Furthermore, a significant proportion of the research has been conducted in populations such as the Japanese and Chinese and thus, extrapolation of these results to the UK population may be confounded by differences in lifestyle, diet, gut microflora, genetic make-up and ADME.

Menopausal symptoms

18.26 Studies examining the effect of soy-based products or isoflavones to relieve menopausal symptoms are inconclusive. Some studies have suggested that soy may be beneficial, especially if basal intake is low, or the vasomotor symptoms severe, but the data are equivocal, as positive results are often not statistically significant and strong placebo responses are observed.

Osteoporosis

18.27 Clinical data on the effects of phytoestrogens on bone density are limited but results of short-term human studies suggest small protective effects in the lumbar spine. The data for protective effects at other sites are equivocal. However, studies using rodent models of the menopause have consistently demonstrated that soy- or isoflavone-rich diets prevent bone loss. Large, long-term intervention studies are required to evaluate these effects in humans.

Cardiovascular disease

18.28 There is a considerable body of evidence to indicate that consumption of soy can have beneficial effects on low-density lipoproteins and total cholesterol levels. There have been attempts to attribute these effects to the isoflavones in soy. However, purified isoflavones appear not to produce the same beneficial effects, and there is little evidence to suggest that this effect is associated with the isoflavone component of soy. The Working Group *noted* that the US Food and Drug Administration reached similar conclusions when examining this issue. The effects of phytoestrogens on other factors important in the risk of cardiovascular disease such as blood pressure, thrombosis or atherosclerosis have not been extensively investigated.

18.29 There is very little epidemiological data on lignans and cardiovascular disease. Such studies would be extremely difficult to design and conduct due to the prevalence of these compounds in fruit and vegetables.

Cancer

18.30 The Working Group *concluded* that there is some evidence for beneficial effects of phytoestrogens on the development of breast and prostate cancer based upon animal experiments. The findings in humans are less convincing. This may be due, in part, to the much higher doses used in the animal studies. The interpretation of epidemiological studies is complicated by a number of confounding factors, including differences in lifestyle and diet and is constrained by the paucity of data on dietary intakes of phytoestrogens.

18.31 It has been suggested that exposure to oestrogens or phytoestrogens during development *in utero*, in infancy or in childhood may play an important role in the programming of hormonal homeostasis and influence the risk of developing cancer later in life. This may, in part, explain why the relatively low risk of certain cancers observed among migrant populations from the East (e.g. Chinese and Japanese) increases in subsequent generations. The Working Group *recommends* that in order to establish the clinical efficacy of phytoestrogens in these conditions in humans, long-term studies should be undertaken.

Breast cancer

18.32 Studies examining the effect of isoflavones on breast cancer incidence are inconclusive. Prospective studies have failed to show significant associations between ingestion of soy or isoflavones and breast cancer incidence. Dietary intervention studies, using phytoestrogen supplements, have indicated changes in biomarkers that may be associated with a decreased risk of breast cancer.

Endometrial and ovarian cancer

18.33 A small number of studies have investigated the effects of phytoestrogens in these conditions and to date there is no evidence to support the suggestion that phytoestrogens have protective effects on the incidence of endometrial or ovarian cancer.

Prostate cancer

18.34 Studies investigating the relationship between phytoestrogens and human prostate cancer are too few to draw conclusions and are limited to studies of soy. Experimental studies in rodents show that diets supplemented with soy or isoflavones may inhibit the development of tumours of the prostate.

Colorectal cancer

18.35 Epidemiological data suggests that consumption of non-fermented soy may lower the risk of colorectal cancer. In contrast, fermented soy products are associated with an increased risk of colorectal cancer. No studies have specifically examined the effects of phytoestrogens. Data from rodent studies are conflicting and there is no firm evidence to suggest that phytoestrogens have beneficial effects on the incidence of colon cancer.

Stomach cancer

18.36 The evidence for protective effects of soy or isoflavones on stomach cancer in humans is inconclusive. Studies in Japanese and Chinese populations have shown higher rates of stomach cancer associated with fermented soy products. However, the high salt concentrations in such foods may contribute to this higher incidence in these populations.

Lung cancer

18.37 Epidemiological studies examining associations between phytoestrogen or soy intake and lung cancer in Chinese populations are inconclusive.

Recommendations for future research

18.38 The Working Group *recommends* further research to address important outstanding issues and to aid future risk assessment of dietary phytoestrogens. The Working Group *considered* that future research should be conducted in humans where possible. The following research priorities were identified.

- Detailed exposure studies of discrete populations in the UK who ingest relatively large amounts of phytoestrogens, such as infants, vegetarians/vegans and users of phytoestrogen-rich foods and supplements, would allow a more informed view of the health implications of phytoestrogens. The Working Group *recommends* that research examining the phytoestrogen content of food, as well as intakes of, and systemic exposure to, phytoestrogens is conducted.

- The extent and nature of soy-based infant formula use in the UK is uncertain. The Working Group *recommends* research to address these areas.

- The Working Group *considers* there is a need for further research on the potential effects of phytoestrogens in infants fed soy-based infant formulae. It may be possible to use established cohorts of infants fed soy-based formula to investigate the possible long-term health effects of exposure to phytoestrogens during infancy.

- The Working Group *considers* there is a need to investigate the potential interaction of phytoestrogens with the thyroid gland in subjects with compromised thyroid function.

- The Working Group *recommends* that further research be conducted to establish whether phytoestrogens act mainly by oestrogen receptor-mediated mechanisms or by alternative mechanisms.

- Large long-term prospective studies are necessary to establish the relationship between dietary phytoestrogens and the development of some diseases, specifically osteoporosis, breast cancer and prostate cancer. Shorter intervention studies are required to assess effects on menopausal symptoms and risk markers of diseases, such as osteoporosis and cancers. Such studies should consider an evaluation of the role of metabolites, especially equol, in the biological effects observed.

The Working Group also identified supplementary areas for future research.

- The health implications of *in utero* exposure to phytoestrogens are unclear. There is a need for research to examine what effects maternal exposure to phytoestrogens may have on the fetus and on subsequent health status of the child.

- The potential for drug-phytoestrogen interactions has not been established. This is of potential importance for individuals consuming phytoestrogen dietary supplements while taking prescribed drugs with hormonal effects.

- The potential differences in the metabolism of phytoestrogens between Western and Eastern populations has not been determined. Knowledge of the potential differences would aid assessment of epidemiological studies.

Appendix 1

Glossary of terms and abbreviations

Aberrant crypt foci	Pre-cancerous lesions of the colon.
Acceptable Daily Intake (ADI)	Estimate of the amount of a substance in food or drink, expressed on a bodyweight basis (e.g. mg/kg bodyweight), that can be ingested over a lifetime by humans without acceptable risk.
Accessory proteins	Proteins involved in the correct assembly of other proteins.
Acetyl	The chemical group (-$COCH_3$).
Acetylation	Addition of an acetyl group.
Acetyltransferase	An enzyme that catalyses addition of acetyl groups.
Activator protein 1	A transcription factor involved in signalling, growth control and apoptosis.
Acute	Short-term, in relation to exposure or effect.
Acute toxicity	Effects that occur over a short period of time (up to 14 days) immediately following exposure.
Adaptor	A short DNA sequence between gene segments.
Adenocarcinoma	A cancerous tumour of the walls of organs.
Adenoma	A benign tumour of the organ wall.
Adenomatous polyps	Small pre-cancerous growths in the colon.
Adenosine triphosphate	A phosphorylated nucleoside used by cells to store energy, which may be released during metabolic reactions.
ADI	See acceptable daily intake.
Adipose tissue	Fatty tissue.

Adverse effect	Change in morphology, physiology, biochemistry, growth, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increase in susceptibility to the harmful effects of other environmental influences.
Aetiology	The cause of disease and mode of operation.
AF-1	See transactivation function.
Affinity	A measure of the strength of binding of one molecule to another, e.g. of a ligand to a receptor or a substrate to an enzyme.
Aglucone	A compound which may be conjugated to form a glucoside.
Agonist	A compound which binds to a receptor to initiate a cellular response.
Alkaline phosphatase	An enzyme present in liver, bone and other tissues that catalyses the removal of phosphate groups from organic compounds.
Amniotic fluid	The fluid that surrounds a developing fetus.
Amygdala	A small region in the brain near the hippocampus.
Anaerobic reaction	A reaction occurring in the absence of oxygen.
Androgen	A male sex hormone.
Androstenedione	A male sex hormone.
Angiogenesis	The formation of new blood vessels.
Anhydrosecoisolariciresinol	A lignan found in plants.
Anogenital distance	Distance between the anus and the external genitalia, used as a measure of rodent development.
Antagonist	A compound that negatively affects the activity of an agonist.

Anti-atherogenic compound	A compound that counteracts degenerative changes in arterial walls.
Antibody	A proteins produced by the immune system which binds to a specific chemical structure.
Antitumor properties	Counteracting tumour formation.
Antral follicle	An ovarian follicle.
AP1	See activator protein 1.
APC^{Min}	A mutation (Min) within the mouse APC gene.
Apigenin	A flavonoid.
Apolipoprotein	A protein involved in lipid transport.
Apoptosis	The process of programmed cell death associated with normal cell turnover in animals. Inappropriate apoptosis may be a toxic response under certain circumstances.
Apoptotic index	The number of apoptotic cells per 1000 tumour cells.
Area under the curve	A mathematical measurement that describes the plasma concentration of a compound over time.
Aromatase	An enzyme which converts androgens to oestrogens.
Aromatase knockout animals	Animals genetically engineered not to possess active aromatase.
Arylhydrocarbon	An aromatic organic compound.
Arylsulfatase	An enzyme that catalyses the removal of sulfate from an aryl compound.
Atherogenic diet	A diet that causes degradation of arterial walls.
Atherosclerosis	A disease of the arteries in which fat-accumulates to obstruct the flow of blood.

Athymic	Having no thymus gland.
Athyreosis	A condition caused by absence of the thyroid gland.
ATP	See adenosine triphosphate.
Atresia	Degeneration of tissue.
Atrophy	A decrease in size or wasting away of a body part or tissue.
AUC	See area under the curve.
Autoimmune disease	A disease caused when the immune system attacks the body's own tissues.
Autonomic nervous system	Part of the nervous system that controls body functions.
Autophosphorylation	The addition of a phosphate group to an enzyme by that enzyme.
Auxiliary protein	A protein that helps another perform its function.
Base pair	Two complementary nucleotide bases joined positioned opposite one another in the DNA double helix.
Bax	Bcl-2 associated protein.
Bcl-2	B-cell lymphoma 2. A member of the Bcl-2 family of proteins that inhibit apoptosis.
Bean curd	See tofu.
Bean sprouts	The young shoots of mung or soy beans.
Benign	Not malignant or life-threatening.
Benzo[a]pyrene	A chemical carcinogen.
Bifidobacteria	Bacteria found in the human intestine.
Bilateral breast cancer	Cancer affecting both breasts.

