

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

Third draft statement on the potential risks from high levels of soya phytoestrogens and soya products 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 and soya products in the infant diet. Soya-based infant formula and weaning food products containing soya are the main source of phytoestrogen exposure in infants. Soya formula is produced using soya protein isolate - a particularly rich source of three isoflavones: genistein, daidzein and to a lesser extent glycitein.
3. The COT produced a report on phytoestrogens and health in 2003¹, 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 relevant evidence that is now available. The criteria that were employed in the literature search are set out in Appendix 3.

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

Introduction

4. 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.

5. 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 17 β -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).

6. 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 aglycone² forms are produced. Bacterial hydrolysis can significantly increase the content of aglycones in fermented soya-based food products such as tofu³ and tempeh⁴. 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).

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

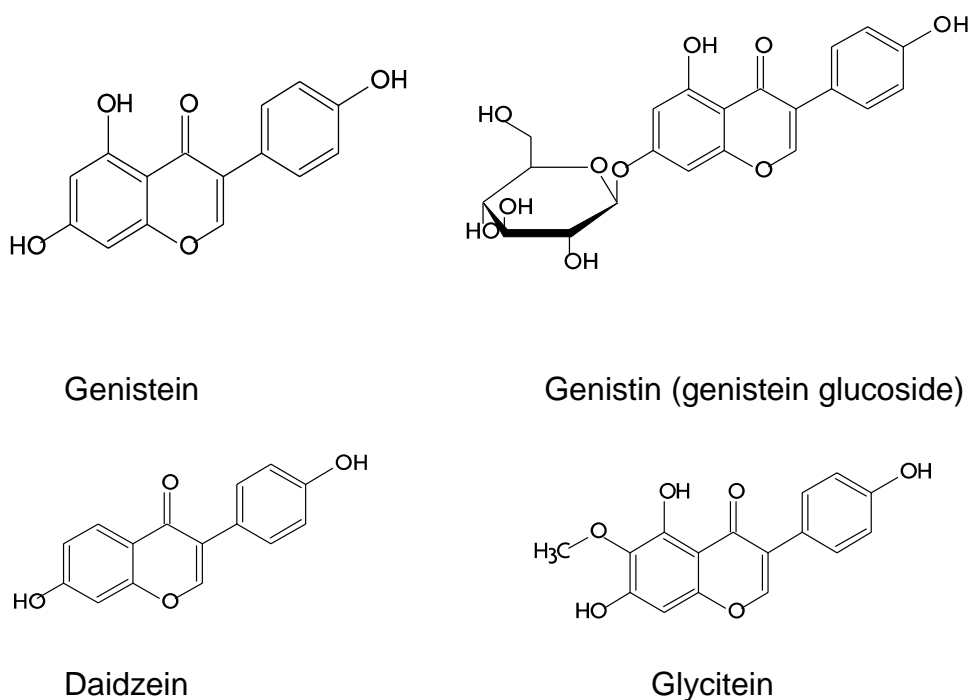


Figure 1. Chemical structures of the isoflavone aglycones genistein, daidzein and glycitein, and the glucoside genistin.

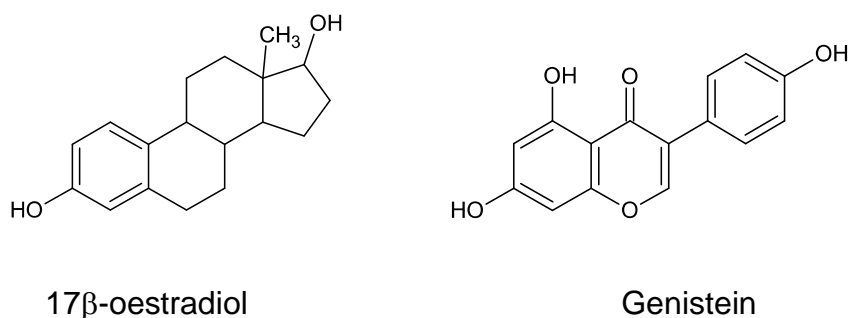


Figure 2. The similarity of the structure of 17β-oestradiol and genistein

Current UK Government recommendations in relation to infant diet

7. 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 formulas⁵. 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,

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>

galactokinase deficiency or galactosaemia, and that they should only be used in exceptional circumstances to ensure adequate nutrition (DH, 2004).

