COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Statement on the potential risks from high levels of soya phytoestrogens in the infant diet

Background

1. The Scientific Advisory Committee on Nutrition (SACN) is undertaking a review of scientific evidence that bears on the Government's dietary recommendations for infants and young children. The review will identify new evidence that has emerged since the Government's current recommendations were formulated, and will appraise that evidence to determine whether the advice should be revised. The recommendations cover diet from birth to age five years, but will be considered in two stages, focussing first on infants aged 0 – 12 months, and then on advice for children aged 1 to 5 years. SACN is examining the nutritional basis of the advice, and has asked that evidence on possible adverse effects of diet should be considered by other advisory committees with relevant expertise. In particular, SACN asked the Committee on Toxicity (COT) to review the risks of toxicity from chemicals in the infant diet.

2. This statement provides an overview of the potential risks from soya phytoestrogens in the infant diet. Soya-based infant formula and weaning food products containing soya are the main source of phytoestrogen exposure in infants. Soya products contain predominantly three isoflavones: genistein, daidzein and to a lesser extent glycitein.

3. The COT produced a report on phytoestrogens and health in 2003[1], which concluded that there was no definitive evidence that phytoestrogens in soya-based formula harm the health of infants. However, there was a possibility of adverse effects, particularly in infants with congenital hypothyroidism, and there might also be impacts on reproductive health, although no definitive conclusions could be drawn. The COT report noted that inter-species differences in absorption, distribution, metabolism and excretion, and in the timing of sexual development, made it difficult to extrapolate effects from animals to humans (COT, 2003).

4. This statement summarises new literature concerning possible health effects from exposure of infants to soya isoflavones, which has become available since the 2003 COT report, and considers the implications for the infant diet of the evidence

[1] COT Report – Phytoestrogens and Health (2003). Available at: http://cot.food.gov.uk/pdfs/phytoreport0503

that is now available. The criteria that were employed in the literature search are set out in Appendix 1

Introduction

5. Phytoestrogens are chemicals of plant origin that have been shown to influence biological processes mainly through their structural similarities to oestrogens, and their ability to bind to oestrogen receptors (ERs). The largest group of phytoestrogens are flavonoids, which can be further divided into three subclasses, coumestans, prenylated flavonoids and isoflavones.

6. The isoflavones, genistein, daidzein and glycitein (Figure 1) share a common structure with two aromatic benzene rings linked by three carbon atoms forming part of an oxygenated heterocyclic ring. The phenolic and hydroxyl moieties (and the distance between them) are key structural similarities between isoflavones and 17β-oestradiol (Figure 2), which allow them to bind to ERs. Numerous studies have indicated that genistein is the isoflavone with greatest oestrogenic activity (NTP, 2010).

7. Isoflavones can be found in many plants, including barley, sunflower, clover, lentils, alfalfa sprout, broccoli and cauliflower. However, the richest sources of isoflavones in the human diet are foods and dietary supplements made from soya bean and soya protein (NTP, 2010). Soya isoflavones in foods occur mainly as carbohydrate conjugates (glycosides), the major group being the glucose conjugates (glucosides), genistin (Figure 1), daidzin and glycitin. When β-glycosidic bonds of glycosides are hydrolysed, the biologically active aglycone[2] forms are produced. Bacterial hydrolysis can significantly increase the content of aglycones in fermented soya-based food products such as tofu[3] and tempeh[4]. In soya infant formula, the aglycones, genistein and daidzein have been reported to constitute 3.2 – 5.8% of the total isoflavones (Chen and Rogan, 2004).

Current UK Government recommendations in relation to infant diet

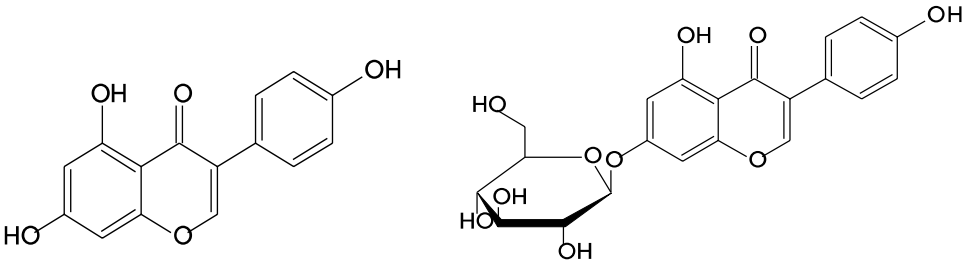
8. Based on the COT (2003) report, the SACN concluded that there was no substantive medical need for, nor health benefit arising from, the use of soya-based infant formulas[5]. In 2004, the Department of Health's Chief Medical Officer advised doctors that soya-based infant formulas should not be the first choice in the management of infants with proven cows' milk sensitivity, lactose intolerance, galactokinase deficiency or galactosaemia, and that they should only be used in exceptional circumstances to ensure adequate nutrition (DH, 2004).

[2] An aglycone derived from a glucoside (where the sugar moiety was a glucose residue) is known as an aglucone. In this statement, the generic terms glycoside and aglycone are used unless referring to specific compounds or in quotations of previous publications.

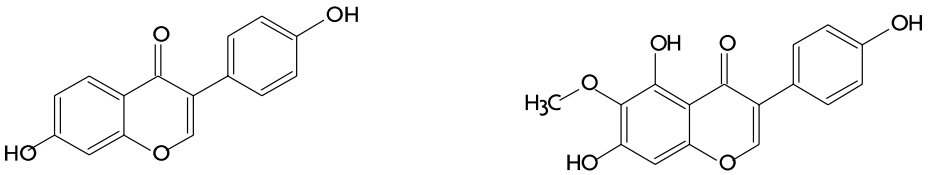
[3] Tofu, also called bean curd, is made by coagulation of soya juice and then precipitation of soya curd into blocks.

[4] Tempeh is a fermented soya product made from whole soya beans, and has a high content of protein, dietary fibre and vitamins

[5] Scientific Advisory Committee on Nutrition response to the COT Working Group on Phytoestrogens draft report on phytoestrogens and health, 2003. Available at: http://cot.food.gov.uk/pdfs/2003-03.pdf

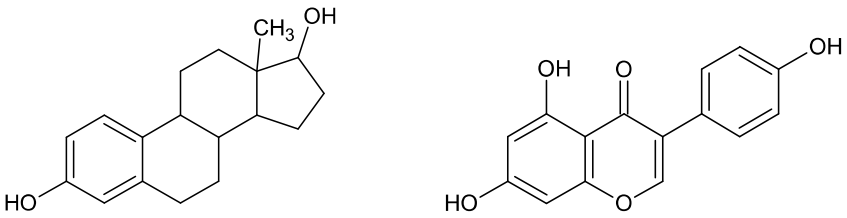


Genistein Genistin (genistein glucoside)



Daidzein Glycitein

Figure 1. Chemical structures of the isoflavone aglycones genistein, daidzein and glycitein, and the glucoside genistin.



17β-oestradiol Genistein

Figure 2. The similarity of the structure of 17β-oestradiol and genistein

Recommendations in other countries

9. In the USA, an expert panel of the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) expressed only minimal concern about possible adverse developmental health effects in infants consuming soya-based infant formula (McCarver *et al.*, 2011). However, the American Academy of Pediatrics (AAP) has highlighted a lack of proven health benefits from soya-based formula or advantages over breastfeeding and cows' milk-based formula, as well as the possibility of health risks (Bhatia and Greer, 2008).

10. In Israel, France and Germany consumption of soya-based infant formula has been recommended only for exceptional medical indications such as galactosaemia, hereditary lactase deficiency and secondary lactose intolerance, or where there is strong preference for a vegetarian/vegan diet (Berger-Achituv *et al*., 2005; AFSSA, 2005; BfR, 2007).

Absorption, distribution, metabolism and excretion

Absorption[6] and metabolism

11. The 2003 COT report reviewed the absorption, distribution, metabolism and excretion (ADME) studies in humans that had been published up to 30th April 2002. The report summarised that "*isoflavones are mainly ingested as glucosides, which undergo hydrolysis most probably in the small intestine through the action of β-glucosidase enzymes associated with the intestinal mucosa and in the lower bowel by the gut microflora. The deglucosylated (aglucone) compounds may be further metabolised by the gut bacteria and/or absorbed, 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). Aglucones are more readily absorbed due to their higher hydrophobicity and lower molecular weight. Once absorbed, these compounds are rapidly and extensively re-conjugated (largely with glucuronic acid or sulphate) 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 re-absorbed or degraded. 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"* (COT, 2003).

12. NTP-CERHR (2010) noted that the principal Phase I metabolism of glycosides and aglycones within the gut includes reduction, deoxygenation, hydroxylation and ring cleavage. Aglycones and their metabolites undergo pre-systemic metabolism by glucuronidation and, to a lesser extent, sulphation, in the intestinal cells and particularly the liver. The conjugated compounds are then transported to tissues, and are excreted in urine or bile (reviewed in NTP, 2010).

13. COT (2003) and NTP-CERHR (2010) noted that because of their high water solubility and high molecular weight, isoflavone glycosides are unlikely to be absorbed through the gut wall in either animals or humans. However, some authors have reported that partial absorption of intact glycosides is possible in the small intestine, either by diffusion (Andlauer *et al*., 2000), or through active transport by a carrier system such as the sodium-dependent glucose transporter (SGLT1) (Gee *et al.*, 1998; Kwon *et al*., 2007; Nemeth *et al*., 2003). Glycosides transported into the cells via SGLT1 are subsequently hydrolysed to aglycones by intracellular cytosolic β-glucosidase (CBG), and then are further metabolised to conjugates (Nemeth *et al*., 2003).

[6] Absorption occurs primarily after isoflavones are hydrolysed to their aglycones. Therefore, in this statement the term absorption refers to the aglycones and their metabolites rather than the parent molecules.

14. In humans, peak concentrations of genistein and daidzein in plasma occur approximately 5.5 and 7.4 hours after oral administration. Equol, the reductive metabolite of daidzein, is detected in plasma 12 – 36 hours following oral administration of isoflavones (Setchell *et al*., 2003; Setchell and Clerici, 2010). Formation of equol depends entirely on intestinal bacterial metabolism (Setchell *et al*., 2002), and it has been shown that only 30% to 50% of adult humans are equol producers (NTP, 2010). Specific bacteria that are responsible for equol production in humans include *Lactobacillus sp*., *Enterococcus faecium*, *Bifidobacterium sp*. and *Finegoldia magna* (reviewed by Setchell and Clerici, 2010). Infants who are exclusively fed soya infant formula from birth, lack the microflora necessary for equol production (Setchell *et al*., 1997 and 1998).

15. Equol, unlike genistein and daidzein, has a chiral centre, and can occur as two diastereoisomers, S- and R-equol. However, only S-equol, which has a high affinity for ER β (the R form is relatively inactive), is found in human plasma and urine (Setchell *et al*., 2005).

16. The bioavailability of genistein following oral administration of genistin (37.5 mg/kg bw/day) or genistein (37.5 mg/kg bw/day) to neonatal mice was investigated by Jefferson *et al*. (2009). Data from a study in which genistein was administered subcutaneously (sc) (50 mg/kg bw/day), as reported by Doerge *et al*. (2002), were assumed to reflect 100% bioavailability, and were used as a reference. The dose-adjusted area under the curve (AUC) measured in serum after oral administration of genistin was 48% (free genistein) and 83% (total of free and conjugated genistein). Following oral dosing of genistein the bioavailability was lower. In support of these findings, when compared with sc genistein, approximately 20-33% more oral genistin was needed to produce similar oestrogenic activity in neonatal female mice (assessed by increase in uterine wet weight) (Jefferson *et al*., 2009). Higher oral bioavailability of genistin than genistein has also been reported in rats (Kwon *et al.,* 2007). The authors attributed this to differences in absorption and metabolism of the two compounds, including a capacity (supported by other evidence) to absorb genistin in both its intact and aglycone forms (Kwon *et al*., 2007).

17. Other experiments comparing the bioavailability of isoflavones consumed as glycosides or aglycones have given conflicting results. Studies reviewed in the COT 2003 report suggested that bioavailability of isoflavones is higher when they are ingested as aglycones, as in fermented foods (COT, 2003). Since then, Rufer *et al*. (2008) reported higher bioavailability of daidzein following ingestion of the glycoside as compared with the aglycone, whereas Izumi *et al*. (2000) found that isoflavone aglycones were absorbed faster and in larger amounts than their glycosides. Zubik *et al*. (2003) reported no difference in bioavailability between the two forms of isoflavone.

18. Bioavailability of soya phytoestrogens was investigated in Caucasian (n=12) and Asian (n=12) male volunteers consuming soya-based cheese (containing approximately 25 mg of genistein and 21 mg of daidzein) once per day. After a single intake, Asians had higher isoflavone plasma concentrations and AUC. In contrast, ingestion for 10 days resulted in higher plasma concentrations in Caucasians, than in

Asians, regardless of whether the background diet was Western or Asian (Vergne *et al.*, 2009).

Distribution and excretion

19. The COT 2003 report summarised that "*isoflavones and their metabolites are widely distributed within body fluids. 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*. *There is evidence of transfer of isoflavones and their metabolites to breast milk via the maternal diet and to the fetal compartment as concentrations similar to those in maternal plasma have been detected in umbilical cord plasma and amniotic fluid. However, definitive tissue distribution studies have not been performed in man"* (COT, 2003).

20. Isoflavones in the mothers' diet can be transferred to babies via breast milk. After mothers who met the criterion of breastfeeding "≥80% of the time" consumed soya protein beverages, levels of isoflavones in their urine and that of their babies were similar (Franke *et al*., 2006).

21. Genistein, daidzein and their metabolites are mainly excreted in urine. Following ingestion, 94% of urinary excretion of isoflavones is complete within approximately 24 hours. Average plasma half-lives have been estimated as 6.1-17 hours for genistein and 3-16 hours for daidzein (NTP, 2010). Hoey *et al.* found that infants aged 4-6 months who were fed soya-based infant formula had significantly higher urinary concentrations of isoflavones than controls. This suggested that the ability to hydrolyse glycosides to aglycones has developed by 4-6 months of age. In most urine samples from older infants who had been fed soya formula in early infancy, levels of isoflavone metabolites were similar to those measured in infants who had not been given soya formula at younger ages (Hoey *et al*., 2004). In another study, genistein and daidzein could not be detected in most blood and saliva samples obtained from infants fed breast or cows' milk. The median urinary concentration of isoflavones in infants fed soya formula was 500 times higher than in the cows' milk-fed group (Cao *et al*., 2009). In accord with the findings of previous studies (Franke *et al*., 2006; Setchell *et al*., 1997), equol was rarely detected (Cao *et al*., 2009).

22. Amniotic fluid samples collected from pregnant women who reported the use of soya products, were found to contain significantly higher concentrations of genistein and daidzein when the fetus was female than when the fetus was male (Jarrell *et al*., 2012). The findings could not be explained by differences in fetal weight as the male and female babies had similar birth weights. There were no sex-related differences in levels of isoflavones measured in breast milk, cord serum and mothers' serum during pregnancy and at birth. The authors suggested that metabolic handling of isoflavones during fetal life may differ between boys and girls. Although

sex differences in fetal levels of glucuronyl transferases have been reported in animals, parallel information is not available for humans.