Bile acids	Acids produced in the liver and secreted into the small intestine to aid in the digestion of fats.
Biliary excretion	Elimination of a chemical from the body in the bile.
Binding affinity	See affinity.
Binding cavity	The site in a receptor or enzyme where the ligand or substrate binds.
Bioassay	A biological assay, using a living organism or tissue.
Bioavailability	The proportion of a substance, which reaches the systemic circulation, unchanged after a particular route of administration.
Biochanin A	An isoflavone phytoestrogen.
Biomarker	Observable change (not necessarily pathological) in an organism, related to a specific exposure or effect.
Biostatistics	Statistical analysis that is used in the to aid the interpretation of biological data.
Biosynthesis	The formation of a biological compound.
Biotransformation	A series of chemical alterations of a compound which occur within the body.
Biphasic	Having two phases.
Blood brain barrier	A barrier that limits the passage of substances between the blood and brain tissue.
BMC	See bone mineral content.
BMD	See bone mineral density.
Body mass index	A measure of body fat that is the ratio of the weight (kg) to the square of its height (m).
Bone mineral content	A measurement of bone mass (g).

Bone mineral density	An indication of bone strength (g/cm^2).
Brain derived neurotrophic factor (BDNF)	A chemical that promotes survival and normal functioning of neurones.
Brassicaceae	A family of plants including cabbage, cauliflower, broccoli and brussel sprouts.
Calbindin	An intracellular protein that transports calcium across intestinal epithelial cells.
Calretinin	A calcium binding protein.
cAMP	See cyclic AMP.
Cancer	Synonym for a malignant neoplasm – this is, a tumour that grows progressively, invades local tissues and spreads to distant sites.
Carcinogenesis	The origin, causation and development of tumours. The term applies to benign as well as malignant neoplasia.
Carcinoma	A malignant tumour arising from epithelial cells lining, for example, the alimentary, respiratory and urogenital tracts and from epidermis, also from solid viscera such as liver, pancreas, kidneys and some endocrine glands (see also 'tumour').
Cardioprotection	Protection of the cardiovascular system from disease.
Cardiovascular system	The circulatory system of the heart and blood vessels.
Case-control study	(Synonyms – case comparison study, case referent study). A study that starts with the identification of persons with the disease of interest and a suitable control group of persons without the disease.
Castration	Removal or destruction of the testes or ovaries by radiation, surgery or drugs.
Cathepsin D	An intracellular protease.
Causality	The relationship between cause and effect.

Cdk2	See cyclin dependent kinase 2.
Cecum	A section of the gastrointestinal system.
Cell culture	A technique for growing cells under laboratory conditions.
Cell Cycle	The sequence of events between cell divisions. The cycle is conventionally divided into G0, G1, (G standing for gap), S (synthesis phase during which the DNA is replicated), G2 and M (mitosis).
Cell differentiation	The process of change from one cell type to another.
Cell proliferation	The process of cellular multiplication or growth.
Cerebellum	A part of the brain involved in muscle co-ordination.
Cerebral cortex	A region of the brain controlling movement and behaviour.
Cerebral ventricle	Ventricles located in the brain.
Cervical adenosis	A disease of the cervix.
c-fos	Proteins involved in intracellular signalling.
CHD	See coronary heart disease.
Chemoprevention	Relating to a compound or activity that will protect an organism from disease.
Cholesterol	A steroid involved in the formation of cell membranes and transport of fat around the body.
Chromatid	One of the usually paired and parallel strands of a chromosome.
Chronic effect	An effect which develops slowly and has a long-lasting course (often but not always irreversible).
Chronic exposure	Continued exposures occurring over an extended period of time, or a significant fraction of the life-time of a human or test animal.

Chrysin	A dietary flavonoid.
CI	See Confidence Interval.
c-jun	Proteins involved in intracellular signalling.
Clastogen	An agent that produces chromosome breaks and other structural aberrations such as translocation (qv). Clastogens may be viruses or physical agents as well as chemicals. Clastogenic events play an important part in the development of some tumours.
Clearance	Volume of blood or plasma, or mass of an organ, effectively cleared of a substance by elimination (metabolism and excretion) in a given time interval.
C_{max}	Concentration of compound giving maximum response in a biological assay.
CMO	Chief Medical Officer.
CNS	Central nervous system.
Cognitive function	Relating to mental functions such as memory, attention and communication.
Cohort	A defined population that continues to exist through time.
Cohort study	(Synonyms – follow-up, longitudinal, prospective study). The method of epidemiological study in which subsets of a defined population can be identified who may be exposed to a factor or factors hypothesised to influence the probability of occurrence of a given disease. An essential feature of the method is observation of the population for a sufficient number of person-years to generate reliable incidence or mortality rates in the population subsets. This generally implies study of a large population and/or study for a prolonged period of time.
Collagen	An insoluble fibrous protein found in animals. It is the chief constituent of connective tissue and bones.

Confidence interval	Statistical term denoting the range of values within which there is a specified probability (e.g. 95%) of the true result falling.
Confounding factors	A variable related to one or more of the variables defined in the study. Confounding factors may mask an actual association or falsely demonstrate an apparent association.
Congenital	Referring to a condition that is present at, and usually before birth, regardless of causation.
Congenital hypothyroidism	Hypothyroidism that is present from birth.
Conjugated	Joined to another chemical.
Corepressor	Molecule that represses a biological response.
Coronal section	A slice or section of the brain made by cutting from side to side.
Coronary heart disease	Disease in which fatty deposits accumulate along the innermost layers of the coronary arteries. The deposits thicken which causes narrowing of the arteries and blocks the flow of blood to the heart.
Corpus luteum	A mature ovum that has been discharged from the ovary.
Cortical bone	Dense bone structure comprising the outer membrane of the bone.
Co-transfection	The introduction of two different DNA molecules into a eukaryotic cells.
Coumestan	One type of phytoestrogen.
Coumestrol	A type of phytoestrogen from the coumestan class.
Craniospinal ganglia	A collection of neurons associated with the spinal cord and cranial nerves.
Creatinine	A waste product of protein metabolism that can be measured in the urine.

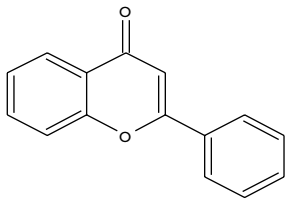
Crossover study/trial	A study comparing the effects of 2 or more treatments in which the subjects, upon completion of one treatment, are switched to another.
Cross-sectional study	A population study based on characteristics of a population at one point in time.
Cross-talk	Communication between two separate signal transduction pathways in the same cell.
Cryptorchidism	A congenital abnormality of the male reproductive system.
c-Src	A member of the non-receptor tyrosine kinase proteins.
C-terminal domain	A region of the oestrogen receptor that contributes to its transactivation capacity.
Cyclic AMP	A cyclic mononucleotide of adenosine responsible for the intracellular mediation of hormonal effects on various cellular processes.
Cyclin A	A protein involved in regulating the cell cycle.
Cyclin D1	A protein involved in cell division.
Cyclin-dependent kinase 2	Involved in regulating the cell cycle.
Cyclins	A group of proteins involved in control of the cell cycle.
Cyclooxygenase 2 (COX-2)	An enzyme involved in prostaglandin synthesis.
Cyst	An abnormal development in a body cavity or structure.
Cytochrome	Any of a group of electron transporting proteins containing a haem iron existing in an oxidised or reduced state.
Cytochrome P450 (CYP)	An extensive family of proteins involved in enzymatic oxidation of a wide range of endogenous and xenobiotic substances and their conversion to forms that may be more readily excreted. In some cases, the metabolites produced may be reactive and may have increased toxicity. In other cases, the substances may be natural precursors of hormones (e.g. steroids).

Cytometry	A method of counting cells.
Cytoplasm	All the living part of the cell inside the membrane, excluding the nucleus.
Cytosol	Component of the cytoplasm excluding membrane bound organelles.
Cytotoxic	Toxic to cells.
Daidzein	An isoflavone phytoestrogen.
Decarboxylation	Loss of a carbon dioxide (CO_2).
Decidualisation	Process by which cells or tissues go through a period of change to another form.
Dehydroepiandrosterone (DHEA)	A male sex hormone.
De-iodinase	An enzyme that catalyses the removal of iodine.
Dementia	A condition of deteriorated mentality characterised by a marked decline from the individual's former intellectual level.
Demethylation	Loss of a methyl group ($-CH_3$).
Deoxyribonucleic acid (DNA)	The carrier of genetic information for nearly all living organisms. DNA is composed of two inter-wound (double helical) chains of linked nucleotides.
Dephosphorylation	Removal of phosphate groups.
DHEA sulfate	The sulfate conjugate of DHEA.
Diastolic blood pressure	The pressure exerted on the walls of the arteries when the heart is in it relaxation phase (diastole).
Diethylstilboestrol	A synthetic oestrogen.
Differentiation	Modification of different cells or tissues of the body to undertake particular functions.

Dimer	A polymer consisting of two parts e.g. a complex of two proteins.
Di-phenolic	A compound containing two phenol groups.
Distal femoral metaphysis	The section of the femur closest to the knee that is actively growing.
DNA binding domain	A section of a receptor which binds to DNA.
Dorsolateral prostate	An area of the prostate.
Dose	Total amount of a substance administered to, taken or absorbed by an organism.
Double knockout animals	Animals which have been genetically engineered so that they do not express either oestrogen receptor.
Double-blind	An experimental procedure in which neither the subjects nor the experimenters know the make up of the test and control groups during the experiment.
Down-regulation	A decrease in number or activity.
E. coli	See *Escherichia coli*.
EC_{min}	The lowest effective concentration of a compound that produces a measurable response.
Efficacy	The ability of a substance to elicit a response following binding to a receptor.
EGF receptor	Epidermal growth factor receptor.
Electrophoresis	A method to separate charged molecules.
Embryo	The developing human individual from the time of implantation to the end of the eighth week after conception.
Endemic	Widespread in a locality or region.