Recommendations in other countries

8. In 1999, the US Food and Drug Administration (FDA) reviewed available human studies, and gave food manufacturers permission to use a health claim on food labels stating that a daily diet containing 25 g of soya protein may help to reduce risk of heart disease. There have been no changes in the FDA recommendations since then. 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).

9. 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).

Absorption⁶ and metabolism

10. 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).

11. NTP-CERHR (2010) noted that the principal Phase I metabolism of glycosides and aglycones within the gut includes reduction, deoxygenation, hydroxylation and

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.

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).

12. 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).

13. 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).

14. 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).

15. 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 (12% for free plus conjugated genistein, and 15% for free genistein). 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).

16. 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).

17. 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.

Distribution and excretion

18. 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).

19. 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).

20. 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).

21. 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 women 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

22. 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

23. 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

24. Effects of supplementation of soya milk with probiotics⁷, such as *Lactobacillus sp.* and *Bifidobacterium sp.*, and prebiotics⁸ (e.g. fructooligosaccharides, inulin, pectin or 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 that the organism may alter isoflavone metabolism by stimulating deconjugation and/or inhibiting degradation.

Age

25. 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). The main factors stimulating gut flora maturation are decreasing gut permeability following initial colonisation with mother's bacteria during vaginal birth. This initial colonisation results in an acidic environment and gut flora dominated by *Bifidobacteria* for the first 6 weeks of life (Catassi *et al.*, 1995). Bacterial β -glucosidase activity, associated with bacteria such as *Lactobacilli*,

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)

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 weaning infants who 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 and sex 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

In vitro studies

31. *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 demonstrated 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).

Genotoxicity

32. *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).

33. 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). 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.

Animal studies

34. 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 (rodents), and alter some parameters of immune function (rodents), as well as sexual development (marmosets) 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).

35. 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).

Effects of phytoestrogens on fertility and development

36. A number of studies have addressed possible adverse effects on development from exposure to isoflavones early in life (see Appendix 1). 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 increased 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) (NOAEL = 5 mg/kg bw/day; LOAEL = 50 mg/kg bw/day) (Jefferson *et al.*, 2002 and 2006) and uterine weights (NOAEL = 12.5 mg/kg bw/day; LOAEL = 20 mg/kg bw/day) (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); delayed (LOAEL = 37.5 mg/kg bw/day) and advanced vaginal opening; ovarian cycle abnormalities; decreased anogenital distance (AGD); and changes in body weight.

37. 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).

Phytoestrogens and behavioural studies

38. A number of animal studies have investigated behavioural effects of dietary exposure to isoflavones (see Appendix 1). 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 height. When reported, the concentrations of isoflavones in feed have been between 0.4 and 600 ppm.

Phytoestrogens and immunosuppression

39. 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 the following health effects: altered thymic size; decreased proliferation; and decreased production of the cytokine IFN- γ (Cooke *et al.*, 2006).

Phytoestrogens and cancer

40. 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 (NTP, 2008b).

41. 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).

42. 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.

Oestrogenic potency of phytoestrogens

43. 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 1). In addition, irregular oestrus cycles have been observed following subcutaneous exposure (10 mg genistein/kg bw/day) (Bateman and Patisaul, 2008).