Modulation of absorption and metabolism of isoflavones

23. The 2003 COT report stated that "*the gut microflora play a crucial role in determining the absorption, metabolism, re-absorption (enterohepatic circulation), degradation and excretion of ingested isoflavones and their metabolites. Data indicate considerable inter-individual variation in the pharmacokinetic and metabolic handling of ingested phytoestrogens. Such differences may be largely attributed to an individual's unique gut microflora, which is influenced by factors such as diet, particularly fibre content, and intestinal transit time, hygiene, antibiotic use, bowel disease, stress, gut motility, gastric pH, mucin and bile secretion. Gender, age, genetics, food matrix and ethnicity may also be determining factors. An initial colonisation of the gut in infants is especially determined by factors such as the composition of maternal gut flora, the mode of delivery (conventional or caesarean birth), hygiene, environment and genetics. The influence of the diet is greater on the gut microflora of babies who were breast fed than those who were fed infant formula*" (COT, 2003).

Food matrix

24. Cassidy *et al*. (2006) observed that the type of food matrix affects the bioavailability of isoflavones in healthy adults. Three soya foods having different isoflavone composition – soya milk, textured vegetable protein (TVP) and tempeh (in which approximately 50% of isoflavones are aglycones) – were studied. Consumption of tempeh resulted in higher peak levels of genistein and daidzein in serum than TVP. However, isoflavones from soya milk were absorbed faster and peak levels were attained earlier than with the other soya foods (Cassidy *et al*., 2006). Another study found no difference in bioavailability of isoflavones following consumption of miso soup as compared with soya milk (Maskariniec *et al.*, 2008).

Pro- and pre- biotics

25. Effects of supplementation of soya milk with probiotics[7], such as *Lactobacillus sp.* and *Bifidobacterium sp*, and prebiotics[8] (e.g. fructooligosaccharides, inulin, pectin and mannitol) were investigated *in vitro* by Yeo and Liong (2010). Using model systems, prebiotics were found to increase growth of probiotics and enhance β-glucosidase activity and proteolysis. As a result an enhanced bioconversion of glucosides to bioactive aglycones, especially of genistin and malonyl genistin to genistein, was observed (Yeo and Liong, 2010). Another study showed that administration of *Lactobacillus sp* as a probiotic supplement in the form of capsules decreased urinary isoflavone excretion Cohen *et al*. (2007). The authors suggested

[7] Probiotics – live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (WHO/FAO, 2001-2002)

[8] Prebiotics – non-viable food components that confer a health benefit on the host associated with the modulation of the microbiota (FAO, 2001)

that the organism may alter isoflavone metabolism by stimulating deconjugation and/or inhibiting degradation.

Age

26. Metabolism of isoflavones may vary with age. Although several studies that have been published since the 2003 COT review have investigated the distribution and metabolism of isoflavones in infants, knowledge of how these substances are handled at young ages is still limited. Halm *et al.* (2007) reported that bioavailability of isoflavones was higher in school age children than in adults consuming the same diet (soya nuts). On the other hand, Cassidy *et al.* (2006) found that men and pre- and postmenopausal women absorbed isoflavones from a range of different soya-rich foods to a similar degree (children were not included in this study). Infants can effectively absorb isoflavones from breast milk, soya infant formula and food products containing soya, but there may be differences in metabolism from adults because of the immaturity of the intestinal flora and/or larger intakes when adjusted for body weight. Also, infants have less ability to glucuronidate isoflavones due to lower activity of uridine diphosphate (UDP)-glucuronosyltransferases activity (NTP, 2010).

Maturity of gut flora

26. The digestive system of the new born infant is immature and takes several weeks to develop. Initially, the gut is colonised by *Enterobacteria*, *Streptococci* and *Staphylococci* capable of oxidative metabolism. Subsequently, they are replaced by strictly anaerobic bacteria, such as *Bifidobacteria*, *Clostridia* and *Bacteroides*. However, it is unclear at what age infants develop gut microflora fully capable of metabolising isoflavones (COT, 2003). Decreasing gut permeability following initial colonisation with mother's bacteria during vaginal birth stimulates gut flora maturation. This initial colonisation results in an acidic environment and gut flora dominated by *Bifidobacteria* for the first 6 weeks of life (Catassu *et al.*, 1995). Bacterial β-glucosidase activity, associated with bacteria such as *Lactobacilli*, *Bifidobacteria* and *Bacteroides*, appears to be lower in infants than in adults, and shows an age-dependent increase (NTP, 2010; Setchell *et al*., 1998).

27. Franke *et al.* (2006) investigated maturity of gut flora, related to age and type of food, as a determinant of the ability of infants and children to take up isoflavones. In a previous publication they had considered isoflavone glucuronides and sulphates (as present in breast milk of mothers eating soya) to be more available to the infant than the glycosides (as in soya food), which require hydrolysis before absorption (Franke and Custer, 1996). In the later study, they observed low isoflavone concentrations in body fluids of breast-fed infants, and higher levels, exceeding those observed in adults eating soya products, in infants who had commenced complementary feeding and were fed tofu. The authors commented that this finding could probably be explained by the very low isoflavone dose from breast milk, and also by the limited ability of the immature gut flora in breast-fed infants to cleave glucuronide and sulphate conjugates in breast milk and produce aglycones that can be taken up. The infants consuming tofu were older, and it was possible that their gut

flora had attained the ability to hydrolyse glycosides (Franke *et al.*, 2006). Although genistein and daidzein have been reported in urine samples from infants, levels of equol have been low or undetectable, indicating limited biotransformation beyond initial hydrolysis (Setchell *et al*., 1998; COT, 2003).

Species, sex and ethnic differences in isoflavone metabolism

28. Gu *et al*. (2006) reported that the ability of female monkeys to produce equol from daidzein was more similar to that of female Sprague-Dawley rats, than to that of women. Rats and monkeys appeared to have intestinal bacterial composition that favoured equol biosynthesis, whereas equol was not detected in the serum of women or pigs (in which genistein and daidzein comprised 88% and 91% respectively of summed isoflavones). Similarly, in urine, the proportion of equol in total isoflavones (including metabolites) was 51% in monkeys and 69% in rats, as compared with 2% and 0% in pigs and women respectively. Monkey and rat urine contained high levels of aglycones, whereas pigs and women excreted isoflavones mainly in the form of glucuronides, with <10% as aglycones. Thus, pigs may provide a better animal model than rats or monkeys for studying the effects of dietary isoflavones in humans (Gu *et al*., 2006).

29. Circulating concentrations of unconjugated isoflavones in rodents and humans were also compared by Setchell *et al.* Based on the steady state percentages of unconjugated isoflavones, the authors concluded that capacity to conjugate isoflavones differs significantly between rats and mice, as well as between rodents and humans, and that humans have a much higher capacity to conjugate isoflavones than either species of rodent (Setchell *et al.*, 2011). Both species and sex differences in tissue distribution of isoflavones were observed by Gilani *et al*. (2011), who reported significantly higher concentrations of isoflavones in the serum of male as compared with female rats when they were fed isoflavone-supplemented diets. In rats, the highest concentrations of isoflavones were detected in serum, with lower levels (sometimes undetectable) in livers and mammary glands. In pigs, isoflavone concentrations in livers were slightly higher than in rats (Gilani *et al*., 2011).

30. It has been reported that some 30% to 50% of individuals are equol-producers, and that 80% to 90% are able to produce *O*-DMA (Atkinson *et al*., 2009). Production of equol has been associated with diets rich in isoflavones, carbohydrates and fibre, but low in fat (NTP, 2010). Song *et al*. studied daidzein metabolism in American women of Korean background (n=91), who consumed one soya protein bar, containing approximately 38 mg of daidzein as aglycone equivalents, daily for three days. The prevalence of equol-producers was 51%, which was higher than reported by Frankenfeld *et al*. (2004) for Caucasian women (36%, n=222). However, the prevalence of the *O*-DMA producer phenotype was lower (84% vs 92% in the earlier study). The Asian women consumed approximately three times more soya products than Caucasians, but soya consumption was not associated with equol-producer phenotype (Song *et al*., 2006).

Hazard identification and characterisation

Toxicity in experimental animals/models

31. Animal studies performed before 2003 indicated that intake of isoflavones in early life can produce oestrogenic effects, affect thyroid function, alter protein concentrations and structures in the brain, and alter some parameters of immune function, as well as sexual development in older animals. Although some animal studies indicated possible risks to humans, overall the results of animal studies were conflicting. The COT 2003 report noted that human data were limited, and that most of the relevant scientific information was derived from experimental studies in animals, mainly rodents. The extrapolation of such studies to humans was difficult because of inter-species differences in ADME, sexual development and reproductive function, and the use of relatively high doses or non-oral routes of administration (COT, 2003).

32. The few available studies evaluating effects of exposure to isoflavones in primates were summarised in COT (2003). Treatment with isoflavones appeared to have no adverse effect on vaginal maturation in ovariectomised cynomolgus macaques; no effect on endometrial or mammary tissue in female macaques; and no effect on plasma hormone concentrations or uterine, prostatic and testicular weights in rhesus monkeys. There was also no significant effect on maternal, fetal or placental weights in rhesus monkeys. Although non-human primates were of particular relevance for investigation of possible adverse health effects in humans, their use in laboratory testing had been limited to a small number of studies, *inter alia* for ethical reasons (COT, 2003).

Genotoxicity and carcinogenicity

33. *In vitro* studies reviewed in the 2003 COT report indicated genotoxic effects of some phytoestrogens. Genistein had been shown to induce DNA strand breaks, mutations and micronuclei (MN). It was also weakly mutagenic in bacterial and mammalian mutation assays. It was noted, however, that the concentrations used in these studies were much higher than would be expected to occur *in vivo* following dietary exposure (COT, 2003). Studies published subsequently have not found genistein to be mutagenic in bacterial tests (McClain *et al.*, 2006; Yee *et al*., 2008). However, several positive results have been observed in mammalian cells. McClain *et al*. (2006) reported mutations in mouse lymphoma cells. Clastogenic (genistein, daidzein) and aneugenic (daidzein, equol) activity was observed in a micronucleus assay with Chinese Hamster V79 cells (Di Virgilio *et al*., 2004); and DNA strand breaks were induced by genistein in the Comet assay, using cultured human lymphocytes (Ullah *et al*., 2009).

34. The COT 2003 report noted that the single *in vivo* study available at that time suggested that genistein at dietary levels was not mutagenic (COT, 2003). This is supported by a more recent study by McClain *et al*. (2006), who reported a lack of mutagenicity of genistein *in vivo* in the micronucleus assay in mice and rats.

35. The National Toxicology Program (NTP) has conducted carcinogenicity studies in which animals were fed diets containing 5, 100, or 500 ppm genistein from the time of conception, through weaning, and then for up to two years. There were no treatment-related increases in tumour incidence in male rats, whereas in female rats the incidence of adenoma/adenocarcinoma of the mammary gland and of pituitary gland adenoma and carcinoma was increased in the top dose group (NTP, 2008b).

36. Spontaneous mammary tumour development was investigated in female Tg.NK mice fed a diet containing soya isoflavones (genistein, daidzein and glycitein) at 0, 11, 39, and 130 mg aglycones/kg diet from post-natal day (PND) 25 for 24 consecutive weeks. The highest concentration increased the number and size of mammary tumours ($p<0.05$). Increased branching of the mammary tree was observed in all treated groups ($p<0.05$) indicating that soya isoflavones can increase epithelial proliferation at an early age (Thomsen *et al*., 2005).

37. Nielsen *et al*. (2011) investigated the influence of *in utero* exposure of rats to isoflavones in cows' milk on susceptibility to induction of mammary tumours by 7,12-dimethylbenz[a]anthracene (DMBA). Maternal intake of cows' milk containing a low level of isoflavones (mean 101, standard error of the mean (SEM) 3.3 ng total phytoestrogens/mL; mean 1.7, SEM 0.6 ng daidzein/mL), resulted in increased levels of circulating oestradiol and IGF-1 in the offspring, but no increase in mammary tumours. In contrast, intake of cows' milk containing a high level of isoflavones (mean 429, SEM 11.9 ng total phytoestrogens/mL; mean 5.8, SEM 0.3 ng daidzein/mL] had no effect on circulating oestradiol and insulin-like growth factor 1 (IGF-1) levels, but significantly increased DMBA-DNA adducts in the mammary gland and the number of mammary tumours per animal.

Fertility and development

38. A number of studies have addressed possible adverse effects on development from exposure to isoflavones early in life (see Appendix 2). The endpoints assessed have included body weight, onset of puberty, and changes in adipose tissue and reproductive organs. Observed effects, although varying between studies, appear consistent with interference in oestrogen-mediated responses. In one study, the lowest dietary dose of genistein (0.42 mg/kg bw/day) that was administered to pregnant and lactating rats, led to increases in thymus weights and subpopulations of T cells (significantly higher proportion of $CD8^+$ T cells in the spleen and $CD4^+CD8^+$ T cells in the thymus) in male offspring, possibly because of reduced gonadal steroid secretion. Reduced serum testosterone was seen in the male offspring of treated mothers (Klein *et al*., 2002). Health effects observed after subcutaneous administration of genistein directly to offspring, included dose-related increases in multi-oocyte follicles (MOFs) (Jefferson *et al*., 2002 and 2006) and uterine weights (Jefferson *et al*., 2009), as well as advanced vaginal opening, prolonged and persistent oestrus cycles, and abnormalities in uteri and ovaries (Kouki *et al*., 2003; Dinsdale *et al*., 2011 and Kaludjerovic *et al*., 2012). Following oral administration, there was an increase in uterine weights (LOAEL = 25 mg/kg bw/day); a dose-dependent increase in percentage of MOFs (NOAEL = 6.25; LOAEL = 12.5); ovarian cycle abnormalities; decreased anogenital distance (AGD); and

changes in body weight. Both advanced (Thigpen *et al*., 2003 and 2007; LOAEL = 51 in NTP, 2008a) and delayed (LOAEL = 37.5 in Jefferson *et al*., 2009) vaginal opening have been reported in different studies.

39. Tan *et al*. (2006) performed a study in seven pairs of male marmoset monkey twins, in which one of each pair of twins was fed standard (cows') milk formula and the co-twin was fed soya formula milk (estimated isoflavone dose: between 1.6 and 3.5 mg/kg bw/day) for 5-6 weeks. Observed effects in the monkeys fed soya formula included increased testicular weight, and increased numbers of Sertoli and Leydig cells (32%; p=0.026). The increase in the number of Leydig cells was especially marked in males with low-normal testosterone levels, indicating possible "compensated Leydig cell failure" in response to neonatal suppression of testosterone secretion (Tan *et al*., 2006). Previously, a 74% increase in Leydig cells, accompanied by decreased levels of testosterone, had been reported in marmosets fed soya formula for 4-6 weeks, (Sharpe *et al*., 2002). In a more recent study, pubertal female cynomolgus monkeys were fed a diet containing soya protein isolate, with a "human-equivalent dose of 120 mg isoflavones/day", for approximately 4.5 years. There were no changes in the onset of menarche, growth or pubertal progression, or in oestradiol or progesterone levels. Treated animals had some changes in breast differentiation (increased numbers of differentiated large-sized lobular units and a lower proportion with immature ducts following menarche) (Dewi *et al*., 2013).

Behavioural studies

40. A number of animal studies have investigated behavioural effects of dietary exposure to isoflavones (see Appendix 2). Behavioural effects commonly observed in treated animals have included decreased time spent in social interactions, increased submissive behaviour, changes in sexual behaviour, increased activity and more frequent episodes of aggressive behaviour, and also lower body weight and length. When reported, the concentrations of isoflavones in feed were between 0.4 and 600 ppm.