Endocrine disruptor	(synonym – endocrine modulator). A chemical, which can be either naturally occurring or man made, that causes alterations in the hormonal activity of an organism.
Endocrine system	A system of glands that secrete a variety of hormones.
Endogenous	Within the body.
Endometriosis	The presence and growth of functioning endometrial tissue in places other than the uterus.
Endometrium	Membrane lining the uterus.
Enterodiol	A lignan metabolite.
Enterocolitis	Inflammation of the small and large intestines.
Enterohepatic circulation	Recycling of a substance by transport through bile via the gut and liver.
Enterolactone	A lignan metabolite.
Enteropathy	A disease of the gastrointestinal tract.
Eosinophilia	An increased number of cell type (eosinophils) in the circulation.
Eosinophils	A type of white blood cell.
Epidemiology	Study of the distribution, and in some instances, the causal factors of disease in communities and populations.
Epidermal growth factor	A polypeptide hormone that stimulates cell proliferation especially of epithelial cells by binding to receptor proteins on the cell surface.
Epididymus	A structure at the back of the testes composed mainly of ductules leading from the testis to the *vas deferens.*
Episodic memory	A type of long term memory.
Epithelial cells	Cells that make up the epithelium.

Epithelium	The tissue covering the outer surface of the body, the mucous membranes and cavities of the body.
Equimolar	At the same molar concentration.
Equol	A metabolite of daidzein.
ER	See oestrogen receptor.
ERE	See oestrogen response element.
ER-negative	Having no oestrogen receptors.
ER-positive	Having oestrogen receptors.
Erythrocyte	Red blood cell.
Escherichia coli	Common bacterium found in human and mammalian digestive tracts.
Ester	A bond between an organic acid and an alcohol.
Ethinyloestradiol	A synthetic oestrogen used in contraceptive pills.
Euthyroid	Having a normally functioning thyroid.
FAC	Food Advisory Committee.
FACS analysis	Fluorescence Activated Cell Sorter – A technique which separates, classifies and quantifies cells and antibodies.
FDA	Food and Drug Administration.
Femur	A bone in the leg.
Fermented soybeans	See natto.
Fetoprotein	Fetal protein that binds to and deactivates maternal hormones to protect the fetus.
FFQ	See food frequency questionnaire.

Flavone	A compound possessing the chemical structure: 
Flavonoid	A general term referring to a compound of similar structure to or derived from a flavone.
Flaxseed	The seeds from the flax plant.
Fluorescence	Luminescence caused by absorption of radiation followed by emission in the form of light.
Focal adhesion kinase (FAK)	A protein required for cell movement and invasion.
Follicle Stimulating Hormone	A hormone (gonadotrophin) secreted by the pituitary gland that promotes sex hormone production in the gonads.
Follicular phase	The first phase of the menstrual cycle. It lasts from the onset of menses until ovulation.
Food frequency questionnaire	A questionnaire used to obtain qualitative descriptive information about usual food consumption patterns.
Forestomach	A specialised part of the stomach consisting of two compartments.
Formononetin	An isoflavone phytoestrogen.
Fos	A transcription factor.
Free androgen index	A measure of the total testosterone:SHBG ratio in men.
Frontal cortex	A part of the brain thought to be where higher level thinking takes place.
FSH	See follicle stimulating hormone.

G_0 phase	Stage of the cell cycle where cells no longer replicate.
G_1 phase	Stage in the cell cycle before S-phase that prepares the cell for replication.
G_2 phase	Stage in the cell cycle before S-phase that prepares the cell for replication.
Galactosaemia	The presence of galactose in the blood and a characteristic of a rare genetic disorder where galactose metabolism is inhibited.
Galactosidase	A class of enzymes that cut the glycosidic bonds between the sugar galactose and another sugar of a different type from galactose.
Gavage	Administration of a liquid via a stomach tube, commonly used as a dosing method in toxicity studies.
GC-MS	Gas chromatography coupled with mass spectrometry.
Gene	A specific sequence of DNA molecule encoding a specific protein product.
Gene expression	The process by which the information in a gene is used to create proteins or polypeptides.
Gene product	A protein or polypeptide coded for by a gene.
Genetic polymorphism	A difference in DNA sequence among individuals, groups, or populations (e.g. a genetic polymorphism might give rise to blue eyes versus brown eyes, or straight hair versus curly hair). Genetic polymorphisms may be the result of chance processes, or may have been induced by external agents (such as viruses or radiation). Changes in DNA sequence, which have been confirmed to be caused by external agents, are generally called 'mutations' rather than 'polymorphisms'.
Genetic predisposition	Having a genotype that increases the risk of developing a disease but does not make it certain that it will develop. Additional factors are required before the disease appears.

Genistein	An isoflavone phytoestrogen.
Genome	All the genetic material in the chromosomes of a particular organism.
Genotoxin	Chemical that damages DNA.
Genotype	The genetic constitution of an organism.
Germ cell	Reproductive cells in the testes.
Gestation	The period from the conception of a fetus until birth.
Glomerular	Relating to glomerulus, particularly renal glomerulus.
Glucose	A type of sugar.
Glucosidase	Enzyme that removes glucose from glucose conjugates.
Glucoside	A compound conjugated to glucose.
Glucuronic acid	A sugar added during metabolism to facilitate excretion of a compound.
Glucuronidase	An enzyme in the gut that hydrolyses conjugated glucuronides.
Glucuronidation	The addition of glucuronic acid to a molecule making it more water-soluble and allowing subsequent elimination.
Glucuronosyl transferase	An enzyme that facilitates addition of glucuronic acid to a compound.
Glycitein	An isoflavone phytoestrogen.
Glycoproteins	Proteins conjugated to carbohydrates.
Glycosylation	Addition of sugar molecules.
GnRH	Gonadotrophin releasing hormone.
Goitre	A non-cancerous enlargement of the thyroid gland.

Goitrin	A compound found in plants from the Brassicaceae family.
Goitrogen	A compound that causes goitre.
Gonadectomized	Having the gonads (ovaries or testes) removed.
Gonadotrophin	Hormone secreted from the pituitary that stimulates production of sex hormones from the gonads.
Gut microflora	Bacteria found in the gut.
Gyrase	An enzyme that catalyses the breaking and rejoining of bonds linking nucleotides in DNA to generate DNA helices.
Half-life ($t_{1/2}$)	Time in which the concentration of a substance will be reduced by half, assuming a first order elimination process.
HDL	See high density lipoprotein.
Heat shock proteins	Proteins synthesised in response to increased temperature.
HeLa cells	A continuously cultured human malignant cell line derived from a cervical carcinoma.
Hepatic	Pertaining to the liver.
HER-2 (human epidermal growth factor receptor 2)	A protein receptor that is produced in excess amounts in some women with breast cancer.
Heterodimer	A complex of two different proteins.
High density lipoprotein	A lipoprotein composed of a high proportion of protein with little triglyceride and cholesterol. HDL is associated with reduced probability of developing atherosclerosis.
Hippocampus	A region of the brain involved in spatial orientation, the functioning of the limbic system. It is also involved in the establishment of memory patterns.
Histology	Study of cells under the microscope.
Homeobox	A conserved DNA sequence that codes for a protein involved in binding to DNA.

Homeostasis	Maintenance of a normal body state.
Homodimer	A complex of two molecules of the same protein.
Homologous	Corresponding or alike in certain critical attributes
Hormone	A molecule secreted into the blood and is carried to specific target cells/organs to produce a specific physiological response.
Hormone replacement therapy (HRT)	The administration of oestrogen to women with reduced levels of the hormone following the menopause or surgical removal of the ovaries.
Hot flushes	A symptom associated with the menopause in which there is a sudden flow of heat to the skin.
HPLC–UV	High performance liquid chromatography coupled with ultra violet detection.
HRT	See hormone replacement therapy.
Hydrolysis	A chemical reaction involving water.
Hydrophilic	Water attracting or attracted to water.
Hydrophobic	Water repelling or repelling water.
Hydroxylation	Addition of hydroxyl group (-OH).
Hydroxyoestradiol	An oestrogen metabolite.
Hydroxyoestrone	An oestrogen metabolite.
Hydroxysteroid dehydrogenase	A class of enzymes that catalyses the conversion of oxo groups to hydroxyl groups on steroids and *vice versa*.
17β-hydroxysteroid oxidoreductase I	An enzyme that converts sex hormones to more potent forms.
17β-hydroxysteroid oxidoreductase II	An enzyme that converts sex hormones to less potent forms.

Hypercholesterolaemia	A condition associated with heart disease, in which abnormally high concentrations of cholesterol are present in the bloodstream.
Hyperlipidemia	An abnormally high amount of lipid (fat) in the circulating blood.
Hyperplasia	An increase in the size of an organ or tissue due to an increase in the number of cells.
Hypertension	High blood pressure.
Hypocholesterolemia	The presence of reduced cholesterol in the bloodstream.
Hypoestrogenic symptoms	See menopausal symptoms.
Hypogonadism	Functional deficiency of the gonads.
Hypolipidaemic	A reduction of lipids in the blood plasma.
Hypophysectomy	Removal of the pituitary gland.
Hypospadias	A congenital abnormality of the male reproductive system.
Hypothalamus	A region of the brain that secretes hormones and regulates the anterior pituitary.
Hypothyroidism	Reduced activity of the thyroid gland.
Idfa	Infant Dietetic Foods Association.
Idiopathic	Denoting a disease or condition for which the cause is not known.
IGF-1	Insulin-like growth factor 1. A hormone involved in muscle growth.
IGFBP3	Insulin growth factor binding protein 3. The major carrier for IGF-1 in human serum.
Immunoassay	A assay system using antibodies to measure the concentrations of analytes.

Immuno(cyto)histochemical	Technique that uses antibodies as a means of detecting molecules in tissues.
Immunosuppressive	Causing or characterised by immunosuppression.
In situ hybridisation	Use of a DNA or RNA to detect the presence of the complementary sequences.
In utero	Within the uterus.
In vitro	Outside the living system.
In vivo	In the living body.
IC_{50}	The concentration of a chemical estimated to cause inhibition of a biological endpoint by 50%.
Inhibin	A hormone that suppresses the release of gonadotrophins from the pituitary.
Inhibitory κB (IκB)	The inhibitory subunit of NF-κB. Removal of IκB activates NF-κB.
Inoculation	Injection of a living or mildly infective pathogen followed by a mild, non-fatal infection resulting in immunity to more virulent forms of the pathogen.
Intelligence Quotient (IQ)	A number, which shows how, a person's intelligence compares with the average.
Intervention study	An epidemiological study in which the experimenter allocates the participants to either an experimental or control group and compares the outcome.
Intraperitoneal	Within the abdominal cavity.
Invasive cancer	See metastasis.
Inverse association	An association in which as one variable increases the other decreases.