44. 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 ER β than ER α (Kuiper *et al.*, 1997 and 1998; COT, 2003)

45. The distribution of ER α 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).

46. Matsumura *et al.* (2005) determined the ER-binding of isoflavones in human breast cancer cells, using radiolabelled oestrogen [$2,4,6,7\text{-}^3\text{H}$]oestrogen at 16×10^{-10} M. Genistein inhibited [^3H]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 ER β (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).

47. 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 ER α and β . In the case of genistein, the concentration must be ≥ 100 fold higher for ER α , and ≥ 2 fold higher for ER β . The relative binding affinities (RBAs) presented in Table 1 illustrate how isoflavones preferentially bind ER β .

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

Compound	ER α		ER β		Ratio of RBAs ER β /ER α	Reference
	IC ₅₀ or [EC ₅₀] (μ M)	RBA (%)	IC ₅₀ or [EC ₅₀] (μ M)	RBA (%)		
Human						
17 β -oestradiol	0.0020		0.0023		0.86	Harris et al., 2002
Diethylstilbestrol	0.0014		0.0011		1.27	
Genistein	0.3340		0.0066		50.60	
Daidzein	>5.0000		0.4100		>12.19	
17 β -oestradiol	0.0043	107.00	0.0057	82.00	0.75	Mueller et al., 2004
Diethylstilbestrol	0.0046	100.00	0.0046	100.00	1.00	
Genistein	0.3000	1.00	0.0150	31.00	20.00	
Equol	1.5000	0.30	0.2000	3.00	7.50	
17 β -oestradiol	[0.021 x 10 ⁻³]	100.00	[0.11 x 10 ⁻³]	100.00	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	
17 β -oestradiol		100.00				Kwok and Cheung, 2010
Diethylstilbestrol		100.00				
Genistein		1.50				
Mouse						
17 β -oestradiol	0.0021		0.0025		0.84	Harris et al., 2002
Diethylstilbestrol	0.0002		0.0023		0.09	
Genistein	0.4000		0.0046		86.95	
Daidzein	4.9930		0.1670		29.89	
Rat						
17 β -oestradiol	0.0018		0.0018		1.00	Harris et al., 2002
Diethylstilbestrol	0.0006		0.0011		0.54	
Genistein	0.2820		0.0058		48.62	
Daidzein	5.1790		0.3450		15.01	
17 β -oestradiol	0.0250	100.00	0.0320	100.00	0.78	Zhao et al., 2009
Genistein	4.7350	0.53	0.0790	41.12	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	20.62	62.87	
17 β -oestradiol	0.0009	100.00				Branham et al., 2002 ^a
Genistein	0.2000	0.45				
Daidzein	4.0000	0.02				
Equol	0.6000	0.15				

IC₅₀ – molar concentration of compound leading to a 50% inhibition of 17 β -oestradiol binding to ER

EC₅₀ – molar concentration of compound producing response equal to 50% of that observed with 17 β -oestradiol

RBA – the Relative Binding Affinity of the compound as a percentage of the binding affinity of 17 β -oestradiol (100%) [or diethylstilbestrol (100%) (Mueller *et al.*, 2004)], calculated as (IC₅₀ of 17 β -oestradiol)/(IC₅₀ of test compound) x 100 [or (IC₅₀ of diethylstilbestrol)/(IC₅₀ of test compound) x 100 (Mueller *et al.*, 2004)].

^a ER type not stated, presumably ER α

Human studies

48. 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.

Effects of phytoestrogens on fertility and development

49. 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).

50. 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.

51. Appendix 2 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).

52. 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.

Effects of phytoestrogens on growth, allergy and immune responses

53. 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.

54. 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).

55. 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).

56. 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).

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

Effects of phytoestrogens on the thyroid gland and thyroid function

57. 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 (T₃) and thyroxine (T₄), and that they might act through the inhibition of thyroperoxidase (TPO) or interactions with thyroid binding globulin (TBG)(COT, 2003).

58. 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).

59. 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 athyreotic¹⁰ 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 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).