Immunosuppression

41. Genistein has been reported to have both inhibitory and stimulatory effects on the immune system in rodents. Several animal studies were reviewed by Cooke *et al.*, who noted altered thymic size; decreased proliferation of leukemic cell lines at higher concentrations; and decreased production of the cytokine IFN-γ (Cooke *et al.,* 2006).

Mechanistic studies

42. *In vitro* experiments reviewed in the 2003 COT report showed that phytoestrogens could modulate the levels of sex hormone binding globulin (SHBG), inhibit enzymes involved in oestrogen biosynthesis and metabolism to modulate concentrations of endogenous oestrogens, and inhibit thyroid peroxidase activity to

reduce the concentrations of thyroid hormones. Genistein was found to interact with topoisomerase II and protein kinases (enzymes involved in cellular proliferation and differentiation) and to inhibit human T-cell proliferation and interleukin-2 production (COT, 2003).

43. DNA methylation, following dietary supplementation of maternal diet with methyl donors such as folic acid, is one of epigenetic modifications that have been associated with a decreased risk of obesity and cancer in the mice offspring. Dolinoy *et al*. (2006) reported that exposure to diet supplemented with genistein (250 mg/kg) *in utero* caused methylation of six cytosine-guanine sites and as a result shifted the coat colour in Agouti mice and reduced incidence of obesity. In another study genistein was shown to counteract a hypomethylation effect of bisphenol A, altered reproductive functions and increased cancer risk (Dolinoy *et al*., 2007).

Oestrogenic potency of phytoestrogens

44. Oestrogenic effects have been reported in studies performed in juvenile and ovariectomised rodents treated with isoflavones through oral and non-oral routes. Increased uterine weights, advanced vaginal opening, and changes in uterine and vaginal epithelium have been observed in mice and rats following oral exposure at doses starting from about 60-100 mg/kg bw/day (see Appendix 2). In addition, irregular oestrus cycles have been observed following subcutaneous exposure (10 mg genistein/kg bw/day) (Bateman and Patisaul, 2008).

45. The cellular and molecular mechanisms of oestrogenic action, and the oestrogenic potency of phytoestrogens, have been extensively investigated. 17β-Oestradiol binds with similar affinities to oestrogen receptors (ER) α and β. Although phytoestrogens are structurally similar to 17β-oestradiol, and have both oestrogen agonist and antagonist activity, genistein and daidzein have been shown to have much higher affinity to ERβ than ERα (Kuiper *et al*., 1997 and 1998; COT, 2003)

46. The distribution of ERα and β varies between tissues, and within tissues between different species. Both subtypes of receptor have been found in humans and rodents in heart, uterus, ovary and bone. They can also be found, although in different ratios, in other tissues such as lung, kidney, prostate, testis, brain, bladder, liver and GI tract. In humans, but not in rodents, the two receptors have been found in vascular, breast and endometrial tissues, as well as in the vagina and fallopian tubes. In rodents, expression of both receptors has been reported in muscle and fat (COT, 2003).

47. Matsumura *et al.* (2005) determined the ER-binding of isoflavones in human breast cancer cells, using radiolabelled oestrogen [2,4,6,7-^{3}H]oestrogen at 16 x 10^{-10} M. Genistein inhibited [^{3}H]oestrogen binding by 50% at 1000- and equol at 4000-fold molar excess. Due to its limited solubility, the maximum inhibition by daidzein that could be demonstrated was 40% (10,000-fold molar excess) (Matsumura *et al*., 2005). ER-binding potencies of isoflavones have also been assessed in other species. In experiments with different isoflavones and their combinations, genistein appeared to have the maximum affinity to both receptors, with approximately 60-fold higher binding preference for ERβ (Zhao *et al.,* 2009). Harris *et al.* tested for

selective affinity of various phytoestrogens to human, rat or mouse ERs, and reported that most compounds were non-selective, which was consistent with the observation that the ligand-binding domains of both ERs are highly conserved across species (Harris *et al*., 2002).

48. Table 1 shows a comparison of the ER-binding potencies of isoflavones in human, mouse and rat. In all species, the oestrogenic binding potency of isoflavones is much weaker than that of oestradiol, and higher molar concentrations are needed to achieve 50% inhibition of oestradiol binding to ERα and β. In the case of genistein, the concentration must be ≥ 100 fold higher for ERα, and ≥ 2 fold higher for ERβ. The relative binding affinities (RBAs) presented in Table 1 illustrate how isoflavones preferentially bind ERβ.

Table 1. The ER binding potency of isoflavones in different species

Compound	ERα		ERβ		Ratio of RBAs ERβ/ERα	Reference
	IC_{50} or [EC_{50}] (μM)	**RBA (%)**	**IC_{50} or [EC_{50}] (μM)**	**RBA (%)**		
Human						
17β-oestradiol	0.0020	*100*	0.0023	*100*	0.86	Harris et al., 2002
Diethylstilbestrol	0.0014	*142*	0.0011	*209*	1.27	
Genistein	0.3340	*0.60*	0.0066	*34.8*	50.60	
Daidzein	>5.0000	*<0.04*	0.4100	*0.56*	>12.19	
17β-oestradiol	0.0043	*100*	0.0057	*100*	0.75	Mueller et al., 2004
Diethylstilbestrol	0.0046	*93*	0.0046	*124*	1.00	
Genistein	0.3000	*1.43*	0.0150	*38.0*	20.00	
Equol	1.5000	*0.29*	0.2000	*2.85*	7.50	
17β-oestradiol	[0.021 x 10^{-3}]	100	[0.11 x 10^{-3}]	100	0.19	Muthyala et al., 2004
Genistein	[0.0800]	0.02	[0.0066]	7.40	12.00	
Daidzein	[0.2500]	0.01	[0.1000]	0.04	2.50	
Equol	[0.2000]	0.20	[0.0740]	1.60	2.70	
17β-oestradiol		100				Kwok and Cheung, 2010
Diethylstilbestrol		100				
Genistein		1.50				
Mouse						
17β-oestradiol	0.0021	*100*	0.0025	*100*	0.84	Harris et al., 2002
Diethylstilbestrol	0.0002	*1050*	0.0023	*109*	0.09	
Genistein	0.4000	*0.53*	0.0046	*54.3*	86.95	
Daidzein	4.9930	*0.04*	0.1670	*1.50*	29.89	
Rat						
17β-oestradiol	0.0018	*100*	0.0018	*100*	1.00	Harris et al., 2002
Diethylstilbestrol	0.0006	*300*	0.0011	*164*	0.54	
Genistein	0.2820	*0.64*	0.0058	*31.0*	48.62	
Daidzein	5.1790	*0.03*	0.3450	*0.52*	15.01	
17β-oestradiol	0.0250	100	0.0320	100	0.78	Zhao et al., 2009
Genistein	4.7350	0.53	0.0790	41.1	60.00	
Daidzein	26.6500	0.09	1.7380	1.87	14.27	
Equol	5.8800	0.43	0.5820	5.57	10.09	
G + D	9.8960	0.26	0.1570	20.6	62.87	
17β-oestradiol	0.0009	100				Branham et al., 2002[a]
Genistein	0.2000	0.45				
Daidzein	4.0000	0.02				
Equol	0.6000	0.15				

IC_{50} – molar concentration of compound leading to a 50% inhibition of 17β-oestradiol binding to ER
EC_{50} – molar concentration of compound producing response equal to 50% of that observed with 17β-oestradiol
RBA – the Relative Binding Affinity of the compound as a percentage of the binding affinity of 17β-oestradiol (100%) calculated as (IC_{50}/EC_{50} of 17β-oestradiol)/(IC_{50}/EC_{50} of test compound) x 100.
Italicised values have been calculated for this statement based on IC_{50}/EC_{50} values reported by authors.
[a] ER type not stated, presumably ERα

Human studies

49. Epidemiological and clinical studies investigating the impacts of phytoestrogens on human health have produced conflicting results (Bernbaum *et al*., 2008; Zung *et al*., 2008; Gilchrist *et al.*, 2010; Adgent *et al*., 2012). This inconsistency may be due in part to differences in the estimation of intakes and in the analytical methods used to calculate levels of isoflavones in foods (Thompson *et al.*, 2006). Furthermore, in studies on soya formula, the reasons for introducing soya into the diet (intolerance of other types of infant formula, its perceived health benefits, or preference for a vegetarian/vegan diet) might independently influence observed health outcomes, but generally have not been reported. Also feeding regimes have been ascertained only through the report of parents or carers, and the exact amounts of soya consumed have not been determined.

Fertility and development

50. The 2003 COT report observed that studies on the effects of phytoestrogens on human development and fertility were limited in number and scope, and that there were no published human studies examining potential effects of exposure to phytoestrogens *in utero,* mainly because of practical and ethical concerns. The human health implications of results obtained in animals are unclear as there are large species differences in sexual development between rodents, non-human primates and humans. Only one human study published before 2003 examined the effects of soya-based formula feeding on development and fertility (Strom *et al*., 2001). No adverse clinical effects were reported with the exception of 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 (COT, 2003).

51. Strom *et al.* (2001) examined the association between consumption of soya formula during infancy and measures of health and development in adulthood. A slightly longer duration of menstrual bleeding (0.37 days) and greater menstrual discomfort was reported by women who had been fed soya formula as infants (n=128) compared to those who had been fed cows' milk formula (n=268). After adjustment for multiple comparisons, these findings were not statistically significant. No differences were observed in either men or women with regard to height, weight, body mass index (BMI), self-reported age at menarche and breast development, reproductive disorders or birth defects in offspring.

52. Appendix 3 summarises studies published since 2003 that have investigated possible oestrogenic effects on infants' development from exclusive consumption of soya formula as compared with breast milk or cows' milk formula. The outcomes investigated include vaginal cell maturation (Bernbaum *et al*., 2008), prevalence of infantile breast tissue in the second year of life (Zung *et al.*, 2008), reproductive health (Gilchrist *et al*., 2010), sexual dimorphism in gender-role play (Adgent *et al*., 2011), early menarche (Adgent *et al*., 2012), and also behavioural development and bone mineral content (Andres *et al*., 2012 and 2013).

53. The balance of evidence from the small number of relevant epidemiological studies is not suggestive of important impacts of soy infant formula on reproductive health and development. Four studies have raised the possibility of subtle minor effects of uncertain clinical significance (early life breast development and minor differences in menarche/menstruation and gender-related play behaviour), but the findings were not conclusive and may have occurred by chance, or because of unrecognised biases in study design or execution.

Growth, allergy and immune responses

54. The Food Standards Agency (FSA) advises that: "*Soya allergy is a common childhood allergy. Most children grow out of it by the age of two, but occasionally adults are allergic to soya. The symptoms of soya allergy are similar to milk allergy and they include rashes, diarrhoea, vomiting, stomach cramps and breathing difficulties. Some people with soya allergy might also react to milk. Very rarely soya can cause anaphylaxis. Infants with other allergic conditions, such as milk allergy, dermatitis etc, are also at higher risk of developing allergy to soya*"[9]. The risks of soya allergy associated with infant-feeding will be addressed in a later COT statement on the infant diet and development of atopic and autoimmune disease. Although there have been reports of allergy to soya, these are likely to relate to the proteins that it contains rather than its oestrogenic activity.

55. The 2003 COT report noted that although one study had indicated that exposure to soya-based infant formula could lead to lower antibody responses, no differences had been observed in the immune response of infants fed soya formula in two other more recent studies (COT, 2003).

56. Klemola *et al*. carried out a study in which infants diagnosed with cows' milk allergy were randomly assigned to hydrolysed formula or soya-based formula. Follow-up was at ages two and four years. The authors reported good tolerance of soya formula by more than 70% of children. The parents of the other 30% suspected adverse reactions. However, the suspicion was sometimes doubtful, and in many cases was not supported by measurements of IgE and skin tests (Klemola *et al*., 2002). A slightly increased risk of sensitisation to soya formula was reported at age four years (Klemola *et al*., 2005).

[9] http://webarchive.nationalarchives.gov.uk/20080910110835; http://eatwell.gov.uk/healthissues/foodintolerance/foodintolerancetypes/soyaallergy

57. No adverse effects on growth and no differences in height or weight were observed in a follow-up study of adults who had consumed soya formula as infants (Strom *et al*., 2001; COT, 2003; Merritt and Jenks, 2004). In a more recent study, infants fed soya formula appeared to have a lower weight gain than others fed casein hydrolysate or rice hydrolysate (Agostoni *et al*., 2007), Similarly, a smaller increase in weight was observed in infants with cows' milk allergy who were fed soy formula as compared with others fed extensively hydrolysed whey formula (Seppo *et al.,* 2005). In contrast, another study suggested that consumption of soya-based formula was associated with a greater risk of becoming overweight (Stettler *et al*., 2005).

Effects on the thyroid gland and thyroid function

58. It has been hypothesised that phytoestrogens may be active in the thyroid due to the similarity of their chemical structure to that of the thyroid hormones, tri-iodothyronine (T_3) and thyroxine (T_4), and that they might act through the inhibition of thyroperoxidase (TPO) or interactions with thyroid binding globulin (TBG)(COT, 2003).

59. The 2003 COT report noted that animal studies showed that dietary soya and isoflavones could affect thyroid function and have a goitrogenic effect in rodents deficient in dietary iodine. Data from human studies suggested that isoflavones were unlikely to affect thyroid function in normal individuals with adequate iodine intake (COT, 2003).

60. A number of scientific publications evaluated by the Committee in 2003 noted the possibility that soya-based infant formula might affect thyroid function in infants. Cases had been reported in the 1950s and 1960s of goitre associated with consumption of soya formula, and of increased faecal loss of orally administered thyroxine in an athyreotic[10] hypothyroid patient when fed soya formula as compared with cows' milk formula. As a consequence, changes were made in the processing and formulation of infant formula (supplementation with iodine, replacement of soya flour with soya protein isolate), and no further reports of goitre were published. No data were found indicating that maternal ingestion of phytoestrogens during pregnancy could influence the development of the thyroid gland. However, the COT considered it possible that together with low iodine intake, increased metabolic demands during pregnancy and increased need for thyroxine, maternal consumption of soya products could adversely influence the neurological development of the fetus (COT, 2003).

61. Conrad *et al*. retrospectively analysed the medical records of infants diagnosed with congenital hypothyroidism and seen at a hospital during their first year of life. Two groups of patients were considered: a soya diet group consuming exclusively soya infant formula, who started on levothyroxine treatment at a median age of 15 days (range: 11 – 22 days) (n=8), and a non-soya diet group, who started treatment at 17 days (range: 12 – 23 days) (n=70). There was no significant difference in serum levels of thyroid stimulating hormone (TSH) and thyroxine (T_4)

[10] Athyreosis is the absence or functional deficiency of the thyroid gland

levels between the groups before the start of treatment with levothyroxine. Levels of $T_{4,}$ measured while on treatment, were similar in the two groups: median 153 nmol/L (soya group) and 188 nmol/L (non-soya group). However, following the start of treatment, normalisation of TSH levels took longer in the soya diet group (Conrad *et al.*, 2004). The authors suggested that soya-based formula impaired absorption of levothyroxine, especially in infants with gastrointestinal problems.

Cancer

62. One study has considered the relationship between consumption of soya-based infant formula and risk of breast cancer later in life. History of feeding was obtained from the mothers of women diagnosed with breast cancer (n=372) and of controls without breast cancer (n=356). The adjusted odds ratio (OR) for exclusive feeding with soya formula during the first 4 months of life was 0.42 (95% CI, 0.13-1.40), based on five exposed cases and 10 exposed controls. For exclusive feeding with soya formula at age 5-12 months, the OR was 0.59 (95% CI, 0.18-1.90), based on seven exposed cases and eight exposed controls (Boucher *et al*., 2008).