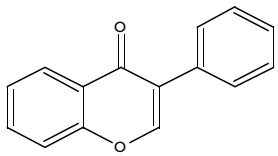
Ion exchange chromatography — A separation method using the ionic properties of molecules and their affinity to an ionic resin.

Ipriflavone — A synthetic drug for the treatment of osteoporosis.

Iso-caloric — Containing the same level of energy value.

Isoenzyme — A physically distinct form of a given enzyme.

Isoflavone — A compound with a 3-phenyl-4H-1-benzopyran-4-one chemical structure:



Isoflavonoid — A general term referring to a compound of similar structure to or derived from an isoflavone.

Isoforms — Proteins from the same gene that have different amino acid sequences.

Isolariciresinol — A lignan phytoestrogen.

Isotopic labelling — Replacement of an atom with a different isotope.

Isoxanthhumol — A prenylated flavonoid phytoestrogen.

IκB — See inhibitory κB.

Jun — A transcription factor.

Ki67 — A marker of cell proliferation.

Kinase — An enzyme that transfers a phosphate group between ATP and another molecule.

Knockout animals — Genetically engineered animals in which one or more genes, usually present and active in the normal animal, are absent or inactive.

Lactase deficiency	Lack of the enzyme lactase.
Lactation	A period of milk production in the female.
Lactoferrin	Protein involved in iron transfer.
Lacto-vegetarian	A person who does not eat meat, meat products or eggs.
Lariciresinol	A lignan phytoestrogen.
Latency	The interval between a stimulus and a response.
LBD	Ligand binding domain.
LC-MS	Liquid chromatography coupled to mass spectroscopy.
LD_{50}	The dose of a toxic compound that causes death in 50% of a group of experimental animals to which it is administered. It can be used to assess the acute toxicity of a compound, but is being superseded by more refined methods.
LDL receptor	Controls the supply of intracellular cholesterol via endocytosis.
LDLC	See low density lipoprotein cholesterol.
Leiomyoma (CHK)	Benign tumour of the smooth muscle.
Leucocytes	White blood cells.
Leukaemia	A group of neoplastic disorders (see tumour) affecting blood-forming elements in the bone marrow, characterised by uncontrolled proliferation and disordered differentiation or maturation. Examples include the lymphocytic leukaemias, which develop from lymphoid cells, and the myeloid leukaemias, which are derived from myeloid cells (producing red blood cells, mainly in the bone marrow).
Leydig cell	A cell type in the testis that produced testosterone.
LH	Lutenising hormone.

Ligand	A molecule which binds to a receptor.
Ligand binding domain	The part of the receptor to which the ligand binds.
Lignan	A compound with a 2,3 substituted 1,4-dibenzylbutane chemical structure.
Linoleic acid	A fatty acid present in some foods that is required by the body.
Linseed	The seeds of flax from which linseed oil is obtained.
Lipoprotein	A complex of protein and lipids.
Lipoprotein (a)	One of a family of lipoproteins.
Locomotor activity	Movement of the body.
Longitudinal study	A study in which the same group of people are observed at intervals over a long period of time.
Lordosis behaviour	A posture adopted by female animals in oestrus in the presence of a male.
Low density lipoprotein cholesterol (LDLC)	A lipoprotein in plasma composed of a moderate proportion of protein with little triglyceride and a high proportion of cholesterol. LDLC is associated with an increased probability of developing atherosclerosis.
Luciferase reporter gene	A gene that encodes an easily assayed product (e.g. CAT) that is coupled to the sequence of another gene introduced into cells. The reporter gene can then be used to see which factors activate response elements in the upstream region of the gene of interest.
Lumbar spine	Lower back.
Lumbar vertebrae	Bones of the lower back region.
Luminal	Within the lumen.
Luteal cells	Cells of the corpus luteum.

Luteal phase	The postovulatory phase of the menstrual cycle.
Luteinising hormone (LH)	A hormone (gonadotrophin) secreted by the pituitary that promotes sex hormone production in the gonads.
Luteolin	A plant derived yellow dyestuff.
Leucocyte	White blood cell.
Lymphocyte	A type of white blood cell that plays central roles in adaptive immune responses.
Lymphoid	Of, relating to, or being a tissue containing lymphocytes.
Macrobiotic diet	A diet based on the principles of yin and yang and comprising mainly brown rice, whole grains and vegetables.
Malignancy	See tumour.
Malonyl	A chemical group ($HOOCCH_2COO$-)
Malonylation	The addition of malonyl group to a molecule.
Mammographic density	Breast tissue that has many glands close together. The level of mammographic (breast) density is highly associated with breast cancer risk.
MAPK	A family of enzymes that are involved in cell signalling.
Matairesinol	A lignan phytoestrogen.
Max_I	Concentration of a compound that gives rise to maximum induction in a biological assay.
MCF-7	Cell line derived from a human breast tumour.
Median basal hypothalamic and preoptic area (MBH-POA)	Specific regions within the hypothalamus.
Melanoma	A malignant skin tumour.
Menarche	Onset of first menstruation.

Menopause	Cessation of menstruation and ovulation.
Menstrual cycle	The process of ovulation and menstruation.
Messenger RNA (mRNA)	A nucleic acid copy of a DAN sequence which serves the instruction for the synthesis of proteins.
Meta-analysis	In the context of epidemiology, a statistical analysis of the results from independent studies, which aims to produce a single estimate of an effect.
Metabolism	Chemical modification of a compound by enzymes within the body, for example by reactions such as hydroxylation (see cytochrome P_{450}), epoxidation or conjugation. Metabolism may result in activation, inactivation, accumulation or excretion of the compound.
Metabolite	Product formed by metabolism of a compound.
Metastasis	The process whereby malignant cells become detached from the primary tumour mass, disseminate (mainly in the blood stream or in lymph vessels) and 'seed out' in distant sites where they form secondary or metastatic tumours. Such tumours tend to develop at specific sites and their anatomical distribution is often characteristic; it is non-random. The capacity to metastasise is the single most important feature of malignant tumours (see tumour).
Methionine	An amino acid.
Methylation	The addition of a methyl group ($-CH_3$) to a molecule.
Micronuclei	Isolated or broken chromosome fragments which are not expelled when the nucleus is lost during cell division but remain in the body of the cell forming micronuclei. Centromere positive micronuclei contain DNA and/or protein material derived from the centromere. The presence of centromere positive micronuclei following exposure to chemicals can be used to evaluate the aneugenic potential of chemicals.
Microsomal enzyme	Enzymes found in the endoplasmic reticulum.

Microsomes	The smallest size particles spun down from cell homogenates in the ultracentifuge, including broken parts of other fractions.
Migrant studies	The epidemiological study of populations that have moved geographical location.
Miso	Miso is a steamed soybean product.
Mitogen	A stimulus which provokes cell division in somatic cells.
Mitosis	The type of cell division which occurs in somatic cells when they proliferate. Each daughter cells has the same complement of chromosomes as the parent cells.
Moiety	One of the portions into which something is, or can be, divided.
Monomer	A molecule consisting of a single unit.
Mono-phenolic	A chemical structure containing one phenol group.
MRC	Medical Research Council.
mRNA	Single stranded RNA molecule that specifies the amino acid sequence required for protein synthesis.
MS/MS	Mass spectrometers used in series that can provide structural information as well as quantitative measurement of the concentration of an analyte.
Mucosal	Regarding the mucosa or mucous membranes.
Multivariate analysis	Statistical analysis that allows simultaneous study of two or more dependent variables.
Mutagens	Compounds capable of causing a change in DNA sequence.
Mycoestrogens	Oestrogenic compounds produced by fungi.
Myelomonocytic leukaemia	Cancer of the bone marrow and white blood cells.
Natto	Food made by fermenting cooked whole soybeans and fried tofu soybean.

Natural killer cell	A type of leucocyte that can recognise and kill virally infected and malignant cells.
Neonatal	The first four weeks of life in humans.
Neoplasm	See tumor.
Neoplastic	Abnormal cells, the growth of which is more rapid than that of other cells.
Neurodegeneration	A loss of nerve cells.
Neurodegenerative	Relating to or characterised by degeneration of nervous tissue.
Neuron	A type of nerve cell.
Neuroprotective factors	Compounds intended to prevent damage to the central nervous system.
Neutrotrophic factor	A protein that promotes nerve cell growth and survival.
Nipple aspirate	Fluid from the mammary gland nipple.
No observed adverse effect level (NOAEL)	The highest administered dose at which no adverse effect has been observed.
Normocholesterolemic	To possess physiological normal cholesterol concentrations.
Normotensive	Normal blood pressure, tension and tone.
Normoxic	At atmospheric pressure.
Northern blot	A technique in molecular biology used to determine mRNA expression.
N-telopeptide	Portions of amino acid sequence of a protein that are removed in maturation.
Nucleotide	The 'building block' of nucleic acids, such as DNA and RNA.
Observational study	Describing the design of a scientific study.

Odds ratio	A figure intended to provide a comparison of the presence of a risk factor for disease in a sample of diseased subjects and non diseased controls.
Oedema	A swelling of tissue through an increase in its fluid volume.
Oestradiol	A female sex hormone.
Oestradiol benzoate	A synthetic analogue of oestradiol.
Oestriol	A female sex hormone.
Oestrogen	Sex hormone or other substance capable of developing and maintaining female characteristics of the body.
Oestrogen receptors	An intracellular protein that binds oestrogen and oestrogen-like compounds and mediates subsequent cell responses.
Oestrogen response element	A DNA sequence in the promoter region of an oestrogen receptor responsive gene that is recognised by and binds to the DNA-binding domain of oestrogen receptors.
Oestrogenicity	The effect of an oestrogen.
Oestrone	A female sex hormone.
Oestrus cycle	Periodic sexual impulse of some animals.
Olfactory bulb	Structure in the central nervous system involved in the sense of smell.
Omnivorous	To eat both animal and plant matter.
Oncogene	A gene carried by a tumour virus or cancer cell, which is solely or partly responsible for tumorigenesis.
Ononin	Isoflavone found in plants.
Ontogeny	The history of development and growth of an individual from the fertilised egg to maturity.
Open field activity	Exercise to study animal behaviour.