60. 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 (T₄) levels between the groups before the start of treatment with levothyroxine. Levels of T₄, 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

61. One study has considered the relationship between consumption of soya-based infant formula and risk of breast cancer in later life. History of feeding was

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

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 (TDI) value 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 breast 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

Soya-based products

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 <i>et al.</i> , 1998 Thompson <i>et al.</i> , 2006 Kuhnle <i>et al.</i> , 2008

*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 weaning (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 (DH, 2013). Since the survey from which these values were derived 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 value)			
	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

Cows' milk-based 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.

Table 4. Estimated total isoflavone exposure (mg/kg bw/day) of infants exclusively fed cows' milk formula for average and high level consumption of milk, based on the highest reported isoflavone content

Daily consumption of milk	Age in months			
	0 - 4	>4 – 6	>6 – 9	>9 - 12
Average (800 mL)	0.038	0.029	0.026	0.023
High level (1200 mL)	0.057	0.043	0.039	0.035

Soya-based infant formula

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 5).

Table 5. Estimated total isoflavone exposure (mg/kg bw/day) of infants fed exclusively soya-based infant formula for average and high level consumption of milk, based on the range of reported isoflavone content

Daily consumption of milk	Age in months			
	0 - 4	>4 - 6	>6 – 9	>9 - 12
Average (800 mL)	2.4 – 6.3	1.8 – 4.8	1.6 – 4.3	1.5 – 3.9
High level (1200 mL)	3.7 – 9.5	2.8 – 7.2	2.5 – 6.4	2.2 – 5.8

Complementary feeding products – exposure assessment

78. Exposures to total isoflavones from infant foods (Table 6) 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 mainly 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 website¹¹ 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 value would be expected to overestimate exposures since it is unlikely that an infant

¹¹ <http://www.annabelkarmel.com/recipes/babies-6-9-months/banana-tofu-puree>

will consume tofu products (such as banana and tofu puree) daily over prolonged periods.

Table 6. 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
Weaning 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, it has been indicated that genistein is the isoflavone with the greatest oestrogenic activity representing approximately 63% of the total isoflavone content of soya formula, when expressed as aglycone equivalents. Thus, taking into consideration animal data, potential health effects in infants exclusively fed soya-based infant formula and exposed to isoflavones at 9.5 mg /kg bw/day (of

which approximately 5.7 mg /kg bw/day could be genistein) cannot be excluded. There is thus some uncertainty about the safety of soya-based formula.

83. Exposures to isoflavones from complementary foods 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 because of 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. 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.

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

90. 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.

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Abbreviations

AAP	American Academy of Pediatrics
ADME	absorption, distribution, metabolism and excretion
AFSSA	L'Agence française de sécurité sanitaire 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
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, Forestry and Fisheries
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

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PR	progesterone receptor
PSAI	Pre-School Activities Inventory
RBA	Relative Binding Affinity
RDI	recommended daily intake
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
TDI	tolerable daily intake
TPO	thyroperoxidase
TSH	thyroid stimulating hormone
TVP	textured vegetable protein
UDP	urine diphosphate

Appendix 1

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
<i>Oestrogenic effects</i>					
<i>Mice</i>					
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	<ul style="list-style-type: none"> • ↓ 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • Advanced vaginal opening at PND 24 in Group 3 (53 to 93%) compared to Group 1 (12 to 37%) (p<0.05) 	Thigpen <i>et al.</i> , 2003

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Pre-pubertal CD-1 mouse pups (n=30)	Chow containing soya protein supplement (200,000 ppm)	Diet (PND 21 to 28)	Mammary gland morphology	<ul style="list-style-type: none"> • ↑ longitudinal gland growth (development beyond lymph node) (p<0.05) 	Alston-Mills <i>et al.</i> , 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	<ul style="list-style-type: none"> • 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 <i>et al.</i> , 2006
Adult ovariectomised 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	<ul style="list-style-type: none"> • ↑ 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • Advanced vaginal opening (5.5 day earlier) in group fed PMI5002 diet at 431 mg/kg compared to PMI5K96 (p<0.05) 	Thigpen <i>et al.</i> , 2007
Female Sprague-Dawley rat				<ul style="list-style-type: none"> • No significant difference 	