Guidance values

63. Soya isoflavones have not been classified as essential nutrients as their absence from the diet does not induce any deficiency syndrome and their presence is not essential for any biological processes. No tolerable daily intake has been established for soya isoflavones.

64. Animal studies indicate that exposure to soya isoflavones in early life could result in oestrogenic effects (increased uterine weights, advanced vaginal opening, changes in uterine and vaginal epithelium, altered development of reproductive organs, decreased fertility, earlier onset of puberty, decreased levels of testosterone, decreased anogenital distance), and effects on body weight, immune function, behaviour, and risk of mammary and pituitary gland tumours. A few human studies provide limited evidence of effects on sexual and reproductive development and gender play behaviour, but the findings are not conclusive. Both, animal and human studies indicate that soya isoflavones can affect thyroid function and have a goitrogenic effect in iodine-deficient but not in healthy individuals.

65. NTP-CERHR (2010) concluded that there was “minimal concern” about adverse developmental effects in infants fed soya infant formula. The term “minimal” was used rather than “negligible” because a number of studies in experimental animals and one study in humans had reported effects on the reproductive system.

66. The COT concludes that it is still not possible to propose health-based guidance values for infants. Reasons for this include the difficulty in extrapolation from animals to humans because of differences in toxicokinetics, uncertainty about differences between adults and infants (particularly those arising from development of the gut microflora), and the lack of dose-response data and possibility of bias and chance effects in the available human studies. Although the pig provides the best animal model with respect to toxicokinetics, few toxicity data are available for pigs.

Occurrence

Levels of isoflavones in human milk

67. The 2003 COT report noted that isoflavones are excreted in human milk at low concentrations reflecting maternal diet, with the highest concentrations in the breast milk of mothers following vegetarian or vegan diets. In one study, means (and ranges in brackets) of total isoflavone concentrations in breast milk samples (sum of genistein and daidzein expressed as mg aglycone/kg) were as follows: mothers consuming omnivorous diet (n=14): 0.001 (0 – 0.002); mothers with vegetarian diet (n=14): 0.004 (0.001 – 0.010) and mothers with vegan diet (n=11): 0.011 (0.002 – 0.032) (MAFF, 1998a).

68. Other research had found total isoflavones in breast milk at concentrations of 0.0016-0.0136 mg aglycone/L in women consuming an omnivorous diet (Setchell *et al*., 1997; Setchell *et al*., 1998). Consumption of foods such as roasted soya beans has been shown to give levels of isoflavones in the breast milk of vegans of up to 0.032 mg/L (Franke and Custer, 1996; MAFF, 1998b).

69. In a study conducted in the US, milk samples were collected from breastfeeding mothers before and after consumption of a soya protein beverage (25 g soya protein/36.5 g of beverage, containing 55 mg isoflavones; daidzein:genistein:glycitein = 1:1:0.1). The mean (SEM) levels of isoflavones in breast milk increased from 5.1 (2.2) nmol/L to 70.7 (19.2) nmol/L after 2-4 days of daily consumption. The daidzein to genistein ratio in breast milk was on average 0.6. Therefore it can be estimated that when expressed as µg/L, levels of genistein increased from 0.00055 to 0.00764, and of daidzein from 0.00078 to 0.01078 (Franke *et al.*, 2006). In Canada, samples of breast milk were collected after delivery from women aged at least 35 years (details of their diets were not reported). The mean concentrations of isoflavones in breast milk samples were 0.00087 µg/L (genistein) and 0.00036 µg/L (daidzein) for women with male infants and 0.00036 µg/L (genistein) and 0.00016 µg/L (daidzein) for women with female infants (Jarrell *et al*., 2012).

Cows' milk-based infant formula

70. As noted in the 2003 COT report, isoflavones were not detected in three different brands of cows' milk formula purchased in the UK (individual isoflavones were below the limit of detection (LOD) of 0.25-0.5 mg/L) (MAFF, 1998b). In another UK study, isoflavones were not detected (LOD = 0.5 mg/kg dry powder) in 6 out of 8 samples of cows' milk infant formula powders. In the other two samples, isoflavones were found at 1.2 mg/kg total isoflavones (0.7 mg genistein/kg and 0.5 mg glycitein/kg) and 2.1 mg genistein/kg, all expressed as aglycone equivalents (Hoey *et al*., 2004).

Soya-based infant formula

71. COT (2003) noted that reported levels of isoflavone in soya-based formulas ranged from 18 to 41 mg aglycone equivalents/L (of made-up formula ready for consumption) (MAFF, 1998a). Levels of isoflavones have also been measured in other soya infant formulas obtained in the UK. All soya based infant formulas analysed by Hoey *et al*. (2004) contained between 34.0 and 46.7 mg aglycone equivalents/L as fed, with genistein comprising 63% (SD 5%), daidzein 27% (SD 1%) and glycitein 10% (SD 5%) of the total. Kuhnle *et al*. (2008) reported the total isoflavone content of a soya infant formula as 1000 times higher than that of a non-soya formula with 25.90 mg aglycone equivalents/L as fed (Kuhnle *et al*., 2008). Concentrations of isoflavones were higher in powdered soya formula (46 – 47 mg/L) than in liquid formula (32 – 45 mg/L) (Setchell *et al*., 1998). Conjugates of genistein accounted for >65% of the total isoflavones, whereas genistein and daidzein made up only 3.2% to 5.8% (Setchell *et al*., 1998).

72. In New Zealand, total isoflavone concentrations (expressed as aglycone equivalents per kg) in a sample of soya-based infant formulas ranged from 81 to 92 mg/kg for genistein and 44 to 55 mg/kg for daidzein (Irvine *et al.,* 1998), and in another study in America the range was 89.5-155.7 mg/kg for genistein, 52.7-101.6 mg/kg for daidzein and 12.8-24 mg/kg for glycitein (Franke *et al*., 1998).

Complementary feeding products[11] containing soya

73. Levels of isoflavones in samples of ready-to-eat and instant foods for infants were previously reported by the COT (Table 2). Table 2 also includes data on isoflavones in other foods, such as vegetables, fruit, cheese and meat products, that are typical components of Western diets, and might be consumed by infants (Thompson *et al*., 2006; Kuhnle *et al*., 2008). However, since the data on these sources came from a very limited number of poorly described samples, and given the uncertainty about the extent to which infants consume such foods, the exposure assessment in this statement is based only on the data presented in the COT report (2003).

Table 2. Isoflavone levels in foods included in infant diet

Food type	**Total isoflavone level, expressed as mg/kg of foods as consumed**	**Source**
Ready-to-eat and instant weaning foods	Range: 18 – 78	References cited in COT, 2003
Firm tofu	275*	References cited in COT, 2003
Other foods with minor levels of isoflavones**	Range: 0.001 – 0.39	Irvine *et al*., 1998 Thompson *et al*., 2006 Kuhnle *et al*., 2008

[11] Solid foods introduced into the diet of infants to complement the milk feed, which remains the predominant part of the diet for most of the first year of life.

*No information was reported on whether the value quoted was a mean or median.
**Vegetables, fruits, bread, pasta and rice, cheese, meat products, fish, biscuits and cakes. The highest value of 0.39 mg total isoflavones/kg was found in green and white beans (Thompson *et al*., 2006).

Exposure

74. In the calculation of infants' potential exposures to phytoestrogens, values of 800 mL and 1200 mL were taken as reasonable estimates of average and high-level daily consumption of breast milk or infant formula before introduction of complementary foods (EFSA, 2012). Average bodyweights of 7.8, 8.7 and 9.6 kg were assumed for infants aged >4.0-6.0, >6.0-9.0 and >9.0-12.0 months respectively. These values were derived from the Diet and Nutrition Survey of Infants and Young Children (DNSIYC), which provided detailed information on the food consumption, nutrient intakes and nutritional status of infants and young children aged 4 to 18 months in the general UK population (DH, 2013). Since the DNSIYC did not include infants younger than 4 months, a mean bodyweight of 5.9 kg was assumed for infants aged 0-4 months, based on findings for infants aged 0-3 months in an earlier survey (DH, 1994).

Breast milk

75. The data for breast milk samples collected in the UK (see paragraph 67) were used to estimate exposures of exclusively breast-fed infants to isoflavones (expressed as a sum of genistein and daidzein) according to maternal diet (Table 3). The estimated exposures ranged from 0.0001-0.0002 mg/kg bw/day for infants whose mothers consume an omnivorous diet up to 0.0065 mg/kg bw/day for infants whose mothers consume a vegan diet.

Table 3. Estimated total isoflavone exposure (mg/kg bw/day) of exclusively breastfed infants

Isoflavones concentration in breast milk	**Age in months (consumption)**			
	0 – 4 (800 mL)	**0 – 4 (1200 mL)**	**>4 – 6 (800 mL)**	**>4 – 6 (1200 mL)**
Omnivorous diet Mean = 0.001mg/L	0.0001	0.0002	0.0001	0.0002
Vegetarian diet Mean = 0.004mg/L	0.0005	0.0008	0.0004	0.0006
Vegan diet Mean = 0.011mg/L	0.0015	0.0022	0.0011	0.0017
Vegan diet Max = 0.032mg/L	0.0043	0.0065	0.0033	0.0049

Infant formula

76. Potential exposures from cows' milk formula were estimated using the maximum level of isoflavone reported in products purchased in the UK (see paragraph 70). This level of 2.1 mg genistein/kg in powdered formula was translated to a value of 0.28 mg/L in reconstituted milk, with the assumption that 0.135 kg of powder was used to prepare 1 L of liquid formula (NTP, 2010). The estimated exposures of average and high level consumers of cows' milk-based infant formula were up to 0.038 and 0.057 mg/kg bw/day, respectively (Table 4). However, since isoflavones were not detected in most samples of cows' milk-based formula, the exposures of most infants fed exclusively with this type of formula, are likely to be substantially lower than these upper values.

77. Based on reported isoflavone levels in reconstituted soya-based infant formulas from the UK (range: 18 – 46.7 mg aglycone equivalents/L) (see paragraph 71), the isoflavone exposure of infants exclusively fed on soya-based infant formula would be up to 9.5 mg/kg bw/day (Table 4).

Table 4. Estimated total isoflavone exposure (mg/kg bw/day) of infants fed exclusively cows' milk formula or soya-based infant formula

Formula (isoflavone level in mg aglycone equivalents/L)	**Age in months (consumption)**			
	0 – 4 (800 mL)	**0 – 4 (1200 mL)**	**>4 – 6 (800 mL)**	**>4 – 6 (1200 mL)**
Cows' milk-based (0.28)	0.038	0.057	0.029	0.043
Soya-based (18 – 46.7)	2.4 – 6.3	3.7 – 9.5	1.8 – 4.8	2.8 – 7.2

Complementary feeding products containing soya

78. Exposures to total isoflavones from infant foods (Table 5) were estimated using the levels reported in such foods (Table 2) and consumption data from the 1986 survey of British Infants (Mills and Tyler, 1992).The "ready-to-eat and instant weaning food" group in Table 2 comprised commercial baby foods (including cereal-based foods and desserts).

79. An exposure estimate for tofu, based on a portion size approach (because actual consumption data were not available) was also calculated, to allow comparison of exposures from a potentially rich source of isoflavones such as tofu with those estimated for ready to eat and instant complementary foods. A baby food recipe website[12] indicated that 50 g of tofu per person per day would be a reasonable portion size. However, it should be noted that calculations based on this

[12] http://www.annabelkarmel.com/recipes/babies-6-9-months/banana-tofu-puree

value would be expected to overestimate exposures since it is unlikely that an infant will consume tofu products (such as banana and tofu puree) daily over prolonged periods.

Table 5. Estimated exposures of UK infants to isoflavones from complementary foods (in mg/kg bw/day)

Product category	Isoflavone level (µg/g food)	Consumption rate (g/kg bw/day)		Exposure (mg/kg bw/day)	
		Mean	97.5th percentile	Mean	97.5th percentile
Complementary foods*	18-78	6	22	0.108-0.468	0.396-1.716
Tofu**	275	5	n/a	1.375	n/a

* Foods tested in the 1986 British Infants Survey (Mills and Tyler, 1992) such as: instant weaning foods and ready-to-eat foods. Most were commercial/retail baby foods (including cereal-based products). Examples included egg/cheese-based dried meals; rice/semolina/chocolate instant puddings; yoghurt-based dried meals.
**In the absence of consumption data on soya-based food products such as tofu, a portion size of 50 g per infant per day was used to estimate exposure levels, together with an average bodyweight of 9.35 kg for infants aged 6-12 months old

Risk characterisation

80. Apart from allergy, the main toxicological concern in infants consuming soya-based products is the oestrogenicity of isoflavones, and their possible effects on reproductive development and function. However, because of limitations in the available data, and particularly uncertainties in extrapolations from animals because of differences in toxicokinetics, it is not possible to set health-based guidance values for soya isoflavones in infants.

81. Based on the maximum reported levels of soya isoflavones in breast milk from mothers consuming different types of diet, exposures of exclusively breastfed infants are likely to be between 0.0001 and 0.0065 mg/kg bw/day; infants consuming only cows' milk formula could have exposures up to 0.057 mg/kg bw/day; and in infants fed exclusively with ready-to-consume soya-based formula, exposures could be up to 9.5 mg/kg bw/day. For each type of milk, the highest potential exposures would be in high-level consumers of milk aged 0-3 months. Limited information is available regarding infants' exposure to isoflavones from complementary foods, but data from one survey suggest intakes of 0.1-1.7 mg/kg bw/day, most being <0.5 mg/kg bw/day.

82. Evidence from the few relevant epidemiological studies does not suggest important impacts of soya-based formula on later reproductive health in humans, although some studies have raised the possibility of subtle effects of uncertain clinical significance. However, animal studies where exposure to isoflavones was at levels similar to those reported in infants exclusively fed soya-based infant formula indicated some developmental and reproductive changes. There is thus some uncertainty about the safety of soya-based formula.

83. Exposures to isoflavones from complementary foods manufactured for infants are lower than those from soya-based formula, and are unlikely to be harmful.

84. Exposures to isoflavones from breast milk (even where mothers consume vegetarian or vegan diets) and cows' milk formula are much lower than those from soya-based formula, and are highly unlikely to cause adverse effects.

Conclusions

85. Soya isoflavones are not essential nutrients.

86. The main toxicological concern regarding consumption of soya isoflavones by infants arises from oestrogenicity and potential to disrupt the development and function of the reproductive system. Other possible adverse effects relate to immune and thyroid function. However, because of limitations in the available data, and particularly uncertainties in extrapolations from animals due to differences in toxicokinetics, it is not possible to set health-based guidance values for soya isoflavones in infants.

87. The highest potential exposures of infants to soya isoflavones are from consumption of soya-based infant formula, and could be as high as 9.5 mg/kg bw/day (expressed as aglycone equivalents).

88. Exposures to isoflavones from complementary foods manufactured for infants are lower than those from soya-based formula, and are unlikely to be harmful.

89. Exposures to isoflavones from breast milk (even where mothers consume vegetarian or vegan diets) and cows' milk formula are much lower than those from soya-based formula, and are highly unlikely to cause adverse effects.

90. Evidence from the few relevant epidemiological studies does not suggest important impacts of soya-based formula on later reproductive health in humans, although some studies have raised the possibility of subtle effects of uncertain clinical significance. However, animal studies where exposure to isoflavones was at levels similar to those reported in infants exclusively fed soya-based infant formula indicate some developmental and reproductive changes. There is thus some uncertainty about the safety of soya-based formula.