Organogenesis	The formation of organs.
Osteoblast	A bone forming cell that secretes the bone matrix.
Osteocalcin	Polypeptide produced by osteoblasts involved in the formation of bone.
Osteoclasts	Cells involved in the degradation of bone.
Osteopenia	Loss of bone.
Osteoporosis	Loss of bony tissue, resulting in bones that are brittle and liable to fracture.
Ovary	Female reproductive organs where eggs are developed.
Ovariectomy	Removal of the ovaries.
Oviduct	A duct that releases eggs from the ovary.
Ovulation	The release of a mature oocyte from the ovary.
Ovum	A female reproductive cell that once fertilised develops into an egg.
Palpitations	Irregular or forceful beating of the heart.
Paracrine factors	Chemicals involved in communication between cells in the same tissue.
Pathogenesis	The biological mechanisms underlying the clinical manifestation of disease.
Pathophysiology	Study of the disease due to the disturbance of the systems of the body.
PCR	Polymerase Chain Reaction. A method to amplify specific sequences of DNA.
Perimenopausal	The period of time before, during and after the menopause.
Perinatal	The period of time before, during and after birth.

Pharmacokinetics	How a chemical interacts with the body in terms of absorption, distribution, metabolism, and excretion.
Phenol	A singly hydroxylated aromatic compound.
Phenotype	The visible properties of an organism.
Phosphorylation	Addition of a phosphate group to a molecule.
Physiological	Of or relating to physiology.
Phytochemical	A chemical derived from a plant.
Phytoestrogen	A phytoestrogen may be defined as any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous oestrogens usually by binding to oestrogen receptors.
Phytosterols	Compounds naturally produced by plants with similar structure to steroids.
Pituitary gland	A gland at the base of the brain that produces hormones.
Placebo	An inert substance used in experiments testing the efficacy of another substance.
Placental transfer	The movement of a compound from the peripheral blood to the placenta.
Plasma	The fluid portion of blood free from red blood cells.
Platelets	Blood cells involved in blood clotting.
Polyclonal antibody	Antibodies derived from a number of lymphocytes.
Polymorphism	The existence of variation of a genetic characteristic in a population.
Polyp	A small growth on a membrane.
Post pubertal	After puberty.

Postmenopausal	After the menopause.
Postnatal	Occurring after birth.
Postnatal day (PND)	Day following birth.
Postpartum	Occurring after birth.
Post-transcriptional	Occurring after genetic transcription.
Potency	Ability of a ligand to elicit a response which is determined by its binding affinity and efficacy.
Pre-implantation	The period between fertilisation and implantation.
Premature thelarche	Premature breast development.
Premenopausal	Before the menopause.
Pre-neoplastic lesions	Abnormal cell growth that may lead to a benign or malignant tumour.
Prenylated flavonoid	A flavonoid including a prenyl group.
6-Prenylnarigenin	A prenylated flavonoid phytoestrogen.
8-Prenylnaringenin	A prenylated flavonoid phytoestrogen.
Preputial separation	Separation of the covering skin of the penis.
Progesterone	A female sex hormone.
Progestin	A female sex hormone.
Prolactin	A hormone secreted by the anterior pituitary to stimulate the production of milk in mammals.
Proliferative index	A measure of the number of dividing cells in a tissue or in culture
Promoter	A site on DNA to which RNA polymerase will bind and initiate transcription.

Prospective study	A method of epidemiological study in which a defined population is identified and then followed up over time with ascertainment of exposures and/or subsequent disease or mortality.
Prostaglandins	A group of fatty acids that have hormone-like actions.
Prostate gland	A male gland that that produces seminal fluid.
Prostate seminal vesicle	Areas in the prostate gland that produce seminal fluid.
Prostate specific antigen	An antigen made by the prostate gland and found in the blood that may indicate prostate cancer.
Prostatic fluid	A fluid produced by the prostate that forms part of semen.
Prostatic hypertrophy	An increase in prostate size.
Proteases	Enzymes which break down proteins.
Protein hydrolysate formula	An infant formula based on cow's milk protein that has been broken down into smaller pieces which are easier to digest and less likely to cause allergic symptoms.
Protein tyrosine kinases	Enzymes which phosphorylate specific tyrosine residues on proteins.
Proto-oncogenes	Normal cell genes involved in the regulation of the cell cycle.
Proximal radius	Relating to part of the forearm.
pS2	An oestrogen-induced protein. pS2 is used as a marker of functioning ER status in breast cancer.
PSA	See prostate specific antigen.
Pulmonary	Of the Lung.
Quartile	One fourth of the total number.
Quercitin	A type of flavanoid.

Quintile	One fifth of the total number.
Raloxifene	Synthetic anti-oestrogen.
Ras	An oncogene involved in signal transduction and gene transcription and capable of causing cancer.
Recall bias	A systematic error in epidemiological studies due to differences in accuracy or completeness of recall to memory of past events or experiences.
Receptor	Proteins that bind ligands to initiate a change in the working of a cell.
Receptor subtypes	Receptors activated by similar ligands but which have sufficient differences in their pharmacological response or molecular structure to justify being classified separately.
5α-Reductase	A enzyme involved in the conversion of testosterone to 5α-dihydrotestosterone.
Regression analysis	The relationship between two variables, which estimates the average increase in one variable that is associated with a change of size of one unit in the other variable.
Relative risk	The proportion of diseased people amongst those exposed to the relevant risk factor divided by the proportion of diseased people amongst those not exposed to the risk factor. This should be used in cohort studies where those with and without disease are followed to observe which individuals become diseased.
Resorption	Loss of a substance through physiologic or pathologic means.
Reticuloendotheliual system	A system in the body that defends against infection and disposes of cell breakdown products.
Retrospective study	A study in which people are enrolled and then have their history of risks, infections or disease determined.
RT-PCR	Reverse-transcriptase polymerase chain reaction. PCR utilising reverse transcriptase (RT), an enzyme that catalyses the synthesis of cDNA from an RNA template.

Running wheel activity	A experimental method used to examine behaviour.
Secoisolariciresinol	A lignan phytoestrogen.
Semen	Fluid which contains sperm.
Seminal vesicles	Small glands located near the prostate that produce seminal fluid which form part of semen.
Seminiferous tubule lumen	The cavity in the testis where sperm cells are formed.
Sensorimotor function	A nerve conveying both sensory and motor signals.
Serine	An amino acid.
Sertoli cell	Cells in the testes that support sperm production.
Serum	The fluid that separates from clotted blood or blood plasma that is allowed to stand.
Sex hormone binding globulin	A hepatic glycoprotein which binds endogenous sex hormones in plasma.
Sex hormones	Steroid hormones that control sexual development, including androgens and oestrogens.
Sexual differentiation	The process a fetus undergoes to become either female or male.
Sexually dimorphic nucleus	An area in the medial preoptic area of the forebrain. It is larger in males than females.
SHBG	See sex hormone binding globulin.
Signal transduction	The process whereby a extracellular signal is transmitted across the plasma membrane of a cell to activate the intracellular biochemical pathways that lead to the cell's response.
Sitosterol	A plant steroid.

Solubility	The extent to which one substance dissolves into another.
Solvent	A liquid that dissolves another substance or substances to form a solution.
Soy	Derived from the soybean; also referred to as 'soya'.
Soybean	The edible seed of *Glycine max*, a dicotyledonous plant of the legume family native to Asia.
Sperm	A reproductive cell produced by males.
Spermatid	An intermediate cell type formed during spermatogenesis.
Spermatogenesis	The production of sperm.
Spermatozoa	Mature male germ cells.
S-phase	Stage of the cell cycle where the cell synthesises DNA prior to mitosis.
Splenic	Of or relating to the spleen.
Spliced isoform	A sequence of mRNA which may change to produce a different gene product.
Squamous metaplasia	An alteration of plate-like cells.
Src	A protein involved in intracellular signalling.
Stem cell	A type of cell from which specific tissue type cells are produced.
Steroid hormone receptors	A family of cellular receptors.
Steroidogenesis	Production of steroids.
Subcutaneous injection	Injection of a compound under the skin.
Sulfatase	An enzyme that catalyses the removal of sulfate.

Sulfotransferases	An enzyme that catalyses the addition of sulfate to a compound.
SULT1A1	Sulfotransferases convert the unconjugated form of oestrone to oestrone sulfate.
SULT1A2	Sulfotransferases convert the unconjugated form of oestrone to oestrone sulfate.
SV40	Symbol for simian vacuolating virus No. 40.
Synergism	Interaction of one agent with another to produce increased activity, which is greater than the sum of the effects of the two agents separately.
Systemic arterial compliance	A measure of the elasticity of the arterial wall.
Systolic blood pressure	The pressure exerted on the walls of the arteries during the contraction phase of the heart.
T_3	See Tri-iodothyronine.
T_4	See Thyroxine.
Tamoxifen	Synthetic anti-oestrogen used in the management of breast cancer.
T-cells	Any of several lymphocytes that differentiate in the thymus.
TEBs	Terminal End Buds present in the mammary gland.
Teleosts	Type of fish.
Tempeh	Cake made by fermenting soybeans with rice or millet.
Terminal end buds	Specialised structures at the end of growing ducts in breast tissue. They are sites of intensive cell proliferation.
Tertile	One third of the total number.
Testes	Male reproductive organs which produce sperm and male sex hormones.

Testosterone	A male sex hormone.
Textured vegetable protein (TVP)	A meat-like substance used to boost the nutritional content of meals. TVP usually contains defatted soy flour.
Threonine	An amino acid.
Thymic	Of or relating to the thymus.
Thyroglobulin (TG)	A protein in the thyroid gland from which the thyroid hormones (thyroxine and tri-iodotyrosine) are synthesised.
Thyroid binding globulin	A plasma protein involved in binding thyroid hormone.
Thyroid gland	An endocrine gland involved in the regulation of metabolism.
Thyroxine (T4)	A hormone secreted by the thyroid gland.
Tofu (soybean curd)	A foodstuff obtained by the coagulation of fresh soymilk.
Tolerable daily intake (TDI)	Regulatory value equivalent to the acceptable daily intake (ADI).
Topoisomerase	An enzyme that reduces supercoiling in DNA by breaking and rejoining the two strands of the DNA molecule either simultaneously or separately.
Toxicodynamics	The interaction of a chemical with its site of toxic action.
Toxicokinetics	The fate of chemicals in the body, including a mathematical account of their absorption, distribution, metabolism and excretion.
TRAMP	Transgenic adenocarcinoma in mouse prostate. Transgenic animal model of prostate cancer.
Transactivation	Stimulation of transcription by factors binding to DNA and activating adjacent proteins.
Transcription	Synthesis of mRNA from DNA.
Transcription factor	A protein involved in the transcription of genes.