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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)		<ul style="list-style-type: none"> 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)				<ul style="list-style-type: none"> 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 19 th day of life)	Uterine weight	↑ uterine weights (significant at different points in all samples)	De Lima Toccafondo Vieira <i>et al.</i> , 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 <i>et al.</i> , 2009

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	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.	<ul style="list-style-type: none"> no detected effects in the offspring (NOAEL = 10 mg/kg bw/day) 	Fielden <i>et al.</i> , 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, <i>in vitro</i> fertilizing ability of sperm in the male offspring.	<ul style="list-style-type: none"> 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 ↑ <i>in vitro</i> fertilisation of sperm (17-18%) on PND 105 and 315 (NOAEL = 2.5 mg/kg bw/day) 	Fielden <i>et al.</i> , 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	<ul style="list-style-type: none"> No detected effects in uterine weights (slight ↑ at 75 mg/kg/day) 	Jefferson <i>et al.</i> , 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	<ul style="list-style-type: none"> ↑ uterine weights at 25 and 37.5 mg/kg bw/day Delayed vaginal opening at 37.5 mg/kg/day 	

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	aglycone equivalents)			<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • ↓ 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 <i>et al.</i> , 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).	<ul style="list-style-type: none"> • 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 	Cimafranca <i>et al.</i> , 2010
Rats					

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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	<ul style="list-style-type: none"> 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 <i>et al.</i> , 2004
	Study II: genistein 15 mg/kg bw/day	Study II: daily gavage (GD 14 through PND 21)		<ul style="list-style-type: none"> 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 – 70 th day of age	Body weight, immune organ masses, testosterone levels.	<ul style="list-style-type: none"> ↑ 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> ↓ body weights and AGD, ↑ vaginal opening, altered oestrous cyclicity at 51 mg/kg bw/d (females) ↓ litter size in F₁ and F₂ 	NTP, 2008a

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		gestation and lactation up to 140 day of age; F ₃ : indirectly (<i>in utero</i> and lactation); F ₄ and F ₅ – no exposure		<ul style="list-style-type: none"> • ↑ rates of mammary gland hyperplasia F₄ and F₅ - 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: <ul style="list-style-type: none"> • ↓ 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • No significant differences in body weight and intestinal weight and length between different groups • ↓ intestinal cell proliferation and ↓ jejunal enterocyte migration 	Chen <i>et al.</i> , 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	<ul style="list-style-type: none"> • Dose dependent ↑ in uterine and cervical tissue mass • ↑ height of uterine and cervix 	Ford <i>et al.</i> , 2006

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	(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 <ul style="list-style-type: none"> • ↑ 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	<ul style="list-style-type: none"> • 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • ↓ 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • ↑ testicular weight (14%) and numbers of Sertoli (7%) and Leydig cells (32%) (p<0.05) 	Tan <i>et al.</i> , 2006

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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	<ul style="list-style-type: none"> • ↑ endometrial area • Changes in breast differentiation • No effect on onset of menarche, growth, pubertal progression, levels of oestradiol and progesterone 	Dewi <i>et al.</i> , 2013
Behavioural effects					
Rats					
8 week old male Lister rats	Diet containing isoflavones at 150 ppm (genistein and daidzein)	Daily oral administration (isoflavone diet <i>ad libitum</i>) 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	<ul style="list-style-type: none"> • ↓ 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • 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 <i>et al.</i> , 2004

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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 <i>ad libitum</i>) 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: <ul style="list-style-type: none"> • ↓ 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: <ul style="list-style-type: none"> • ↓ 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 <i>et al.</i> , 2008
5 week old Wistar rats	Diet containing fermented (FSM)	Daily oral administration (diet	Voluntary wheel running activity in	<ul style="list-style-type: none"> • ↑ running activity (p<0.05; p<0.01) observed 2 and 3 	Sato <i>et al.</i> , 2010