91. There is no scientific basis for a change in the current government advice that there is no substantive medical need for, nor health benefit arising from the use of soya-based infant formula and it should only be used in exceptional circumstances to ensure adequate nutrition.

December 2013

References

Adgent MA, Daniels JL, Edwards LJ, Siega-Riz AM and Rogan WJ (2011) Early-life soy exposure and gender-role play behavior in children. *Environ Health Perspect* **119**:1811-1816.

Adgent MA, Daniels JL, Rogan WJ, Adair L, Edwards LJ, Westreich D, Maisonet M and Marcus M (2012) Early-life soy exposure and age at menarche. *Paediatr Perinat Epidemiol* **26**:163-175.

Agence Française de Sécurité Sanitaire des Aliments (French Food Safety Agency). Report of the working group on phytoestrogens. 2005.

Agostoni C, Fiocchi A, Riva E, Terracciano L, Sarratud T, Martelli A, Lodi F, D'Auria E, Zuccotti G, Giovannini M (2007) Growth of infants with IgE-mediated cow's milk allergy fed different formulas in the complementary feeding period. *Pediatric allergy and immunology* **18**(7):599-606.

Alston-Mills B, Lepri JJ and Martin CA (2011) Modulation of mammary gland development in pre-pubertal mice as affected by soya and milk protein supplements. *Br J Nutr* **106**(4):502-9.

Andlauer W, Kolb J, Furst P (2000) Absorption and metabolism of genistin in the isolated rat small intestine *FEBS Lett* **475**: 127-130

Andres A, Cleves MA, Bellando JB, Pivik RT, Casey PH and Badger TM (2012) Developmental status of 1-year-old infants fed breast milk, cow's milk formula, or soy formula. *Pediatrics* **129**(6):1134-40

Andres A, Casey PH, Cleves MA and Badger TM (2013) Body fat and bone mineral content of infants fed breast milk, cow's milk formula, or soy formula during the first year of life. *The Journal of Pediatrics* **163**(1):49-54

Atkinson C, Newton KM, Aiello Bowles EJ, Lehman CD, Stanczyk FZ, Westerlind KC, Li L, Lampe JW (2009) Daidzein-metabolizing phenotypes in relation to mammographic breast density among premenopausal women in the United States. *Breast Cancer Res Treat* **116**:587-594

Badger TM, Gilchrist JM, Pivik RT, Andres A, Shankar K, Chen JR, Ronis MJ (2009) The health implications of soy infant formula. *The American Journal of Clinical Nutrition* **89**(suppl):1668S-72S.

Basaria S, Wisniewski A, Dupree K, Bruno T, Song MY, Yao F, Ojumu A, John M and Dobs AS (2009) Effect of high-dose isoflavones on cognition, quality of life, androgens, and lipoprotein in post-menopausal women. *J Endocrinol Invest* **32**:150-155.

Ball ER, Caniglia MK, Wilcox JL, Overton KA, Burr MJ, Wolfe BD, Sanders BJ, Wisniewski AB and Wrenn CC (2010) Effects of genistein in the maternal diet on reproductive development and spatial learning in male rats. *Hormones and behaviour* **57**(3): 313-22

Bateman HL, Patisaul HB (2008) Disrupted female reproductive physiology following neonatal exposure to phytoestrogens or estrogen specific ligands is associated with

decreased GnRH activation and kisspeptin fiber density in the hypothalamus. *NeuroToxicology* **29**:988-997

Berger-Achituv S, Shohat T, Romano-Zelekha O, Ophir E, Rachmani S, Malovizky D and Garty BZ (2005) Widespread use of soy-based formula without clinical indications. *J Pediatr Gastroenterol Nutr* **41**:660-666.

Bernbaum JC, Umbach DM, Ragan NB, Ballard JL, Archer JI, Schmidt-Davis H and Rogan WJ (2008) Pilot studies of estrogen-related physical findings in infants. *Environ Health Perspect* **116**:416-420.

Bhatia J and Greer F (2008) Use of soy protein-based formulas in infant feeding. *Pediatrics* **121**:1062-1068.

Blake C, Fabick KM, Setchell KDR, Lund TD and Lephart ED (2011) Neuromodulation by soy diets or equol: anti-depressive & anti-obesity-like influences, age & hormone-dependent effects*. BMC Neuroscience* **12**:28

Boucher BA, Cotterchio M, Kreiger N, Thompson LU (2008) Soy formula and breast cancer risk. *Epidemiology* **19**(1):165-166

Bowey E, Adlercreutz H, Rowland I (2003) Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. *Food and Chemical Toxicology* **41**:631-636.

Branham WS, Dial SL, Moland CL, Hass BS, Blair RM, Fang H, Shi L, Tong W, Perkins RG, Sheehan DM (2002) Phytoestrogens and mycoestrogens bind to the rat uterine estrogen receptor. *Biochemical and Molecular Action of Nutrients* **132**:658-664

Bundesinstitut für Risikobewertung (The German Federal Institute for Risk Assessment). Isolated isoflavones are not without risk. 2007.

Cao Y, Calafat AM, Doerge DR, Umbach DM, Bernbaum JC, Twaddle NC, Ye X and Rogan WJ (2009) Isoflavones in urine, saliva, and blood of infants: data from a pilot study on the estrogenic activity of soy formula. *J Expo Sci Environ Epidemiol* **19**:223-234.

Cassidy A, Bingham S and Setchell KD (1994) Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr* **60**:333-340.

Cassidy A, Brown JE, Hawdon A, Faughnan MS, King LJ, Millward J, Zimmer-Nechemias L, Wolfe B and Setchell KD (2006) Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. *J Nutr* **136**:45-51.

Catassu C (1995) Intestinal permeability changes during the first month: effects of natural versus artificial feeding. *J Pediatr Gastroenerol Nutr* **21**: 383-386

Cederroth CR, Zimmermann C, Beny JL, Schaad O, Combepine C, Descombes P, Doerge DR, Pralong FP, Vassalli JD and Nef S (2010) Potential detrimental effects

of a phytoestrogen-rich diet on male fertility in mice. *Molecular and Cellular Endocrinology* **321**(2):152-160

Chen A, Berhow MA, Tappenden KA, Donovan SM (2005) Genistein inhibits intestinal cell proliferation in piglets. *Pediatric Research* **57**(2):192-200.

Chen A and Rogan WJ (2004) Isoflavones in soy infant formula: a review of evidence for endocrine and other activity in infants. *Annu Rev Nutr* **24**:33-54.

Cicero AF, Derosa G and Arletti R (2004) Effect of oral chronic isoflavones supplementation on male rat sexual performances and sexual hormone plasma levels. *Phytotherapy research* **18**:849-852

Cimafranca MA, Davila J, Ekman GC, Andrews RN, Neese SL, Peretz J, Woodling KA, Helferich WG, Sarkar J, Flaws JA, Schantz SL, Doerge DR and Cooke PS (2010) Acute and chronic effects of oral genistein administration in neonatal mice. *Biol Reprod* **83**:114-121.

Cohen L, Crespin JS, Wolper C, Zang EA, Pittman B, Zhao Z, Holt PR (2007) Soy isoflavone intake and estrogen excretion patterns in young women: effect of probiotic administration. *in vivo* **21**: 507-512

Conrad SC, Chiu H and Silverman BL (2004) Soy formula complicates management of congenital hypothyroidism. *Arch Dis Child* **89**:37-40.

Cooke PS, Selvaraj V and Yellayi S (2006) Genistein, estrogen receptors, and the acquired immune response. *J Nutr* **136**:704-708.

Day AJ, Gee JM, DuPont MS, Johnson IT, Williamson G (2003) Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochemical Pharmacology* **65**:1199-1206.

De Lima Toccafondo Vieira M, Ferreira Duarte R, Moreira Campos LM, de Aguiar Nunan E (2008) Comparison of the estrogenic potencies of standardized soy extracts by immature rat uterotrophic bioassay. *Phytomedicine* **15**:31-37

Department of Health's Chief Medical Officer. Advice issued on soya-based infant formulas. CMO Update 37. 2004.

Dewi FN, Wood CE, Lees CJ, Wilson CJ, Register TC, Tooze JA, Franke AA, Cline JM (2013) Dietary soy effects on mammary gland development during the pubertal transition in nonhuman primates. *Cancer Prev Res (Phila)* **6**(8):832-42.

De Wilde A, Rassi CM, Cournot G, Colin C, Lacroix HC, Chaumaz G, Coxam V, Bennetau-Pelissero C, Pointillart A, Lieberherr M (2007) Dietary isoflavones act on bone marrow osteoprogenitor cells and stimulate ovary development before influencing bone mass in pre-pubertal piglets. *Journal of Cellular Physiology* **212**:51-59

DH (Department of Health) (1994). The COMA report on weaning and the weaning diet. Report on Health and Social Subjects 45. The Stationary Office London.

DH (Department of Health) (2013). Diet and nutrition survey of infants and young children, 2011. Available at: http://transparency.dh.gov.uk/2013/03/13/dnsiyc-2011/

Dinsdale EC, Chen J and Ward WE (2011) Early life exposure to isoflavones adversely affects reproductive health in first but not second generation female CD-1 mice. *J Nutr* **141**:1996-2002.

Di Virgilio AL, Iwami K, Watjen W, Kahl R, Degen GH (2004) Genotoxicity of the isoflavones genistein, daidzein and equol in V79 cells. *Toxicology Letters* **151**: 151-162

Doerge DR, Twaddle NC, Banks EP, Jefferson WN, Newbold RR (2002) Pharmacokinetic analysis in serum of genistein administered subcutaneously to neonatal mice. *Cancer Letters* **184**(1):21-27

Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* **114**(4):567-572.

Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *PNAS* **104**(32):13056-13061.

EFSA (2012) Scientific opinion on brominated flame retardants (BFRs) in food: brominated phenols and their derivatives. *EFSA Journal* **10**(4): 2634

Fielden MR, Fong CJ, Haslam SZ and Zacharewski TR (2002) Normal mammary gland morphology in pubertal female mice following in utero and lactational exposure to genistein at levels comparable to human dietary exposure. *Toxicol Lett* **133**:181-191.

Fielden MR, Samy SM, Chou KC and Zacharewski TR (2003) Effect of human dietary exposure levels of genistein during gestation and lactation on long-term reproductive development and sperm quality in mice. *Food Chem Toxicol* **41**:447-454.

Fletcher RJ (2003) Food sources of phyto-oestrogens and their precursors in Europe. *Br J Nutr* **89 Suppl 1**:S39-S43.

Ford JA, Clark SG, Walters EM, Wheeler MB, Hurley WL (2006) Estrogenic effects of genistein on reproductive tissues of ovariectomised gilts. *Journal of Animal Science* **84**:834-842

Franke AA and Custer LJ (1996) Daidzein and genistein concentrations in human milk after soy consumption. *Clin Chem* **42**:955-964.

Franke AA, Custer LJ and Tanaka Y (1998) Isoflavones in human breast milk and other biological fluids. *Am J Clin Nutr* **68**:1466S-1473S.

Franke AA, Halm BM, Custer LJ, Tatsumura Y and Hebshi S (2006) Isoflavones in breastfed infants after mothers consume soy. *Am J Clin Nutr* **84**:406-413.

Frankenfeld CL, McTiernan A, Tworoger SS, Atkinson C, Thomas WK, Stanczyk FZ, Marcovina SM, Weigle DS, Weiss NS, Holt VL, Schwartz SM, Lampe JW (2004) Serum steroid hormones, sex hormone-binding globulin concentrations, and urinary hydroxylated estrogen metabolites in post-menopausal women in relation to daidzein-metabolizing phenotypes. *The Journal of Steroid Biochemistry and Molecular Biology* **88**:399-408

Fritz MA, Speroff L (2010) Clinical Gynecologic Endocrinology and infertility. Eight edition. Lippincott Williams & Wilkins, p.752

Gee JM, DuPont MS, Rhodes MJC, Johnson IT (1998) Quercetin glucosides interact with the intestinal glucose transport pathway. *Free Radic Biol Med* **25**:19 – 25

Gilani GS, Farmer C, Dyck M, Robertson P, Dahiya J, Sepehr E, Fan L, Nicolidakis H, Curran I (2011) Distribution of isoflavones in samples of serum, liver and mammary glands of rats or pigs fed dietary isoflavones. *Annals of Nutrition and Metabolism* **58**:171-180

Gilchrist JM, Moore MB, Andres A, Estroff JA and Badger TM (2010) Ultrasonographic patterns of reproductive organs in infants fed soy formula: comparisons to infants fed breast milk and milk formula. *J Pediatr* **156**:215-220.

Golub MS, Hogrefe CE, Germann SL, Tran TT, Beard JL, Crinella FM, Lonnerdal B (2005) Neurobehavioral evaluation of rhesus monkey infants fed cow's milk formula, soy formula, or soy formula with added manganese. *Neurotoxicology and Teratology* **27**:615-627

Gu L, House SE, Prior RL, Fang N, Ronis MJ, Clarkson TB, Wilson ME and Badger TM (2006) Metabolic phenotype of isoflavones differ among female rats, pigs, monkeys, and women. *J Nutr* **136**:1215-1221.

Halm BM, Ashburn LA and Franke AA (2007) Isoflavones from soya foods are more bioavailable in children than adults. *Br J Nutr* **98**:998-1005.

Harris HA, Bapat AR, Gonder DS, Frail DE (2002) The ligand binding profiles of estrogen receptors α and β are species dependent. *Steroids* **67**:379-384

Hartley DE, Edwards JE, Spiller CE, Alom N, Tucci S, Seth P, Forsling ML and File SE (2003) The soya isoflavone content of rat diet can increase anxiety and stress hormone release in the male rat. *Psychopharmacology* **167**(1):46-53.

Hertrampf T, Schleipen B, Offermanns C, Velders M, Laudenbach U, Diel P (2009) Comparison of the bone protective effects of an isoflavone-rich diet with dietary and subsutaneous administrtions of genistein in ovariectomized rats. *Toxicology Letters* **184**:198-203

Hoey L, Rowland IR, Lloyd AS, Clarke DB and Wiseman H (2004) Influence of soya-based infant formula consumption on isoflavone and gut microflora metabolite

concentrations in urine and on faecal microflora composition and metabolic activity in infants and children. *Br J Nutr* **91**:607-616.

Hughes CL, Liu G, Beall S, Foster WG and Davis V (2004) Effects of genistein or soy milk during late gestation and lactation on adult uterine organization in the rat. *Exp Biol Med (Maywood)* **229**:108-117.

Irvine CH, Shand N, Fitzpatrick MG and Alexander SL (1998) Daily intake and urinary excretion of genistein and daidzein by infants fed soy- or dairy-based infant formulas. *Am J Clin Nutr* **68**:1462S-1465S.

Izumi T, Piskula MK, Osawa S, Obata A (2000) Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J Nutr* **130**: 1695-9

Jarrell J, Foster WG and Kinniburgh DW (2012) Phytoestrogens in human pregnancy. *Obstet Gynecol Int* **2012**:850313.

Jefferson WN, Couse JF, Padilla-Banks E, Korach KS and Newbold RR (2002) Neonatal exposure to genistein induces estrogen receptor (ER)alpha expression and multioocyte follicles in the maturing mouse ovary: evidence for ERbeta-mediated and nonestrogenic actions. *Biol Reprod* **67**:1285-1296.