Transfection	The incorporation of exogenous DNA into a cell.
Transgenic	Any animal into which cloned genetic material has been transferred.
Translocation	The transfer of cellular components to different positions.
Translocator	A membrane protein controlling the transfer of a substance across a cell membrane.
Triacylglycerol	Glycerol esterified at three hydroxyl groups by a fatty acid.
Triglycerides	The form in which fats are stored in the body.
Tri-iodotyrosine (T_3)	A hormone secreted by the thyroid gland.
TRPM2	Testosterone repressed prostate message 2, a gene involved in apoptosis.
TSH	Thyroid stimulating hormone.
Tumorigenesis	The development of tumour.
Tumour	An abnormal mass of tissue that results from excessive cell division that is uncontrolled and progressive.
Tumour T-antigen	Proteins coded by viral genes that are expressed early in the replication cycle.
TVP	See textured vegetable protein.
Tyrosine	An amino acid.
Ulna	One of the bones that comprise the forearm, below the elbow.
Unopposed oestrogen therapy	HRT products containing only oestrogen.
Uterine adenocarinoma	A cancer that involves cells in the lining of the uterus.
Uterotrophic assay	Biological assay used to measure oestrogenic activity, in which the ability of chemicals to stimulate uterine growth is monitored.

Vaginal adenocarinoma	A cancer that involves cells in the lining of the vagina.
Vaginal canalisation	Formation of channels in the vagina during fetal development.
Vaginal cornification	Formation of keritin in cells of the vagina.
Vaginal epithelium	Cells covering the surfaces of the vagina.
Vasculature	Blood vessels.
Vasorelaxation	Relaxation of blood vessels.
Vegan	A person who consumes no animal products.
Vegetarian	A person living on a diet of grains pulses, nuts, seeds, vegetables and fruits with or without the use of dairy products and eggs. A vegetarian does not eat meat, poultry, game fish, shellfish or crustacea and avoids all animal by-products. In addition, a lacto-vegetarian does not eat eggs.
Veno-occlusive disease	A disease in which blood vessels in the liver become swollen and clogged.
Ventral prostate	Part of the prostate gland.
Vertebrate	Organisms characterised by the possession of a well formed bony or cartilaginous vertebral column or backbone enclosing the spinal cord.
Very Low Density Lipoprotein (VLDL)	A plasma lipoprotein produced primarily by the liver.
Vitellogenin	A protein synthesised in hepatocytes following oestrogen stimulation.
Vulvar carcinoma	Cancer of the external female genitalia.
Westernisation	Describes the adoption of a lifestyle typically seen in USA and Europe.
Whey	Watery liquid left when milk forms curds.
WHO	World Health Organisation.

Xanthohumol	A prenylated flavonoid phytoestrogen.
Xenobiotic	A foreign chemical not normally found in the body.
Xenoestrogen	A compound with oestrogenic properties not normally found in the body.
Zearalanol	A mycoestrogen.
Zearalenone	A mycoestrogen.
Zinc finger	A protein structure that binds zinc, often found in proteins that bind to DNA.

Units of measurement

mg	Milligram (10^{-3} grams or 0.001 grams)
µg	Microgram (10^{-6} grams or 0.000,001 grams)
ng	Nanogram (10^{-9} grams or 0.000,000,001 grams)
pg	Picogram (10^{-12} grams or 0.000,000,000,001 grams)
mole (mol)	Molecular weight of a compound expressed in grammes.
µM	Micromolar (10^{-6} moles or 0.000,001 moles)
nM	1 nanomolar (1 nanomole (10^{-9} moles or 0.000,000,001 moles)
pM	1 picomolar (10^{-12} moles or 0.000,000, 000, 001 moles)
mU	MilliUnits, of enzyme activity, expressed in terms of the turnover of the appropriate substrate of the enzyme
mV	Millivolts
ppb	Parts per billion (1 part in a thousand million)
ppm	Parts per million
w/w	Weight/weight, to indicate that measures of weight are used in the preparation of a mixture.

Appendix 2

Submissions to the Working Group

Submissions of evidence received

Date Received	Received From	Contents
04/07/00	S Fallon Weston A Price Foundation (USA)	• Article from The Third International Soy symposium (November 1999) – Tragedy and Hype • Leaflet – Wise Traditions in Food, Farming and the Healing Arts
12/07/00	Dr M Fitzpatrick (New Zealand)	• Food Commission Briefing Paper – Soya Infant Formula: The Health concerns and addendum by Dr M Fitzpatrick and Ms S Dibb (1998) • Article for the Weston A Price Foundation – Soy Isoflavones: Panacea or Poison? by Dr M Fitzpatrick
24/7/00	R James (New Zealand)	Letter and various printed articles: • Correspondence from Dr CR Sirtori to the Lancet (March 2000) – Dubious benefits and potential risk of soy phytoestrogens • Correspondence from Drs Doerge and Sheehan (February 1999) – Scientists protest soy approval • Article in Food Labelling and Nutrition News (March 1999) – Proposed soy protein/CHD health claim criticised by FDA's toxicology centre Three abstracts from The Third International Soy Symposium (November 1999): • Genistein toxicity from dietary exposure from early pregnancy through puberty (Sheehan *et al*) • Long term adverse effects after developmental exposure to genistein (Newbold *et al*) • Association of high midlife tofu consumption with accelerated brain ageing (White L) Abstract from the British Journal of Urology International (January 2000): • Vegetarian diet in pregnancy linked to birth defect Article: Scientists protest Soy approval (February 1999). Published on ABC News website
07/08/00	Dr S Milligan King's College, London (UK)	Two phytoestrogen research proposals: • The effect of phytoestrogens and flavonoids on membrane transport mechanisms • The development of time-resolved fluorescence immunoassays for the measurement of hop phytoestrogens in biological fluids and tissues

Date Received	Received From	Contents
07/08/00	R James (New Zealand)	Letter and various printed articles: • Fractionation and characterization of alcohol extractables associated with soybean proteins. Non-protein components (J Agr Food Chem, Nash *et al*, 1967) • US Department of Commerce – Scientific literature reviews on generally recognised as safe (GRAS) food ingredients – soybean protein-isolated (1974) • Evaluation of the health aspects of soy protein isolates as food ingredients – prepared by the Select Committee of GRASD Substances (1979) • CODEX general guidelines for the utilization of vegetable protein products (VPP) in foods – CAC/GL 4-1989 • Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women (Am J Clin Nutr, Cassidy *et al*, 1994) • Anti-thyroid isoflavones from soybean (Biochem Pharmacol, Divi *et al*, 1997) • Frequency and characteristics of silent dementia among elderly Japanese-American men (JAMA, Ross *et al*, 1997) • Article from the Honolulu Star-Bulletin (1999) – Too much TOFU induces 'brain aging', study shows • Genotoxicity of estrogens (Z Leb Unt Forsch A, Metzler et al, 1998) • Induction of micronuclei, DNA strand breaks and HPRT mutations in cultured Chinese hamster V79 cells by the phytoestrogen coumestrol (Fd Chem Toxicol, Kulling *et al*, 1997) • A maternal vegetarian diet in pregnancy is associated with hypospadias (BJU International, North *et al*, 2000) • Dietary estrogens stimulate human breast cells to enter the cell cycle (EHP, Dees *et al*, 1997) • Phytoestrogen interaction with estrogen receptors in human breast cancer cells (Endocrinology, Martin *et al*, 1978) • Exposure of infants to phyto-oestrogens from soy-based infant formula (Lancet, Setchell et al, 1997) • Article from Food Labelling and Nutrition News – Proposed soy protein/CHD health claim criticized by FDA's toxicology letter (March 1999)

Date Received	Received From	Contents
		• Correspondence from Dr CR Sirtori to the Lancet (March 2000) – Dubious benefits and potential risk of soy phytoestrogens • Premature thelarche in Puerto Rico (AJDC, Feni-Titulaer *et al*, 1986) • Soybeans and related products: an investigation into their toxic effects – by Dr MG Fitzpatrick (March 1994)
11/8/00	P MacQueen Novogen Limited (UK)	The submission contained background information and a number of printed articles: • Effect of dietary components, including lignans and phytoestrogens on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG) (J Ster Biochem, H Adlercreutz *et al*, 1987) • The effect of isoflavone phytoestrogens in relieving hot flushes in Peruvian postmenopausal women (9th International Menopause Society World Congress on Menopause, Japan, Jeri *et al*, 1999, abstract) • The effects of isoflavones derived from red clover on vasomotor symptoms and endometrial thickness (9th International Menopause Society World Congress on Menopause, Japan, Nachtigall *et al*, 1999) • Randomised placebo-controlled trial of an isoflavone supplement and menopausal symptoms in women (Climacteric, Baber *et al*, 1999) • The effect of Promensil™, an isoflavone extract on menopausal symptoms (Climacteric, Knight *et al*, 1999) • Isoflavones from red Clover improve systematic arterial compliance but not plasma lipids in menopausal women (J Clin Endocrinol Metab, Nestel *et al*, 1999) • The effects of Isoflavone phytoestrogens on bone; preliminary results from a large randomised controlled trial (ENDO 2000 The Endocrine Society 82nd annual meeting, Atkinson *et al*, 2000) • The effect of an isoflavone dietary supplement (Rimostil™) on serum lipids, forearm bone density and endometrial thickness in post-menopausal women (10th annual meeting of the North American Menopause Society, New York, Baber *et al*, 1999)