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(n=7/group)	or non-fermented (SM) soya milk (isoflavone content not specified)	<i>ad libitum</i>): 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 <ul style="list-style-type: none"> 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: <ul style="list-style-type: none"> No differences in behavioural parameters between groups Effects observed in HS group: <ul style="list-style-type: none"> ↓ 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: <ul style="list-style-type: none"> ↓ body weight following equol sc STUDY 5: Behaviour: <ul style="list-style-type: none"> ↑ 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: <ul style="list-style-type: none"> ↓ body weights ($p<0.05$; a 	Blake <i>et al.</i> , 2011

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		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: <ul style="list-style-type: none"> • ↓ 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: <ul style="list-style-type: none"> • ↑ 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 <i>et al.</i> , 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	<ul style="list-style-type: none"> • ↓ 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 <i>et al.</i> , 2005

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				behaviours (p<0.05)	
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#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 2

Human studies of reproductive and developmental outcomes associated with exposure to isoflavones.

Participants (Reference)	Isoflavone exposure	Endpoint	Observed health outcome	Comments
Children <48 hours to 6 months of age (7 age intervals), 37 boys and 35 girls – 2 boys and two girls in each age interval and three feeding regimes: breast milk, cows' milk-based formula and soya formula AIM: to study whether soya formula prolongs maternal oestrogenisation of newborns (Bernbaum <i>et al.</i> , 2008)	Cross-sectional pilot study. 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 as part of regular physical examinations over a period from birth to 6 months	<ul style="list-style-type: none"> • ↑ vaginal cell maturation in newborns and older girls (the curvature was statistically significant: $p < 0.0001$; feeding regimen influenced the trajectory of maturation index: $p = 0.07$). The lowest maturation index of vaginal wall cells was observed in 1 month olds. • No changes in breast and genital anatomy over the time 	Reported change was based on approximately 2 girls per feed type. The results could have been influenced by 2 higher values in girls fed soya-based formula at the 6 months period, which could be a chance finding. This study does not allow conclusions regarding possible adverse effects

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Female infants aged 3 to 24 months AIM: to evaluate the oestrogenic effect of soya-based formulas in female infants (Zung <i>et al.</i> , 2008)	Cross-sectional study. At least 3 months of soya-based formula feeding either exclusively (n=92) or in combination with breastfeeding or cows' milk formula (n=602) (as reported by parents). Reasons for preference for soya formula unknown.	Measurements of breast buds and breast tissue as part of regular physical examinations over a period of 2 years	<ul style="list-style-type: none"> • Higher prevalence of breast buds during the 2nd year of life when compared to cows' milk fed infants ($p<0.02$; 95% CI 1.11 – 5.39) • Preserving effect on infantile breast tissue and slower waning in the 2nd year of life compared to children fed breast milk or cows' milk formula ($p<0.001$) 	This study raises the possibility that early life soya in an early life may affect natural development of breast tissue in a small percentage of children. The significance is not clear as changes were subtle rather than pathological. Findings would need to be confirmed in further studies.
Infants fed soya formula (n=39), cows' milk formula (n=41) and breast milk (n=40). Approximately 20 boys and 20 girls per diet group. AIM: to determine differences in hormone-sensitive organ size between infants on different diets. (Gilchrist <i>et al.</i> , 2010)	Nested study within longitudinal cohort Soya formula, cows' milk formula or breastfeeding either exclusively or for the majority of the first 4 months of life, as reported by parents. No obvious selection for feeding decision.	Measurements of body weights, ovaries, number of follicles, testicular volume	<ul style="list-style-type: none"> • ↓ body weight in boys (comparing to cows' formula fed boys) ($p<0.05$) • ↓ ovarian volume in girls (comparing to cows' formula fed girls) ($p<0.09$) • ↑ number of follicles per cyst per ovary compared to breastfed girls ($p<0.01$) • ↓ testicular volume compared to breastfed boys ($p<0.04$) • No differences in infant weight, length, or body surface area 	Well designed and conducted study with thorough statistical analysis. Investigators were blind as to diet group. Moderately small number of children in each group. No adverse effects of soya feeding on reproductive organ development were demonstrated by age 4 months.