Jefferson WN, Doerge D, Padilla-Banks E, Woodling KA, Kissling GE and Newbold R (2009) Oral exposure to genistin, the glycosylated form of genistein, during neonatal life adversely affects the female reproductive system. *Environ Health Perspect* **117**:1883-1889.

Jefferson WN, Padilla-Banks E and Newbold RR (2006) Studies of the effects of neonatal exposure to genistein on the developing female reproductive system. *J AOAC Int* **89**:1189-1196.

Kaludjerovic J, Chen J and Ward WE (2012) Early life exposure to genistein and daidzein disrupts structural development of reproductive organs in female mice. *J Toxicol Environ Health A* **75**:649-660.

Kaludjerovic J and Ward WE (2009) Neonatal exposure to daidzein, genistein, or the combination modulates bone development in female CD-1 mice. *J Nutr* **139**:467-473.

Klein SL, Wisniewski AB, Marson AL, Glass GE and Gearhart JP (2002) Early exposure to genistein exerts long-lasting effects on the endocrine and immune systems in rats. *Mol Med* **8**:742-749.

Klemola T, Vanto T, Juntunen-Backman K, Kalimo K, Korpela R, Varjonen E (2002) Allergy to soy formula and to extensively hydrolysed whey formula in infants with cow's milk allergy: a prospective, randomized study with a follow-up to the age of 2 years. *The Journal of Pediatrics* **140**(2):219-24.

Klemola T, Kalimo K, Poussa T, Juntunen-Backman K, Korpela R, Valovirta E, Vanto T. (2005) Feeding a soy formula to children with cow's milk allergy: the development of immunoglobulin E-mediated allergy to soy and peanuts. *Pediatric Allergy and Immunology* **16**(8):641-6.

Kouki T, Kishitake M, Okamoto M, Oosuka I, Takebe M and Yamanouchi K (2003) Effects of neonatal treatment with phytoestrogens, genistein and daidzein, on sex difference in female rat brain function: estrous cycle and lordosis. *Horm Behav* **44**:140-145.

Kuhnle GG, Dell'Aquila C, Aspinall SM, Runswick SA, Mulligan AA and Bingham SA (2008) Phytoestrogen content of foods of animal origin: dairy products, eggs, meat, fish, and seafood. *J Agric Food Chem* **56**:10099-10104.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138**(3): 863-870.

Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **139**(10):4252-4263.

Kwok KC, Cheung NH (2010) Measuring binding kinetics of ligands with tethered receptors by fluorescence polarization and total internal reflection fluorescence. *Analitical Chemistry* **82**:3819-3825.

Kwon SH, Kang MJ, Huh JS, Ha KW, Lee JR, Lee SK, Lee BS, Han IH, Lee MS, Lee MW, Lee J, Choi YW (2007) Comparison of oral availability of genistein and genistin in rats. *International Journal of Pharmaceutics* **337**: 148-154

Liang S, Byers DM, Irwin LN (2008) Sex and diet affect the behavioural response of rats to chronic mild stressors. *Physiology and behaviour* **93**:27-36

Maskariniec G, Watts K, Kagihara J, Hebshi S, Franke AA (2008) Urinary isoflavonoid excretion is similar after consuming soy milk and miso soup in Japanese-American women. *Br J Nutr* **100**: 424-429

Matsumura A, Ghosh A, Pope GS, Darbre PD (2005) Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **94**:431-443.

McCarver G, Bhatia J, Chambers C, Clarke R, Etzel R, Foster W, Hoyer P, Leeder JS, Peters JM, Rissman E, Rybak M, Sherman C, Toppari J and Turner K (2011) NTP-CERHR expert panel report on the developmental toxicity of soy infant formula. *Birth Defects Res B Dev Reprod Toxicol* **92**:421-468.

McClain RM, Wolz E, Davidovich A, Bausch J (2006) Genetic toxicity studies with genistein. *Food and Chemical Toxicology* **44**: 42-55

Merritt RJ, Jenks BH (2004) Safety of soy-based infant formulas containing isoflavones: the clinical evidence. *The Journal of Nutrition* **134**:1220S-1224S.

Mills A and Tyler H. Food and Nutrient Intakes of British Infants Aged 6-12 months. HMSO. London. 1992.

Ministry of Agriculture Fisheries and Food Report. FS2829 - Levels of oestrogens in the diets of infants and toddlers. University of Reading. 1998a.

Ministry of Agriculture Fisheries and Food Report. Plant oestrogens in soya-based infant formula. Food Surveillance Paper No. 167. London. UK. HMSO. 1998b.

Mosquette R, de Jesus Simoes M, da Silva IDCG, Oshima CTF, Oliveira-Filho RM, Haidar MA, Simoes RS, Baracat EC, Junior JMS (2007) The effects of soy extract on the uterus of castrated adult rats. *Maturitas* **56**:173-183.

Mueller SO, Simon S, Chae K, Metzler M, Korach KS (2004) Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor α (ERα) and ERβ in human cells. *Toxicological Sciences* **80**:14-25.

Muthyala RS, Ju YH, Sheng S, Williams LD, Doerge DR, Katzenellenbogen BS, Helferich WG, Katzenellenbogen JA (2004) Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta. *Bioorganic and Medicinal Chemistry* **12**:1559-1567.

Naaz A, Yellayi S, Zakroczymski MA, Bunick D, Doerge DR, Lubahn DB, Helferich WG and Cooke PS (2003) The soy isoflavone genistein decreases adipose deposition in mice. *Endocrinology* **144**:3315-3320.

Nemeth K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, Williamson G, Swallow DM, Kroon PA (2003) Deglycosylation by small intestinal epithelial cell β-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *European Journal of Nutrition* **42**:29-42.

Nielsen TS, Purup S, Warri A, Godschalk RW and Hilakivi-Clarke L (2011) Effects of maternal exposure to cow's milk high or low in isoflavones on carcinogen-induced mammary tumorigenesis among rat offspring. *Cancer Prev Res (Phila)* **4**:694-701.

NTP (2008a) Multigenerational reproductive study of genistein (Cas No. 446-72-0) in Sprague-Dawley rats (feed study). *Natl Toxicol Program Tech Rep Ser*1-266.

NTP (2008b) Toxicology and carcinogenesis studies of genistein (Cas No. 446-72-0) in Sprague-Dawley rats (feed study). *Natl Toxicol Program Tech Rep Ser*1-240.

NTP. Final CERHR Expert Panel Report on Soy Infant Formula. 2010.

Patisaul HB, Luskin JR and Wilson ME (2004) A soy supplement and tamoxifen inhibit sexual behaviour in female rats. *Hormones and behaviour* **45**(4):270-277

Rozman KK, Bhatia J, Calafat AM, Chambers C, Culty M, Etzel RA, Flaws JA, Hansen DK, Hoyer PB, Jeffery EH, Kesner JS, Marty S, Thomas JA and Umbach D (2006a) NTP-CERHR expert panel report on the reproductive and developmental toxicity of genistein. *Birth Defects Res B Dev Reprod Toxicol* **77**:485-638.

Rozman KK, Bhatia J, Calafat AM, Chambers C, Culty M, Etzel RA, Flaws JA, Hansen DK, Hoyer PB, Jeffery EH, Kesner JS, Marty S, Thomas JA and Umbach D

(2006b) NTP-CERHR expert panel report on the reproductive and developmental toxicity of soy formula. *Birth Defects Res B Dev Reprod Toxicol* **77**:280-397.

Rufer CE, Bub A, Moseneder J, Winterhalter P, Sturtz M, Kulling SE (2008) Pharmacokinetics of the soybean isoflavone daidzein in its aglycone and glucoside form: a randomized, double-blind, crossover study. *J Clin Nutr* **87**: 1314-23

Sato T, Shinohara Y, Kaneko D, Nishimura I and Matsuyama A (2010) Fermented soymilk increases voluntary wheel running activity and sexual behaviour in male rats. *Appl. Physiol. Nutr. Metab*. **35**:749-754

Schmidt S, degen GH, Seibel J, Hertrampf T, Vollmer G, Diel P (2006) Hormonal activity of combinations of genistein, bisphenol A and 17β-estradiol in the female Wistar rat. *Arch Toxicol* **80**:839-845.

Seppo L, Korpela R, Lonnerdal B, Metsaniitty L, Juntunen-Backman K, Klemola T, Paganus A, Vanto T (2005) A follow-up study of nutrient intake, nutritional status, and growth in infants with cow milk allergy fed either a soy formula or an extensively hydrolysed whey formula. *The American Journal of Clinical Nutrition* **82**:140-5

Setchell KD, Brown NM, Zhao X, Lindley SL, Heubi JE, King EC and Messina MJ (2011) Soy isoflavone phase II metabolism differs between rodents and humans: implications for the effect on breast cancer risk. *Am J Clin Nutr* **94**:1284-1294.

Setchell KD, Clerici C, Lephart ED, Cole SJ, Heenan C, Castellani D, Wolfe BE, Nechemias-Zimmer L, Brown NM, Lund TD, Handa RJ and Heubi JE (2005) S-equol, a potent ligand for estrogen receptor beta, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am J Clin Nutr* **81**:1072-1079.

Setchell KD, Zimmer-Nechemias L, Cai J and Heubi JE (1997) Exposure of infants to phyto-oestrogens from soy-based infant formula. *Lancet* **350**:23-27.

Setchell KD, Zimmer-Nechemias L, Cai J and Heubi JE (1998) Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. *Am J Clin Nutr* **68**:1453S-1461S.

Setchell KDR, Brown NM, Desai PB, Zimmer-Nechimias L, Wolfe B, Jakate AS, Creutzinger V, Heubi JE (2003) Bioavailability, disposition, and dose response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. *J Nutr* **133**: 1027-35

Setchell KDR, Clerici C (2010) Equol: pharmacokinetics and biological actions. *J Nutr* **140**: 1363S-8S

Setchell KDR, Brown NM, Zimmer-Nechemias L, Brashear WT, Wolfe BE, Kirschner AS, Heubi JE (2002) Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am J Clin Nutr* **76**: 447-53

Sharpe RM, Martin B, Morris K, Greig I, McKinnell C, McNeilly AS and Walker M (2002) Infant feeding with soy formula milk: effects on the testis and on blood

testosterone levels in marmoset monkeys during the period of neonatal testicular activity. *Human Reproduction* **17**(7):1692-1703

Simon NG, Kaplan JR, Hu S, Register TC and Adams MR (2004) Increased aggressive behaviour and decreased affiliative behaviour in adult male monkeys after long-term consumption of diets rich in soy protein and isoflavones. *Hormones and Behavior* **45**(4):278-284

Song KB, Atkinson C, Frankenfeld CL, Jokela T, Wahala K, Thomas WK, Lampe JW (2006) Prevalence of daidzein-metabolizing phenotypes differs between Caucasian and Korean American women and girls. *The Journal of Nutrition* **136**:1347-1351.

Steensma A, Bienenmann-Ploum ME, Noteborn HPJM (2004) Intestinal uptake of genistein and its glycoside in the rat using various isolated perfused gut segments. Environmental Toxicology and Pharmacology **17**: 103-110

Stettler N, Stallings VA, Troxel AB, Zhao J, Schinnar R, Nelson SE, Ziegler EE, Strom BL. (2005) Weight gain in the first week of life and overweight in adulthood. *Epidemiology* **111**:1897-1903.

Strom BL, Schinnar R, Ziegler EE, Barnhart KT, Sammel MD, Macones GA, Stallings VA, Drulis JM, Nelson SE and Hanson SA (2001) Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood. *JAMA* **286**:807-814.

Tan KA, Walker M, Morris K, Greig I, Mason JI and Sharpe RM (2006) Infant feeding with soy formula milk: effects on puberty progression, reproductive function and testicular cell numbers in marmoset monkeys in adulthood. *Hum Reprod* **21**:896-904.

Thigpen JE, Haseman JK, Saunders HE, Setchell KDR, Grant MG, Forsythe DB (2003) Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. *Comparative Medicine* **53**(6):607-615.

Thigpen JE, Setchell KDR, Padilla-Banks E, Haseman JK, Saunders HE, Caviness GF, Kissling GE, Grant MG, Forsythe DB (2007) Variations in phytoestrogen content between different mill dates of the same diet produces significant differences in the time of vaginal opening in CD-1 mice and F344 rats but not in CD Sprague-Dawley rats. *Environmental Health Perspectives* **115**(12):1717-1726.

Thomsen AR, Mortensen A, Breinholt VM, Lindecrona RH, Penalvo JL, Sorensen IK (2005) Influence of Prevastein, an isoflavone-rich soy product, on mammary gland development and tumorigenesis in Tg.NK (MMTV/c-neu) mice. *Nutrition and Cancer* **52**(2):176-88.

Thompson LU, Boucher BA, Liu Z, Cotterchio M and Kreiger N (2006) Phytoestrogen content of foods consumed in Canada, including isoflavones, lignans, and coumestan. *Nutr Cancer* **54**:184-201.

Ullah MF, Shamim U, Hanif S (2009) Cellular DNA breakage by soy isoflavone genistein and its methylated structural analogue biochanin A. *Mol Nutr Food Res* **53**: 1676-85

Vergne S, Sauvant P, Lamothe V, Chantre P, Asselineau J, Perez P, Dyrand M, Moore N, Bennetau-Pelissero C (2009) Influence of ethnic origin (Asian v. Caucasian) and background diet on the bioavailability of dietary isoflavones. *British Journal of Nutrition* **102**:1642-1653.

Wada K, Nakamura K, Masue T, Sahashi Y, Ando K and Nagata C (2011) Soy intake and urinary sex hormone levels in preschool Japanese children. *Am J Epidemiol* **173**:998-1003.

Walsh KR, Haak SJ, Bohn T, Tian Q, Schwartz SJ, Failla ML (2007) Isoflavonoid glucosides are deconjugated and absorbed in the small intestine of human subjects with ileostomies. *Am J Clin Nutr* **85**:1050-6.

Yee S, Burdock GA, Kurata Y, Enomoto Y, Narumi K, Hamada S, Itoh T, Shimomura Y, Ueno T (2008) Acute and subchronic toxicity and genotoxicity of SE5-OH, an equol-rich product produced by Lactococcus garvieae. *Food Chem Toxicol* **46**: 2713-20

Yeo SK and Liong MT (2010) Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics. *Int J Food Sci Nutr* **61**:161-181.

Zhao L, Mao Z, Diaz Brinton R (2009) A select combination of clinically relevant phytoestrogens enhances estrogen receptor β-binding selectivity and nauroprotective activities in vitro and in vivo. *Endocrinology* **150**:770-783.

Zubik L, Meydani M (2003) Bioavailability of soybean isoflavones from aglycone and glucoside forms in American Women. *Am J Clin Nutr* **77**: 1459-65

Zung A, Glaser T, Kerem Z and Zadik Z (2008) Breast development in the first 2 years of life: an association with soy-based infant formulas. *J Pediatr Gastroenterol Nutr* **46**:191-195.