Date Received	Received From	Contents
		• The effect of supplementation with isoflavones on plasma lipids and oxidisability of low density lipoprotein in pre-menopausal women (Atherosclerosis, Samman *et al*, 1999) • A double-blind randomised controlled trial of isoflavones in the treatment of cyclical mastalgia (Med J Aust, Ingram D *et al*, 2000) • Acute and chronic Pharmacokinetics of an extract of isoflavones from red clover (Novogen Ltd, Howes JB *et al*) • Hormone replacement. Risk-benefit relation differs between populations and individuals (BMJ, Khaw KT, 1998)
14/08/00	D Welsby Applied Technology Protein Technologies International Ltd (UK)	Letter and various published papers: • Genistein suppresses mammary cancer (Carcinogenesis, Lamartiniere *et al*, 1995) • Neonatal genistein chemoprevents mammary cancer (PSEBM, Lamartiniere *et al*, 1995) • Xenoestrogens alter mammary gland differentiation and cell proliferation in the rat (EHP, Brown *et al*, 1995) • Protection against breast cancer with genistein: a component of soy (Am J Clin Nutr, Lamartiniere CA, 2000) • Isoflavones, soy-based infant formulas, and relevance to endocrine function (Nutrition Reviews, Klein KO, 1998) • Follow up study of a cohort fed soy-based formula during infancy (FASAB J, Sammel *et al*, 2000 – abstract) • Food intake and growth of infants between six and twenty-six weeks of age on breast milk, cow's milk formula or soy formula (Acta Paediatr Scand, Kohler *et al*, 1984) • A soy protein formula and a milk-based formula (Clin Pediatr, Jung *et al*, 2000) • Growth of newborn, term infants fed soy formulas for 1 year (Clin Pediatr, Lasekan *et al*, 1999) • Vitamin D metabolism, mineral homeostasis and bone mineralization in term infants fed human milk, cow milk-based formula (J Pediatr, Hillman *et al*, 1998)

Date Received	Received From	Contents
		• Soy-based infant formulas (Sarett HP) • Bone mineralization in the first year of life in infants fed human milk, cow-milk formula, or soy-based formula (J Pediatr, Mimouni *et al*, 1993) • Exposure of infants to phyto-oestrogens from soy based infant formula (Lancet, Setchell *et a*l, 1997) • Isoflavone content of infant formulas and the metabolic fat of these phytoestrogens in early life (Am J Clin Nutr, Setchell *et al*, 1998) • Soy protein isolates in infant feeding (Soy Protein and Human Nutrition, Fomon *et al*, 1979) • Requirements for protein and essential amino acids in early infancy (Acta Paediat Scand, Fomon *et al*, 1973) • Dietary protein quality in infants and children (Amer J Dis Child, Graham *et al*, 1970) • Requirements for sulfur-containing amino acids in infancy (J Nutr, Fomon *et al*, 1986) • A study of normal infants fed a soya protein isolate formula (Med J Aust, Dean *et al*, 1973) • Growth and protein status of term infants fed soy protein formulas differing in protein content (J Am Coll Nutr, Churella *et al*, 1994) • Isoflavones in human breast milk and other biological fluids (Am J Clin Nutr, Franke *et al*, 1998) • Diets containing whey proteins or soy protein isolate protect against 7, 12-dimethylbenz(a)anthracene-induced mammary tumors in female rats (Can Epidemiol Biomark Prev, Hakkak *et al*, 2000) • Isoflavone content of infant foods and formulas (J Paediatr Child Health, Knight *et al*, 1998)
14/08/00	S Jacobs Infant and Dietetic Foods Association (UK)	Letter and document: 'Phytoestrogens and soya infant formulas'

Date Received	Received From	Contents
15/8/00	Dr E Underwood Wyeth Nutritionals International (UK)	Copy of a research paper on Follow-up study of a cohort fed soy-based formula during infancy by Strom *et al* submitted to the New England Journal of Medicine for publication upon completion of peer reviewing (subsequently published in JAMA as Strom *et al*, 2001)
15/8/00	Dr P Albertazzi Hull and East Yorkshire Hospitals NHS Trust (UK)	Review of the health implications of phytoestrogen supplementation in postmenopausal women and suggestions for future area of research
15/08/00	Dr M Jones ADM Nutraceuticals (Europe)	ADM Nutraceuticals: Standard operating procedure for determination of soy isoflavones
15/08/00	Dr E Underwood SMA Nutrition (UK)	Draft research paper: Follow up study of a cohort fed soy based infant formula during infancy. Published in JAMA by Strom *et al*, 2001
19/12/00	Y Le Bail-Collet Eridania Beghin-Say (Belgium)	The submission contains: • Conclusions of an expert panel convened to examine the safety of the isoflavone products Prevastein™ and Prevastein HC™. • Literature review covering ADME, toxicity and clinical trials of isoflavones to support the GRAS status of Prevastein™ and Prevastein HC™ for use in food • Assessment of isoflavone consumption in the Netherlands (March 2000) • Assessment of isoflavone consumption in France (September 2000)
23/01/01	R James (New Zealand)	The submission contains a number of responses from members of the public to advertisements in 4 New Zealand newspapers. The advert suggests ingestion of soy infant formulae may result in thyroid and reproductive disorders and compensation claims may be made
06/02/01	R James (New Zealand)	The submission contains a letter and a number of printed articles: • Article in Food Labelling and Nutrition News (March 1999) – Proposed soy protein/CHD health claim criticized by FDA's toxicology center • Article for the Weston A Price Foundation – Soy Isoflavones: Panacea or Poison? by Dr M Fitzpatrick

Date Received	Received From	Contents
14/02/01	N Worthington PitRok Ltd (UK)	The submission contains a number of documents on the company's Tofupill™ line of products. The submission also contains testimonials from users of the products
19/02/01	R James (New Zealand)	The submission contains a letter and a copy of a submission made to the US FDA in opposition to claims made by Protein Technologies Inc, that soy protein is generally recognised as safe (GRAS)

List of those who commented on the draft report following the consultation exercise

The following individuals and organisations submitted comments on the draft report in response to the consultation exercise (9 October to 3 December 2002). All the comments made were considered by the Working Group and, where appropriate, the draft report was amended to reflect the comments made.

Name of individual (Organisation)
Dr P Albertazzi (Hull Royal Infirmary)
Anon (2)
T Arditi (Solbar Ltd)
Dr T Badger (Arkansas Children's Nutrition Centre)
Prof S Barnes (University of Alabama)
Prof P Bateson (Royal Society)
J Battershill (Committees on Mutagenicity and Carcinogenicity)
S Bennet
T Carr
Prof T Clarkson & Dr M Anthony (Wake Forest University)
L Dorcik
N Duffin (So Good International Ltd)
D Dyer (Food & Drink Federation)
E Eubank
S Fallon (Weston A Price Foundation)
B Frazer-Smith

Name of individual (Organisation) *(Continued)*

J Gross

C Harris

N Howe

R & V James

M Jones (ADM Natural Health & Nutrition Europe)

H Killick

Dr C Kirk (University of Birmingham)

A Long (Vegetarian Economy & Green Agriculture)

J Lowe

Dr J MacLeod

G Markham (Infant & Dietetic Foods Association)

R Martins

Dr M McKinley (Dairy Council)

Prof A Milner (Royal Society of Edinburgh)

J Pingree

Dr S Potter (Health & Nutrition, Du Pont)

P Purdy (National Council for Women)

P Rundall (Baby Milk Action)

Dr J Travis

P Viner (Health Food Manufacturers' Association)

G Ward

B Watzl (Federal Research Centre for Nutrition)

L West (Novogen)

Dr D Woodhams

Dr W Wong

K Zelinski

Appendix 3

Literature searches conducted and search terms used

The following internet search engines were used to conduct searches of the scientific literature: PubMed, BioMedNet & Infotrieve. Searches were conducted using the following search terms either individually or in combination.

*= open term

estrogen*, oestrogen*, estrogenic potency, oestrogenic potency

estrogen receptor*, oestrogen receptor*, ER alpha, ER beta

phytoestrogen*, phyto-oestrogen*, phyto-estrogen*, isoflavonoid*, flavone*, isoflavanone*, isoflavone*, coumestan*, lignan*

daidzein, daidzin, daidzein glucuronide, enterodiol, enterolactone, matairesinol, secoisolariciresinol, genistein, genistin, genistein glucuronide, biochanin A, equol, formononetin, coumestrol, dihydroxyflavone, isopentenylnaringenin, isopentenylgenistein, isopentenylapigenin, isopentenylquercetin, isopentenylnaringenin, prenylnaringenin, naringenin, narigenin

soy*, soya*, flax*, flaxseed*, linseed*, infant*, formula*

antioxidant, brain, central nervous system, immune system, immune function, menopause, hot flush, hot flash, hormone, hormonal, cancer*, lung, prostate, endometrial, ovarian, breast, stomach, colon, colorectal, rectal, thyroid, T3, T4, tri-iodothyronine, triiodothyronine, thyroxine, hypothyroidism, hyperthyroidism, goitre, goitrogenic, bone density, bone mineral density, bone loss, bone mineral content, osteoblast, osteoclast, hip fracture, osteopenia, osteoporosis, cardiovascular, arterial compliance, arterial stiffness, lipoproteins, lipids, nitric oxide, cholesterol, cardiac disease, coronary heart disease, lipid metabolism, vasculature, heart disease, hypertension, hypotension, hypocholesterolemia, hypocholesterolaemia

Appendix 4

List of those who made presentations at Working Group meetings

Professor A Boobis (Imperial College School of Medicine, London)

Dr S Boobis (Imperial College School of Medicine, London)

Dr S Cotterell (Imperial College School of Medicine, London)

Professor H Makin (St Barthlomew's and the London School of Medicine, Queen Mary University of London, London)

Advice was also sought and received from **Professor M Dowsett** (Institute of Cancer Research, London).