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Preschool boys (n=230) and girls (n=198), aged 3-6 years (Wada <i>et al.</i> , 2011)	Mean soya intake was recorded by parents over 3 days (24.4 g/day for boys and 22.8 g/day for girls)	Sex differences	<ul style="list-style-type: none"> • Soya intake negatively related to oestrone and oestradiol in boys ($p < 0.05$) • Soya intake positively related to testosterone and 5-androstene-3β,17α diol (3β,17α-AED) levels in girls ($p < 0.01$) • The average levels of oestrone, oestradiol, testosterone, and DHEA in girls were higher than those in boys 	Significant results show biological activity of soya in food in this age group. The relevance to infants and soya formula is unclear
ALSPAC study* 42 month old children (3,664 boys and 3,412 girls). (Adgent <i>et al.</i> , 2011)	Prospective, cohort study. Early-life feeding plan was reported by parents at 1, 6, 15 and 24 months postpartum as primarily breast (n=1428), early formula (n=5185), early soya (n=157) and late soya (n=306) Reasons for soy feeding were not given.	Sexual dimorphism in gender-role play behaviours assessed between infant feeding and scores of the Pre-School Activities Inventory (PSAI) test. Mother's response was scored on a 5-point Likert scale ("never" to "very often"). Higher scores indicated masculine typical behaviour, and lower scores indicated feminine typical behaviour.	<ul style="list-style-type: none"> • Modest increase in masculine typical behaviour among girls at 42 months of age fed soya in early life (but not 30 or 57 months). Mean [CI] PSAI score: 40.8 [38.6, 43.0] in early soya feeding and 36.7 [36.4, 37.1] in early formula feeding. • No significant effects in boys • Authors noted that the results were within the range of normal behaviour. 	Bias or residual confounding by social class may explain the soya findings. There may be residual confounding by social class (better educated mothers were more likely to report both masculine and feminine traits in children). Dividing play into masculine and feminine could also be socially determined
ALSPAC study* Girls (n=2920) for	Prospective, cohort study. Early-life	Timing of menarche in relation to infant feeding	<ul style="list-style-type: none"> • Median age at menarche was earliest for girls 	The study found a non-statistically significant

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whom at least one puberty questionnaire was available between the ages of 8 and 14.5 years if age (at approximately 8, 9.5, 10.5, 11.5, 13 and 14.5 years of age) (Adgent <i>et al.</i> , 2012)	feeding plan was reported by parents at 1, 6, 15, and 24 months either as primarily breast (n=631), early formula (n=2124), early soya (n=54) and late soya (n=111)		receiving an early soya diet (149 months; IQR 140-159), and latest among those who were primarily breastfed (154 months; IQR 145-165) <ul style="list-style-type: none"> • Early soya-fed girls were at 25% higher risk of early menarche throughout the course of follow-up (up to 14.5 years of age) (HR 1.25 [95% CI 0.92, 1.71]), compared to girls fed non-soya-based or milk formula 	trend for slightly earlier menarche. This contrasts with study by Strom <i>et al.</i> (2001) described above, which found no difference in reported age at menarche between 128 females fed soya and 268 fed cows' milk formula.
Infants (n=391) fed breast milk (BF), cows' milk formula (MF), or soya formula recruited between ages 1 and 2 months. Assessment at age 3, 6, 9, and 12 months. (Andres <i>et al.</i> , 2012)	One-third (n=129) of children exclusively fed soya-based infant formula (SF)	Infant behavioural development (Bayley Scales: MDI** and PDI***; PLS-3****)	<ul style="list-style-type: none"> • ↓ MDI at 6 months (compared to BF and MF) and at 9 and 12 months (compared to BF) (p<0.05) • ↓ PDI at 6 months compared to BF infants (p<0.05) • NS differences between different diets 	Although significant the differences in the MDI were very small and within expected normal range. PLS-3 were the lowest in the MF group – with small differences and all within range.
Infants (n=207) fed breast milk (BM), cows' milk formula (CMF), or soya formula during 1 st	One-third of children exclusively fed soya-based infant formula (SF)	Growth, fat mass and bone mineral content	<ul style="list-style-type: none"> • ↑ fat-free mass at 6 and 9 months compared to CMF (p<0.001) • ↑ bone mineral content by 12 months (lower at 3 	