Abbreviations

AAP	American Academy of Pediatrics
ADME	absorption, distribution, metabolism and excretion
AFSSA	L'Agence française de sécurite sanitarie des aliments (French Food Safety Agency)
AGD	anogenital distance
AGDI	anogenital distance index
ALSPAC	Avon Longitudinal Study of Parents and Children
AUC	area under the curve
BfR	Bundesinstitut für Risikobewertung (German Federal Institute for Risk Assessment)
BMI	body mass index
BW	body weight
CERHR	Center for the Evaluation of Risks to Human Reproduction
CI	confidence interval
COT	Committee on Toxicity
DH	Department of Health
DHEA	dehydroepiandrosterone
DMBA	7,12-dimethylbenz[a]anthracene
DNSIYC	Diet and nutrition survey of infants and young children
DTH	delayed-type hypersensitivity
EE	ethinyl oestradiol
ER	oestrogen receptor
ESPGHAN	European Society for Paediatric Gastroenterology, Hepatology and Nutrition
FDA	Food and Drug Administration
FSA	Food Standards Agency
GD	gestational day
GI	gastrointestinal
HIM	high isoflavone levels milk
HR	hazard ratio
IGF-1	insulin-like growth factor-1
IQR	interquartile range
LIM	low isoflavone levels milk
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of Detection
LOQ	Limit of Quantification
LQ	lordosis quotients
MAFF	Ministry of Agriculture, Fisheries and Food
MDI	Mental Developmental Index
MN	micronuclei
MOF	multi-oocyte follicles
NOAEL	no-observed-adverse-effect level
NOF	natural ovarian failure
NS	not significant
NTP	National Toxicology Program
O-DMA	*O*-demethylangolensin
PDI	Psychomotor Development Index
PLS-3	Preschool Language Scale-3

PND	postnatal day
PR	progesterone receptor
PSAI	Pre-School Activities Inventory
RBA	Relative Binding Affinity
SACN	Scientific Advisory Committee on Nutrition
sc	subcutaneous
SD	standard deviation
SE	standard error
SGLT1	sodium dependent glucose transporter
SHBG	sex hormone binding globulin
SPI	soy protein isolate
SPT	skin prick test
T_3	tri-iodothyronine
T_4	thyroxine
TBG	thyroid binding globulin
TPO	thyroperoxidase
TSH	thyroid stimulating hormone
TVP	textured vegetable protein
UDP	urine diphosphate

Appendix 1
Search strategy

General isoflavones/genistein/daidzein exposure search

Websites interrogated

- EFSA
- COT
- FSA
- JECFA

Also searched PubMed from May 2002 to June 2013.

Specific search terms:

Isoflavone/phytoestrogens/genistein/daidzein/soy AND breast milk
Exclusion Criteria:

- Supplementation research in developing countries
- Supplementation programs in developing countries
- Deficiency related research

Isoflavone/genistein/daidzein/soy AND infant formula
Exclusion Criteria:

- Supplementation studies in developing countries
- Supplementation programs in developing countries
- Infant formulas in non-EU countries

Isoflavone/genistein/daidzein/soy AND infant diet
Exclusion Criteria:

- Supplementation studies in developing countries
- Supplementation programs in developing countries
- Infant diet in developing countries
- Children's diet (above >2 years) in developed countries

Isoflavone/genistein/daidzein/soy AND weaning
Exclusion Criteria:

- Supplementation studies in developing countries
- Supplementation programs in developing countries
- Infant weaning in developing countries
- Children's diet (above >2 years) in developed countries

Soya/soya formula/phytoestrogens AND animals AND diet AND oestrogenic effect/behaviour/reproduction
Exclusion Criteria:

- Studies in pre-menopausal females and mature animals
- *In vitro* studies

- Studies in which administered levels of isoflavones were not reported
- Studies in which routes of administration were other than oral
- Studies performed in lower organisms, sheep and rabbits
- Studies in developing countries
- Co-treatment with other chemicals
- Studies investigating therapeutic effect of soya in relation to other diseases

Soya/soya formula/phytoestrogens AND exposure
Exclusion Criteria:

- Supplementation studies in developing countries
- Supplementation programs developing countries

The above mentioned search terms were also used in Google. This identified additional publications and latest government advice and opinions.

Some papers published before 2002 were also retrieved if they were considered particularly relevant –e.g. because they reported levels of isoflavones in food, levels of exposure to isoflavones, or doses leading to toxicity.

Appendix 2

Oestrogenic, developmental and behavioural effects reported in animal studies following oral exposure to isoflavones

Treated population	Dose/ compound	Route of administration/ duration	Endpoint	Observed health effects in treated animals/the offspring	Reference
Oestrogenic effects					
Mice					
25-27 day old C57/BL6 female mice	Genistein 300, 500, 1000 and 1500 ppm, equivalent to 60, 100, 200 and 300 mg/kg bw/day	Diet (starting a week after ovariectomy for 12 days)	Uterine weights, effects on adipose tissue: - body weight - fat pad weight - adipocyte circumference	• ↓ in parametrial fat pad weight ($p<0.05$) and adipocyte circumference at 100-300 mg/kg bw/day • ↑ uterine weights at all doses ($p<0.05$).	Naaz *et al*., 2003
Female CD-1 mouse pups (n=5/group)	Diets containing genistein and daidzein: **Group 1**: low (0-20 ppm) **Group 2**: medium (101-210 ppm) **Group 3**: high (270-370 ppm)	Diet (PND 15 to 30)	Vaginal opening	• Advanced vaginal opening at PND 24 in Group 3 (53 to 93%) compared to Group 1 (12 to 37%) ($p<0.05$)	Thigpen *et al*., 2003

Pre-pubertal CD-1 mouse pups (n=30)	Chow containing soya protein supplement (200,000 ppm)	Diet (PND 21 to 28)	Mammary gland morphology	• ↑ longitudinal gland growth (development beyond lymph node) ($p<0.05$)	Alston-Mills *et al*., 2011
Rats					
Juvenile female Wistar rats	Genistein 100 mg/kg bw/day; EE 30 µg/kg bw/day	Daily gavage for 3 days (starting 2 weeks after ovariectomy)	Uterine wet weight, uterine and vaginal epithelium	• Combination of both compounds led to increased uterine weight/height of the uterine epithelium/height of the vaginal epithelium compared to EE alone ($p<0.05$)	Schmidt *et al*., 2006
Adult ovariectomi-sed female Wistar rats (n=8/group)	Soya extracts: 10; 50; 100; 300; 600 mg/kg bw/day corresponding to dosing of 4.3; 21.3; 42.6; 127.8; and 255.6 mg isoflavones/kg bw/day, respectively.	Daily gavage for 21 days (starting 1 month after ovariectomy)	Uterine weight, morphometric analysis	• ↑ uterine weights at soya extract >100 mg/kg bw/day • Changes in endometrial and myometrial morphometry at soya extract >300 mg/kg bw/day	Mosquette *et al.*, 2007
Female F334 rat pups (n=16-20 pups/diet)	Diets (genistein and daidzein): PMI5K96 (7 ppm); PMI5002 (98; 223; 431 ppm)	Diet (starting on PND19 until the time of vaginal opening)	Vaginal opening	• Advanced vaginal opening (5.5 day earlier) in group fed PMI5002 diet at 431 mg/kg compared to PMI5K96 ($p<0.05$)	Thigpen *et al*., 2007
Female Sprague-Dawley rat				• No significant difference	

pups (n=16-20 pups/diet)					
Female F334 rat pups (n=16-20 pups/diet)	Diet AIN-76A spiked with genistein at 0 (control), 150, 300, and 450 ppm	Diet (PND 19 to 40)		• Advanced vaginal opening in group fed diet containing 300 (PND 34) and 450 (PND 26.8) mg/kg compared to control (PND 36.8) ($p<0.05$)	
Female Sprague-Dawley rat pups (n=16-20 pups/diet)				• Advanced vaginal opening in group fed diet containing 450 (PND 27.1) mg/kg compared to control (PND 29.6) ($p<0.05$)	
19 day old female Wistar rats (n=10)	5 samples of soya dry extract at doses: 125; 300; 720; 1730; 4150 mg/kg bw/day. Approximate contents: total isoflavone: 44-52%; genistein: 9-11% (not detected in samples 3-5); daidzein: 7-46%	Daily gavage for 3 days (starting on the 19th day of life)	Uterine weight	↑ uterine weights (significant at different points in all samples)	De Lima Toccafondo Vieira *et al*., 2008
12 week old adult female rats	Low isoflavone diet (daidzein and genistein<10 ppm) enriched with genistein at 700 ppm (corresponding to 42 mg/kg bw/day);	Daily oral administration (diet) for 12 weeks (starting 2 weeks after ovariectomy)	Uterine weights	No effects	Hertrampf *et al*., 2009

	High isoflavone diet (daidzein: 232 ppm; genistein: 240 ppm; corresponding to 14 mg genistein/daidzein/kg bw/day).				
Effects on fertility and development					
Mice					
C57BL/6 mice n≥9	Genistein 0.1, 0.5, 2.5, 10 mg/kg bw/day	Daily gavage of dams GD 12 to PND 21.	Body weight, AGD (PND 7 and 21), mammary glands (PND 49) in the female offspring.	• no detected effects in the offspring (NOAEL = 10 mg/kg bw/day)	Fielden *et al*., 2002
$B6D2F_1$ mice n=10-13	Genistein 0.1, 0.5, 2.5, 10 mg/kg bw/day	Daily gavage of dams GD 12 to PND 21.	Body weight, AGD (PND 7 and 21), testis and seminal vesicle weight, sperm count and motility, *in vitro* fertilizing ability of sperm in the male offspring.	• Small but significant ↓ in AGD (<5%) at 10 mg/kg bw/day on PND 21 • No effects on sperm count, motility, testis, or body weight • Significantly ↑ *in vitro* fertilisation of sperm (17-18%) on PND 105 and 315 (NOAEL = 2.5 mg/kg bw/day)	Fielden *et al*., 2003
Pups: CD-1 female mice	Genistein 25, 37.5 or 75 mg/kg bw/day	Daily oral administration using a pipette (PND 1-5)	Uterotropic response	• No detected effects in uterine weights (slight ↑ at 75 mg/kg/day)	Jefferson *et al*., 2009
	Genistin 6.25, 12.5, 25 or 37.5 mg/kg bw/day (as genistein	Daily oral administration using a pipette (PND 1-5)	Uterotropic response, ovarian histology, vaginal opening	• ↑ uterine weights at 25 and 37.5 mg/kg bw/day • Delayed vaginal opening at 37.5 mg/kg/day	

	aglycone equivalents)			• Abnormal oestrous cycles and • significant ↓ in delivering live pups at 37.5 mg/kg bw/day • Dose dependent ↑ percentage of MOFs (NOAEL = 6.25; LOAEL = 12.5 mg/kg bw/day)	
Male mice		Daily oral administration (diet) from conception to adulthood	Testicular and reproductive functions	• ↓ proportion of haploid germ cells in testes • ↓ by 25% in epididymal sperm counts • ↓ by 21% in litter size • ↓ size of seminal vesicle • No changes in fertility and behaviour	Cederroth *et al*., 2010
Pups: C57BL/6 female and male mice n≤8	Genistein 50 mg/kg bw/day	Daily oral administration using a pipette (PND 1-5)	Body weight (PND 1-5, 7, 14, 21), vaginal opening, thymic and uterine weight, ovarian histology (PND 5 and 4 months old).	• Body weights and timing of vaginal opening no different when compared to controls • 28% ↓ in thymic weight • 41% ↑ in uterine weight • ↑number of MOFs • Ovarian cycle abnormalities at 6 months of age LOAEL = 50 mg/kg bw/day	Cimafranca *et al*., 2010
Rats					

Pregnant and lactating Long-Evans hooded rats	**Study I:** soya milk = isoflavones (genistein:daidzein, 3:2). Single dose level estimated to be between 10-30 mg/kg bw/day	**Study I:** daily gavage (PND 1-21)	Body weight, AGDI, age of puberty, oestrus cycling, reproductive organ weights	• Significantly ↑ body weight and ↓ AGDI (female pups) • No detected effects regarding reproductive endpoints, except in male pups - ↓ epididymal weight • ↑ in PR expression in glandular epithelial cells	Hughes *et al*., 2004
	Study II: genistein 15 mg/kg bw/day	**Study II**: daily gavage (GD 14 through PND 21)		• Significant ↑ in PR expression in glandular epithelial cells (20%) • Earlier onset of puberty in genistein treated males (LOAEL = 15 mg/kg bw/day)	
Part I: Pregnant and lactating Long Evans rats n=12 **Part II**: Male offspring	Genistein (5 and 300 ppm, corresponding approximately to 0.42 and 25 mg/kg bw/day)	**Part I**: Daily oral administration (diet); from 2 weeks before breeding to PND 21 **Part II**: Weaning – 70th day of age	Body weight, immune organ masses, testosterone levels.	• ↑ thymus masses ($p<0.05$) and ↑ subpopulations of T cells in the spleen at both doses ($p<0.05$) ↓ testosterone concentrations at both doses ($p<0.05$) (LOAEL = 0.42 mg/kg bw/day) • PART II: no additional effect over perinatal exposure	Klein *et al*., 2002
Sprague Dawley rats: n=35/group	Genistein 0.3, 7, 35 mg/kg bw/day (male) and 0.5, 10, 51 mg/kg bw/day (females)	Daily oral administration (diet); from 3 (F_1 and F_2) and 6 (F_0) wk of age, through	Body weights, vaginal opening, AGD, oestrous cyclicity, litter size	• ↓ body weights and AGD, ↑ vaginal opening, altered oestrous cyclicity at 51 mg/kg bw/d (females) • ↓ litter size in F_1 and F_2	NTP, 2008a

		gestation and lactation up to 140 day of age; F_3: indirectly (*in utero* and lactation); F_4 and F_5 – no exposure		• ↑ rates of mammary gland hyperplasia F_4 and F_5 - no effects (NOAEL = 0.5 mg/kg bw/day)	
Female Sprague-Dawley rats	Exposure to genistein as 5 ppm feed: **Group 1.** none (n=8) **Group 2**. during gestation and lactation (n=9) **Group 3.** during gestation (n=8) **Group 4**. during lactation (n=8)	Daily oral administration (diet) for 2 weeks prior to mating	AGD (measured on PND 2, 7, 14, 21), weight of reproductive organs, spatial learning and memory in the Morris water maze (latency to find a hidden platform and swim speed), cued and contextual fear conditioning (emotional learning)	Effects observed in male offspring: • ↓ mean AGD on PND 14 and 21 (p=0.001) in Group 2 • ↓ mean body mass on PND 21 in Group 2 (p=0.02) and Group 4 (p<0.001) • No effect on organs weight measured on PND 70 • No impact on emotional learning • Impaired spatial learning in Group 2	Ball *et al*., 2010
Pigs					
Piglets (gender not specified); n=8/group	Milk-replacer with low genistein content (1 mg/L) and high genistein content (14 mg/L)	Daily self-feeding from a nipple attached to tubing, from 48h to day 10 (approximately 360 ml/kg bw per day)	Body weight, small intestinal weight and length	• No significant differences in body weight and intestinal weight and length between different groups • ↓ intestinal cell proliferation and ↓ jejunal enterocyte migration	Chen *et al*., 2005
Postpubertal gilts – 180 days of age	Genistein injections: 50 (n=4); 100 (n=5); 200 (n=5); or 400	15 days post-ovariectomy gilts were assigned to intramuscular	Uterine and cervical mass/weight; height of uterine epithelial cells; percentage of	• Dose dependent ↑ in uterine and cervical tissue mass • ↑ height of uterine and cervix	Ford *et al*., 2006