List of those who made presentations at the Phytoestrogen Programme Workshop in November 2000

FSA funded project	Contractor (Organisation)
Synthesis of Labelled and Unlabelled Isoflavonoid Phytoestrogen Standards	Dr N Botting (University of St Andrews)
Analysis of Natural Toxicants and Inorganic multi-elements in a Vegetarian Duplicate Diet Study	Dr D Clarke (Central Science Laboratory)
The effects of isoflavone phytoestrogens on bone: preliminary results from a large randomised controlled trial	Dr C Atkinson (MRC – Biostatistics Unit)
Identification and Quantification of Dietary Lignans by Liquid Chromatography Mass Spectroscopy	Dr L Howells (Veterinary Laboratories Agency)
Results of two phytoestrogen quality assurance check sample rounds	Dr D Clarke (Central Science Laboratory)
Development and Application of Screening Assays for the Beneficial and Adverse Effects of Phytoestrogens in Food	Dr N Coldham (Veterinary Laboratories Agency)
Diet, Phytoestrogen and Gene-nutrient Interactions in Relation to Cancer: A Prospective Study	Dr S Bingham (MRC-Dunn Nutrition Laboratory)

FSA funded project	Contractor (Organisation)
Dietary Phytoestrogens: Possible Effects on Prostate Cancer and 5-Alpha Reductase Activity	Professor F Alexander (University of Edinburgh)
Levels of Plant Oestrogens in the Diets of Infants and Toddlers	Professor H Nursten (Reading University)
Absorption, Distribution, Metabolism and Excretion of [^{14}C]-Labelled Genistein	Dr A-Q Zhang (VLA)
Absorption, Distribution, Metabolism and Excretion of Isoflavones in vivo	Dr M Faughnan (University of Surrey)
Absorption and Metabolism of Dietary Phytoestrogens in Humans – Effect of Age, Gender, Food Matrix and Chemical Composition	Dr A Cassidy (University of Surrey)
Influence of Human Gut Microflora on Dietary Soya Isoflavone Phytoestrogen Bioavailability in Adults and Children	Dr H Wiseman (King's College London)
Investigation of the Post-natal Developmental Toxicity of an Isoflavone in Rats	Dr R Lewis (Syngenta-CTL)
Activity of Phytoestrogens in Rats and Rabbits	Professor J Ashby (Syngenta-CTL)
Genomics Microassays and Transgenic Reporter Mice in the Study of Phytoestrogen Effects on Estrogen Receptor Mediated Gene Transcription	Dr G Orphanides (Syngenta-CTL)
Do Dietary Phytoestrogens Protect Against Cancer by Disrupting Metabolism of Endogenous Estrogens?	Dr C Kirk (University of Birmingham)

Appendix 5

Membership of the Working Group on Phytoestrogens

Chairman

Professor H F Woods BSc(Hons) BM BCh DPhil FFPM FRCP (Lon & Edin) CBE

Members

Professor S A Bingham BSc(Hons) MA PhD
Professor N A Brown BSc(Hons) PhD
Professor J K Chipman BSc(Hons) PhD CBiol FIBiol MRCP
Ms Sue Dibb BSc(Hons)
Dr P Hindmarsh BSc MB BS MRCP MD FRCP FRCPCH
Professor I A Hughes MA MD FRCP FRCP(C) FRCPCH
Dr M Joffe BA MB BChir MSc MD FRCP FFPHM MSc(Econ) PhD
Professor I Kimber BSc(Hons) MSc PhD FIBMS CBiol MIBiol
Professor I R Rowland BSc(Hons) PhD
Ms J Salfield BSc(Hons) MSc MIFST CertED RPHN
Dr R M Sharpe BSc(Hons) MSc PhD

Secretariat

Dr C Boyle BSc(Hons) MSc Dip Tox	(Scientific Secretary to August 2002)
Mrs S Hattersley BSc(Hons) Dip Tox	(Scientific Secretary from October 2002)
Miss J Lamothe	(Administrative Secretary until October 2000)
Mrs A Nathan BSc(Hons)	(Administrative Secretary from January 2001)
Dr T Barlow BSc(Hons) PhD	
Dr J Ince BSc(Hons) PhD	(until June 2000)
Dr B Jeffery BSc(Hons) PhD	(from October 2000)
Mrs K Moizer BSc(Hons) MSc	(from September 2000 to August 2002)
Miss S Paul BSc(Hons) MSc	(from September 2000 to January 2003)

Appendix 6

Declared interests of the Working Group on Phytoestrogens

	Personal Interests		Non Personal Interests	
Member	Company	Interest	Company	Interest
Professor H F Woods (Chairman)	Halifax Bank HSBC Ipsen Pharmaceuticals	Shares Fee Lecture fee	University of Sheffield, Faculty of Medicine Wide range of national and international food and chemical companies.	Has extensive activity in teaching and research in nutrition and toxicological and in topics related to and supported by may companies in the food and chemical industries. Trustee of the Harry Bottom Charitable Trust and Special Trustee for the former United Sheffield Hospitals.
Professor S A Bingham	None	None	None	None
Professor N A Brown	Merck Glaxo Wellcome Searle Styrene Information Research Centre Du Pont	Consultancy Consultancy Consultancy Consultancy Consultancy	EC(DGXI and DGXII) Glaxo Welcome US EPA	Research Support Research Support Research Support
Professor J K Chipman	Sequani AstraZeneca Inamed Unilever Syngenta	Training/ Consultancy Consultancy Consultancy Consultancy Lecture fees	AstraZeneca Glaxo Smith Kline ICI HSE CEFIC – LRI Dept. Health Inamed	Research Support Research Support Research Support Research Support Research Support Research Support Research Support
Ms S E Dibb	None	None	None	None
Dr P Hindmarsh	Glaxo Wellcome Serono	Consultancy Consultancy	None	None

	Personal Interests		Non Personal Interests	
Member	Company	Interest	Company	Interest
Professor I A Hughes	Pharmacia	Education Adviser	Academy of Med	IAH – Fellow
	BP Amco	Shares	Sciences	
	BP Amoco	Daughter is an employee of this company	Soc for Endocrinology	IAH – Council Member
			Royal College of Paediatrics and child Health	IAH – Fellow, Senior Examiner, Regional Academic Adviser
	Topical Endocrinology		Medical Res Council	IAH – Member of Advisory Board
			Pharmacia Aventis NovoNordisk Diabetes UK Wellcome Trust Juvenile Diabetes Fund	Funds received from all these sources for Departmental research and education in medicine and health related topics
Dr M Joffe	None	None	None	None
Professor I Kimber	British Airways	Share Holder	Unilever plc	Grant for Research
	British Petroleum-Amoco	Share Holder		
	ICI	Share Holder		
	Halifax	Share Holder		
	Astra-Zeneca	Share Holder		
	Syngenta	Share Holder		
	Lloyds TSB	Share Holder		
	Syngenta	Employee		
Professor I R Rowland	Colloids Naturels International Rouen (CNI)	Consultancy	Various	Departmental teaching and research funded by various food companies
	Cerestar	Consultancy		
	Halifax	Shares		
	Woolwich	Shares		
Ms J Salfield	None	None	None	None
Dr R M Sharpe	None	None	AstraZeneca European Centre for Ecotoxicology of Chemicals (ECETOC)	Research Support Research Support

Appendix 7

Membership of the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment

Chairman

Professor I A Hughes MA MD FRCP FRCP(C) FRCPH F Med Sci

Members

Professor P Aggett OBE MB ChB FRCP MSc DCh FRCPCH
Dr S Ariyanayagam MBBS MRCGP MRCOG MFFP DCh Lond
Dr P Carthew BSc(Hons)MSc PhD FRCPath
Professor J K Chipman BSc(Hons) PhD CBiol FIBiol MRCP
Dr P Jackson BA(Oxon) MA(Oxon) MB ChB MRCP PhD FRCP
Dr M Joffe BA MB BChir MSc MD FRCP FFPHM MSc(Econ) PhD
Professor I Kimber BSc(Hons) MSc PhD FIBMS CBiol MIBiol
Professor J Lunec BSc(Hon) PhD FRC Path
Dr A Piersma MSc PhD
Professor I R Rowland BSc(Hons) PhD
Dr L Rushton BA(Hons) MSc PhD CStat
Ms J Salfield BSc(Hons) MSc MIFST CertED RPHN
Dr A G Smith BSc(Hons) PhD CChem FRSC
Dr L Stanley BA PhD
Professor S Strobel MD PhD FRCP FRCPCH
Professor J A Timbrell BSc(Hons) PhD DSc MRCPath FRFC FIBiol
Dr M Tucker BSc(Hons) PhD FRCPath

Secretariat

Dr D Benford BSc(Hons) PhD	Scientific Secretary
Mr K Butler	Administrative Secretary
Dr D Gott BSc(Hons) PhD	
Ms C A Mulholland BSc(Hons)	
Dr C Tahourdin BSc(Hons) PhD	
Dr N Thatcher BSc(Hons) PhD	
Mr B Maycock BSc(Hons) MSc	
Mr N Ball BSc(Hons) MSc	
Mrs K Moizer BSc(Hons) MSc	
Dr S Sivapathasundaram BSc(Hons) PhD	

Appendix 8

The Food Standards Agency research projects on phytoestrogens

This table shows projects that have been completed or have yet to be completed.

Project code	Project Title	Contractor
T05001	Synthesis of labelled and unlabelled isoflavonoid phytoestrogen standards	University of St Andrews
T05002	Possible beneficial and adverse effects of dietary phytoestrogens in men	Rowett Research Institute
T05003	Possible effects of dietary phytoestrogens on prostrate cancer and 5-α-reductase activity	University of Edinburgh
T05004	Effects of phytoestrogens and related dietary components on bone metabolism	Rowett Research Institute
T05005	Development and application of screening assays for the beneficial/adverse effects of phytoestrogens in food	Veterinary Laboratories Agency
T05006	Investigation of the post-natal developmental toxicity of an isoflavone-genistein in rats	Syngenta-CTL
T05009	Use of ^{13}C isomers as analytical standards and tracers of phytoestrogens in humans	MRC-Human Nutrition Research
T05010	Absorption and metabolism of dietary phytoestrogens in humans- effect of age, gender, food matrix and chemical composition	University of Surrey
T05011	Influence of human gut microflora on dietary soya isoflavone phytoestrogen bioavailability in adults and children	King's College London & University of Ulster
T05013	Do dietary phytoestrogens protect against cancer in genetically susceptible groups by disrupting the metabolism of endogenous oestrogens?	University of Birmingham
T05014	The effect of phytoestrogen ingestion on menopausal women	University of Manchester
T05015	Diet, phytoestrogen and gene nutrient interactions in relation to cancer: a prospective study	MRC Dunn Human Nutrition Unit
T05016	Genomics microassays and transgenic reporter mice in the study of phytoestrogen effects on estrogen receptor mediated gene transcription	Syngenta-CTL

Project code	Project Title	Contractor
T05018	Consensus review of the Food Standards Agency phytoestrogens programme	Ashwell Associates
T05019	Absorption, distribution, metabolism and excretion of isoflavones *in vivo*	University of Surrey
T05020	Absorption, distribution, metabolism and excretion of ^{14}C labelled genistein	Veterinary Laboratories Agency
T05021	Quality assurance scheme for phytoestrogens	Central Science Laboratory
T05022	Biological effects of phytoestrogens	MRC Dunn Human Nutrition Unit
T05023	Synthesis of phytoestrogen standards in labelled and unlabelled form	University of St Andrews
T05024	Quality assurance scheme for the Food Standards Agency's phytoestrogen research programme	Institute of Food Research
T05025	Effect of ERβ over-expression on the molecular action of phytoestrogens	University of Reading
T06001	Identification and quantitation of dietary lignans by liquid chromatography and mass spectrometry	Veterinary Laboratories Agency
T06002	Phytoestrogen dietary supplement survey	Veterinary Laboratories Agency
T06003/T06004	Duplicate diet study of vegetarians and analysis of total diet study samples	Central Science Laboratory