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year of life. Assessment at age 3, 6, 9, and 12 months. (Andres <i>et al.</i> , 2013)			months comparing to BM)	
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*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 livebirths 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

Appendix 3

Search strategy

General isoflavones/genistein/daidzein exposure search

Websites interrogated

- EFSA
- COT
- FSA
- JECFA

Scientific publications literature search in PubMed

Specific search terms:

Isoflavone/phytoestrogens/genistein/daidzein/soy AND breast milk

Search Dates (From/To) - From May 2002 to present*

*Some papers pre-2002 were included if it felt they added value to the paper, particularly with regards to papers which identified previous cases of chronic and acute isoflavone toxicity and where a dose which lead to toxicity was identifiable.

Exclusion Criteria:

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

Isoflavone/genistein/daidzein/soy AND infant formula

Search Dates (From/To) - From May 2002 to present*

*Some papers pre-2002 were included if it felt they added value to the paper, particularly with regards to papers which identified previous cases of chronic and acute isoflavone toxicity and where a dose which lead to toxicity was identifiable.

Exclusion Criteria:

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

Isoflavone/genistein/daidzein/soy AND infant diet

Search Dates (From/To) - From May 2002 to present*

*Some papers pre-2002 were included if it felt they added value to the paper, particularly with regards to papers which identified levels of isoflavones in foods

Exclusion Criteria:

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

Isoflavone/genistein/daidzein/soy AND weaning

Search Dates (From/To) - From May 2002 to present*

*Some papers pre-2002 were included if it felt they added value to the paper, particularly with regards to papers which identified levels of isoflavones in foods

Exclusion Criteria:

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

Soya/soya formula/phytoestrogens AND animals AND diet AND oestrogenic effect
Search Dates (From May 2002 to present)

Exclusion Criteria:

- Studies in pre-menopausal females
- *In vitro* studies

Soya/soya formula/phytoestrogens AND animals AND diet AND behaviour
Search Dates (From May 2002 to present)

Exclusion Criteria:

- Studies where levels of isoflavones in the treatment are not given
- Studies where ways of administration are other than oral

Soya/soya formula/phytoestrogens AND animals AND diet AND reproduction
Search Dates (From May 2002 to present)*

*Some papers pre-May 2002 were included if they added value to the paper, particularly if studies published after May 2002 were sparse

Exclusion Criteria:

- Studies in pre-menopausal women and mature animals
- *In vitro* studies
- Studies performed in lower organisms, sheep and rabbits
- Studies in undeveloped countries
- Co-treatment with other chemicals
- Studies investigating therapeutic effect of soya in relation to other diseases

Soya/soya formula/phytoestrogens AND exposure
Search Dates (From/To) - From January 2002 to present*

*Some papers pre-2002 were included if they added value to the paper, particularly with regards to papers which identified isoflavone exposure values

Exclusion Criteria:

- Supplementation studies in undeveloped countries
- Supplementation programs in undeveloped countries
- Deficiency related research

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

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