	(n=7) mg/day	injections containing genistein (at 12 hours intervals for 10 days)	cells stained positive for progesterone receptor	epithelial cells at 400 mg/day • ↑ in % of cells staining positive for progesterone receptor in the uterine glands and cells lining the vaginal cervix	
Pre-pubertal piglets (n=8/group)	Diet containing soya bean extract (approx. 48 mg daidzein, 22 mg genistein and 0.5 mg equol)	Daily oral administration (diet) for 6 weeks	Strength and density of bones, body weight, effects on genital tracts	• No effects on growth rate, body weight, plasma bone markers, bone mineral density and strength • Heavier ovaries and ↑ number of follicles. No effects on uterus weight.	De Wilde *et al.*, 2007
Male and female piglets n=4/group	Soy formula (content not specified).	Daily oral administration from 48h through 21st day	Body and testicular weight, bone mineral density	• No differences in testicular development and body weight in males • ↑ trabecular bone mineral density/total mineral content and cortical thickness (measured in tibia bone) in males and females	Badger *et al*., 2009
Monkeys					
Marmoset monkeys n=13 pairs	Isoflavones in soya formula (range: 1.6 – 3.5 mg/kg bw/day)	Daily oral administration (syringe) from PND 4-5 until PND 35-45	Body weight, organ weights, testosterone levels, testicular cell composition	• ↓ levels of testosterone at PND 35-45 ($p<0.004$) • ↑ numbers of Leydig cells (74%) ($p=0.006$) • No differences in numbers of Sertoli and germ cells	Sharpe *et al*., 2002
Marmoset monkeys n=7 pairs of	Isoflavones in soya formula (range: 1.6 – 3.5	Daily oral administration (syringe) from PND	Body weight, organ weights, testicular cell counts	• ↑ testicular weight (14%) and numbers of Sertoli (7%) and Leydig cells (32%) ($p<0.05$)	Tan *et al*., 2006

twins	mg/kg bw/day)	4-5 until PND 35-45			
Pubertal female cynomolgus monkeys (n=17)	Diet containing soya protein isolate: 120 mg isoflavones per day	Daily oral administration (diet) for 4.5 years	Pubertal breast development, sex hormones, growth	• ↑ endometrial area • Changes in breast differentiation • No effect on onset of menarche, growth, pubertal progression, levels of oestradiol and progesterone	Dewi *et al*., 2013
Behavioural effects					
Rats					
8 week old male Lister rats	Diet containing isoflavones at 150 ppm (genistein and daidzein)	Daily oral administration (isoflavone diet *ad libitum*) for 2 weeks (n=32) followed by further 2 weeks of isoflavone diet (n=16) and control diet (without isoflavones, n=16). Then the rats were housed singly for 4 days and examined	Social interaction; anxiety measured as entries into the open arms of the elevated plus-maze apparatus; level of corticosterone and vasopressin	• ↓ time spent in active social interaction (p=0.02) • ↓ number of entries onto the open arms of the plus-maze (p=0.002) indicating an increase in anxiety • ↑ level of stress-induced corticosterone (p<0.01) and plasma vasopressin (p<0.04)	Hartley *et al*., 2003
Sexually experienced Sprague-Dawley rats (n=20/group)	Treatment with saline or isoflavones (0.4 or 0.8 mg/kg)	Daily oral administration (gastric tube) for 40 days	Sexual behaviour	• No significant differences in sexual performance • ↑ intromission frequencies in group fed 0.8 mg isoflavones/kg (p=0.13) • ↓ LH and testosterone plasma levels (p<0.05)	Cicero *et al*., 2004

7-8 week old male and female Sprague-Dawley rats Study 1: n=23 Study 2: n=72	Diets: **Study 1**: 1. Cookies 2. Peanuts 3. Lab chow 4. Soya beans (isoflavone content not specified) **Study 2:** 1. Lab chow (n=36) 2. Mixture of cookies and soya beans (n=36)	Daily oral administration (diet *ad libitum*) **Study 1**: for 7 days before exposure to stressors rats (n=23) were fed mixture of food types in order to determine their food preference, then diet continued in all rats but stressors were applied in half of the animals **Study 2**: There were 2 groups of animals: exposed to stressors (n=36) and left undisturbed (n=36). Half of each group was fed lab chow and the other half received mixture of cookies and soya beans.	Interaction between stressors, diet, and sex. **Study 1**: <u>Stressors</u> were applied singly or in combination for 25 days to half of the rats (loud static, flashing light, tilted cages, wet bedding, water deprivation, photoperiod reversal) **Study 2**: Behaviour of rats exposed to stressors (n=36) was analysed before and after the period of stressor exposure using following methods: dark avoidance activity, open field activity, elevated plus-maze test, forced swim test	**Study 1**: • ↓ consumption of lab chow and peanuts in both sexes • ↑ consumption of soya beans in males before and after stressors comparing to females ($p=0.0036$) • Interaction between sex and exposure to stressors in the consumption of cookies ($p=0.004$) **Study 2**: • ↓ total caloric intake of all diets in both sexes exposed to stressors (in males $p<0.08$) • No significant influence of diet on open field rearing • ↑ attention to novelty among males exposed to stressors ($p<0.05$) correlated with diet • In control females mixed diet lead to ↓ time in the closed arm ($p<0.01$), ↑ time in the open arm ($p<0.01$) and ↑ moving time ($p<0.05$) compared to control females fed lab chow. • ↑ time of immobility in females fed the mixed diet ($p=0.0065$)	Liang *et al.*, 2008
5 week old Wistar rats	Diet containing fermented (FSM)	Daily oral administration (diet	Voluntary wheel running activity in	• ↑ running activity ($p<0.05$; $p<0.01$) observed 2 and 3	Sato *et al.*, 2010

(n=7/group)	or non-fermented (SM) soya milk (isoflavone content not specified)	*ad libitum*): weeks 1-3 and 7-9: normal diet; weeks 4-6: diet containing FSM or SM	rats measured over 9 weeks	weeks after starting a diet in FSM group • No changes in running activity in SM rats • ↑ in mounting behaviour (by 10 days) in FSM rats (p<0.01)	
Female Long-Evans rats (n=3/4 per treatment): **Study 3**: ovariectomy (OV) before day 45 **Study 4**: Ovariectomy on day 100	Low-soya (**LS**) and soya-rich (**HS**) diet containing 10 and 600 ppm of isoflavones. In Study 3 additionally medium soya (**MS**) diet was used – 200 ppm of isoflavones. **Equol** sc at 5 mg/kg bw/day	**STUDY 1**: LS diet (n=23; conception=day 0 to 120 days), then 50% switched to HS diet until day 200. Equol sc in LS group (days 194-200). **STUDY 2**: LS (n=8) or HS (n=7) diet from day 0 to 145. Equol sc in LS group (days 136-145) **STUDY 3**: MS (n=22; 0 to 45 day), LS (n=22; 46 to 85 days), HS (n=11; 86 to 100 days). **STUDY 4**: LS or HS diet (n=8/group; 0 to 200 days). Equol sc in LS group (days 197-200). **STUDY 5**: LS (n=6) or HS (n=8)	Depressive-like behaviours measured by PFST*, weight gain and white adipose tissue deposition (WAT)	**STUDIES 1-4:** **Behaviour:** • No differences in behavioural parameters between groups **Effects observed in HS group:** • ↓ body weight gain (p<0.05; in Study 1 after diet change; and Study 4 after OV) and ↓ WAT (p<0.01 to p<0.001) • ↓ body weight following ovariectomy (p<0.01) **Effects observed in LS group:** • ↓ body weight following equol sc **STUDY 5:** **Behaviour:** • ↑ mobility, swimming distance and speed (p<0.005) • ↑ mobility in LS group when compared to pre-equol injection values (p<0.05) **Effects observed in HS group:** • ↓ body weights (p<0.05; a	Blake *et al*., 2011

		diet from 0 to 365 days. Natural ovarian failure (NOF) occurred approximately on day 295.		month after NOF) and ↓ WAT ($p<0.05$) **Effects observed in LS group:** • ↓ body weight following equol sc	
Monkeys					
Male cynomolgus macaques (n=44)	Soy protein isolate diet containing 0.94 and 1.88 mg isoflavones/g protein	Daily oral administration (diet) for 15 months	Spontaneous social behaviour	Effects observed at highest concentration of isoflavones: • ↑ frequencies of intense aggressive (67%) and submissive (203%) behaviour ($p<0.05$) • ↓ time of physical contact (68%), time in proximity to other animals (50%)($p<0.02$) • ↑ time spent alone (30%)($p<0.02$)	Simon *et al*., 2004
Male newborn rhesus monkeys (n=8/group)	Diet: cows' milk formula, soya formula (SF), soya formula with Mn (SMF)	Daily oral administration (diet) from birth to 4 months	Body weight, motor, cognitive and social parameters, rest and activity cycles	• ↓ in weight ($p<0.01$) and height ($p<0.05$) in control vs SF group at 9 months of age • ↑ number of total behaviour initiations, climbing behaviour (SF) and walking initiations (SMF)(ns) • ↓ duration of wake periods at 8 months ($p<0.01$, SF) ($p<0.05$, SMF) • ↓ total duration of play	Golub *et al.*, 2005

				behaviours ($p<0.05$)	

#The mixture of genistein and daidzein comparable to quantity and ratio of each isoflavone in soy protein based infant formula (as reported by Dinsdale *et al*., 2011)

*PFST – Porsolt forced swim test – quantifies animals' mobility (swimming speed, distance, time of activity) as a sign of a less depressive behaviour

AGD – anogenital distance; AGDI – AGD Index (ratio of AGD/BW); bw – body weight; CL – corpora lutea; EE – ethinyl oestradiol; LQ – number of lordosis behaviours displayed/number of mounts x 100); ER – oestrogen receptor; MOFs – multi-oocyte follicle; NOF – natural ovarian failure; ns – not significant; OV – ovariectomy; PR – progesterone receptor

Appendix 3

Human studies of reproductive and developmental outcomes associated with *e*xposure to isoflavones.

Study design (Reference)	Isoflavone exposure	Outcomes	Findings	Comments
Cross sectional study of children (37 boys and 35 girls) aged <48 hours to 6 months. Stratified by age (in 7 bands) and feeding regime (breast milk, cows' milk-based formula and soya formula). (Bernbaum *et al*., 2008)	One-third of children exclusively fed soya-based infant formula (as reported by parents). Reasons for preference for soya formula unknown.	Measurements of breast adipose tissue, breast buds, and testicular volume; breast and genital development; vaginal cell changes; vaginal discharge.	• ↑ vaginal cell maturation index in girls older than 30 days who were fed soya formula.	Difference by feeding regime was not statistically significant.

Cross-sectional survey of 694 female infants aged 3 to 24 months. (Zung *et al*., 2008)	At least 3 months of continuous soya-based formula feeding (n=92).	Measurement of breast buds.	• In the second but not the first year of life, there was a higher prevalence of breast buds when compared to breast-fed and cows' milk-fed infants ($p<0.02$; 95% CI 1.11 – 5.39).	This study raises the possibility that soya in early life can affect breast tissue in a small percentage of children. The findings would need to be confirmed in further studies.
Cross-sectional survey of infants aged 4 months from an ongoing longitudinal study. (Gilchrist *et al*., 2010)	Infants fed soya formula largely or exclusively from birth (n=39) compared with others fed cows' milk formula (n=41) or breast milk (n=40). Approximately 20 boys and 20 girls per diet group Parents chose feeding method.	Measurements of body weight, and of breast bud, ovary, prostate and testicular volumes assessed by ultrasonography.	• ↓ body weight in boys (compared to those fed cows' milk formula) ($p<0.05$). • No differences in breast bud or uterine volume in girls. • No differences in prostate or breast bud volumes in boys.	Well designed and conducted study with thorough statistical analysis. Investigators were blind as to diet group. Modest sample size. No adverse effects of soya feeding on reproductive organ development were demonstrated by age 4 months.
Cross-sectional survey of boys (n=230) and girls (n=198), aged 3-6	Dietary intake of soya and isoflavone assessed from 3-day dietary records	Levels of sex hormones in urine, adjusted for creatinine.	• Soya intake negatively related to oestrone and oestradiol in boys ($p<0.05$).	Suggests an influence of soya intake on secretion or metabolism of sex

years. (Wada *et al.*, 2011)	completed by parents.		• Soya intake positively related to testosterone and 5-androstene-3β,17α diol (3β,17α-AED) levels in girls ($p<0.01$). • Isoflavone intake negatively associated with oestradiol in boys and positively associated with testosterone in girls	steroids in age group studied, but implications for infants consuming soya formula is unclear.
Longitudinal study (ALSPAC*) of 3,664 boys and 3,412 girls followed up from birth to age 42 months. (Adgent *et al*., 2011)	Use of soya early in infancy (n=157) and only later in infancy (n=306) compared with primarily breastfed (n=1428) and early formula-fed (n=5185). Reasons for soya feeding were not reported.	Gender-role play behaviour assessed by Pre-School Activities Inventory (PSAI).	• Early soya (vs early formula) feeding associated with less feminine PSAI scores in girls. • No significant differences in boys. • Authors noted that scores were within the range of normal behaviour.	Bias or residual confounding by social class might explain the positive findings. (better educated mothers were more likely to report both masculine and feminine traits in children).
Longitudinal study (ALSPAC *) of 2920 girls followed up from birth to puberty.	Use of soya early in infancy (n=54) and only later in infancy (n=111) compared with primarily breastfed (n=631)	Timing of menarche assessed by approximately annual questionnaires from age 8 to 14.5.	• Compared with girls fed formula early, early soya-fed girls had a higher risk of menarche throughout follow-up (hazard ratio	The non-significant association with earlier menarche contrasts with the study by Strom *et al*. (2001) described above,

(Adgent *et al*., 2012)	and early formula-fed (n=2124).		1.25, 95%CI 0.92-1.71).	which found no difference in reported age at menarche between 128 females fed soya and 268 fed cows' milk formula.
Longitudinal study of 391 infants assessed at ages 3, 6, 9 and 12 months. (Andres *et al*., 2012)	129 children fed soya-based infant formula (SF) from 2 to 12 months compared with 131 fed milk formula (MF) from 2 to 12 months and 131 breastfed (BF) (some of whom changed to milk formula between 6 and 12 months.	Infant behavioural development (Bayley Scales: MDI** and PDI***; PLS-3****).	• ↓ MDI at 6, 9 and 12 months (compared to BF) ($p<0.05$). • ↓ PDI at 6 months compared to BF infants ($p<0.05$). • No differences between SF and MF groups.	Although significant, the differences in the MDI were small and scores were within the expected normal range. PLS-3 scores were lowest in the MF group.
Longitudinal study of 207 infants assessed at ages 3, 6, 9, and 12 months. (Andres *et al*., 2013)	Children fed soya-based infant formula (SF) from 2 to 12 months (approximately one third of total) compared with groups fed milk formula (MF) from 2 to 12 months and breastfed (BF) (some of whom changed to milk	Growth, fat mass and bone mineral content	• ↑ fat-free mass at 6 and 9 months compared to MF ($p<0.001$) • ↑ bone mineral content by 12 months (but lower at 3 months compared to BF)	

	formula between 6 and 12 months.			

*ALSPAC study – Avon Longitudinal Study of Parents and Children; ongoing study that enrolled pregnant women residing in the Avon region of the UK expecting to deliver between 1 April 1991 and 31 December 1992. During pregnancy 14 062 live births were recruited into the study.
**MDI – Mental Developmental Index measures performance in sensory perception, knowledge, memory, problem solving and early language with tasks adapted to age
***PDI – Psychomotor Development Index assesses fine and gross motor development with tasks adapted to age
****PLS-3 – Preschool Language Scale-3 assesses receptive, expressive language skills and language precursors
CI – confidence interval; DHEA – dehydroepiandrosterone; IQR – interquartile range; HR – hazard ratio